European Commission

Combined Draft Renewal Assessment Report prepared according to Regulation (EC) N° 1107/2009
and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

Glyphosate

Volume 3 – B.7.5 - B.7.8 (AS)

Rapporteur Member State : Assessment Group on Glyphosate (AGG) consisting of FR, HU, NL and SE

Version History

When	What
2021/06	Initial RAR

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

Table of contents

B.7. RESIDUE DATA

B.7.1. STORAGE STABILITY OF RESIDUES

Refer to separate Volume 3 B.7.1 – B.7.4.

B.7.2. METABOLISM, DISTRIBUTION AND EXPRESSION OF RESIDUES

Refer to separate Volume 3 B.7.1 – B.7.4.

B.7.3. MAGNITUDE OF RESIDUE TRIALS IN PLANTS

Refer to separate Volume 3 B.7.1 – B.7.4.

B.7.4. FEEDING STUDIES

Refer to separate Volume 3 B.7.1 – B.7.4.

B.7.5. EFFECTS OF PROCESSING

B.7.5.1. Nature of the residue

The nature of residues of glyphosate and its metabolites AMPA and N-acetyl AMPA was investigated in hydrolysis studies. The first study (CA 6.5.1/001) is submitted for the first time in the frame of this active substance renewal, whereas the second (CA 6.5.1/002) and third (CA 6.5.1/003) studies were already evaluated in the previous assessment (RAR, 2015).

B.7.5.1.1. Study 1

1. Information on the study

Data point:	CA 6.5.1/001
Report author	[redacted]
Report year	2020
Report title	AMPA and *N*-Acetyl AMPA Hydrolysis under typical conditions (pH, temperature and time) of processing
Report No	S19-22457
Document No	M-680101-01-1
Guidelines followed in study	OECD Guideline for the Testing of Chemicals, 507 European Commission Working Document SANCO 3029/99 rev. 4
Deviations from current test guideline	Test material AMPA and *N*-acetyl AMPA used in the study were not radiolabelled. This deviation is deemed acceptable since no significant degradation is observed (≥ 10%) and therefore, no degradation product needs to be identified or characterised.
Previous evaluation	New study for AIR5
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 1 **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

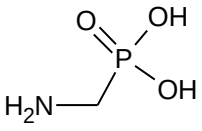
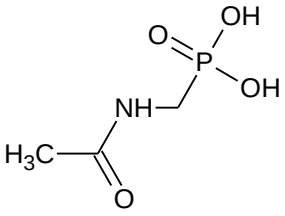
The purpose of this study was to investigate the hydrolytic transformation/degradation of AMPA and *N*-acetyl AMPA. The experiments were carried out under laboratory conditions, which are representative for food processing operations of raw agricultural commodities (RACs). The nature of test items during hydrolysis was tested separately, i.e. not as a mix of both compounds. The following conditions were tested in duplicate:

Pasteurisation:	90°C at pH 4 for 20 min
Baking, brewing, boiling:	100°C at pH 5 for 60 min
Sterilisation:	120°C at pH 6 for 20 min

The recovery (not corrected for the T_0 recovery) for the high temperature hydrolysis tests ranged from 90.8 % to 100 % for AMPA and from 101.9 % to 103.1 % for *N*-acetyl AMPA. For all the tests conducted, no significant change in the concentration of AMPA and *N*-acetyl AMPA was detected in all samples at the end of the incubation period. Based on these results, AMPA and *N*-acetyl AMPA were found to be stable to hydrolysis in the pH range tested when subjected to high temperatures. AMPA and *N*-acetyl AMPA are expected to be stable during common processing practices such as pasteurisation, sterilisation and baking/brewing/boiling.

I. Materials and Methods

A. Materials

Test material	Aminomethylphosphonic acid (AMPA)	*N*-Acetyl AMPA
Chemical structure:		

B. Study design

1. Preparation of stock and test solutions

All aqueous buffered solutions were prepared using citric acid monohydrate dissolved in demineralised water and adjusted to pH 4.0, 5.0 and 6.0 with 2 M sodium hydroxide. For sterilisation, all buffers were autoclaved after preparation.
The test solutions for hydrolysis were prepared by adding buffer solution to the test vessel, followed by adding the respective 100 mg/L test item stock solution to yield a final concentration of 1.0 mg/L. The test vessel was closed with a PTFE sealed cap. Two replicate samples were prepared per test item and set of hydrolytic conditions.
Sterility of the test solutions (before hydrolysis) was checked by their application to sterile agar plates and incubation at 37 °C for 2 days. In addition, a negative and a positive control were incubated under the same conditions. The colonies developed on the plates were counted.

2. Experimental conditions

Two samples at pH 4 ± 0.1 were placed in an oven and maintained at 90 °C ± 5 °C for 20 minutes. Two samples at pH 5 ± 0.1 were placed in an oven and maintained at 100 °C ± 5 °C for 60 minutes. Two samples at pH 6 ± 0.1 were placed in an autoclave and maintained at sterilizing conditions at 120 °C ± 5 °C for 20 minutes.

Table B.7.5.1.1-1: Parameters for hydrolysis conditions

pH	Temperature [°C]	Test period [min]	Representative Process
4.0 ± 0.1	90 ± 5	20 min	Pasteurisation
5.0 ± 0.1	100 ± 5	60 min	Baking, brewing and boiling
6.0 ± 0.1	120 ± 5	20 min	Sterilisation

3. Sampling

Duplicate samples were collected before (T_0) and after the respective hydrolytic condition. The pH of the solution was measured and recorded. Aliquots of 0.05 mL were taken from the test vessel before and after the respective processing and were diluted 20-fold with water + 0.1 % formic acid prior to LC-MS/MS analysis. After analysis, samples were stored in a freezer at ≤ -18 °C.

4. Analytical phase

The buffer solutions were analysed for AMPA and *N*-Acetyl AMPA residues by high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode using AMPA and *N*-Acetyl-AMPA standards in diluted buffer solutions for calibration. Quantification was performed by using linear regression with additional correction for bracketing standards. Injections of diluted samples were interspersed with injections of standard solutions after maximum 5 samples to verify the detector response and to adjust the calculated concentration.
The method was validated within this study, and fortification experiments with AMPA and *N*-Acetyl AMPA at fortification levels of 0.05 mg/L (LOQ) and 1.1 mg/L were performed. The results are summarised in the table below.

Table B.7.5.1.1-2: Method recovery results

Matrix	Analyte	Fortification level (mg/L)	Recovery			
			Range (%)	Mean (%)	Relative standard deviation (%)	Number analyses (n)
Buffer pH 4	AMPA	0.05	87.4 - 107.2	96.9	8.8	5
		1.1	89.0 - 100.0	95.5	5.6	5
		Overall	**87.4 - 107.2**	**96.2**	**7.1**	**10**
	N-Acetyl AMPA	0.05	102.4 - 108.4	105.0	2.3	5
		1.1	94.4 - 100.7	99.0	2.7	5
		Overall	**94.4 - 108.4**	**102.0**	**3.9**	**10**
Buffer pH 5	AMPA	0.05	89.4 - 105.6	96.7	6.3	5
		1.1	93.4 - 110.9	100.3	7.0	5
		Overall	**89.4 - 110.9**	**98.5**	**6.6**	**10**
	N-Acetyl AMPA	0.05	96.4 - 100.4	98.5	1.4	5
		1.1	94.0 - 101.8	98.6	2.9	5
		Overall	**94.0 - 101.8**	**98.6**	**2.2**	**10**
Buffer pH 6	AMPA	0.05	89.2 - 108	103.1	1.6	5
		1.1	86.4 - 105.4	94.5	7.4	5
		Overall	**86.4 - 105.4**	**98.8**	**6.7**	**10**
	N-Acetyl AMPA	0.05	100 - 105.2	102.5	1.9	5
		1.1	100.0 - 104.4	102.4	2.1	5
		Overall	**100.0 - 105.2**	**102.4**	**1.9**	**10**

II. Results and Discussion

The pH of the samples was measured at each sampling time. The pH results for all sets of samples indicated that the buffering capacity was maintained in the solution during the study period. Sterility assay for pH 4, 5 and 6 samples showed no growth for any of the samples tested, indicating that sterility was preserved throughout the study. The hydrolysis of AMPA and *N*-Acetyl AMPA test substances were examined at pH 4, pH 5 and pH 6 at 90 °C, 100 °C and 120 °C, respectively. Aliquots of all test solutions were analysed in duplicate by LC-MS/MS before and after hydrolysis. Under the tested conditions representative for food processing, no hydrolysis of AMPA and *N*-Acetyl AMPA was observed.

The recoveries for the high temperature hydrolysis tests ranged from 90.8 % to 100 % for AMPA and from 101.9 % to 103.1 % for *N*-acetyl AMPA of the applied dose for all solutions showing that AMPA and *N*-Acetyl AMPA did not degrade at temperatures ranging from 90 °C to 120 °C in any of the buffer systems tested. Detailed results are provided below.

Table B.7.5.1.1-3: Recovery results for AMPA and *N*-acetyl AMPA before and after processing

Hydrolysis conditions	AMPA			*N*-Acetyl AMPA		
	Recovery before processing [%][1]	Recovery after processing [%][1]	Recovery corrected for T_0 [%]	Recovery before processing [%][1]	Recovery after processing [%][1]	Recovery corrected for T_0 [%]
pH 4, 90 °C, 20 min	97.0	90.8	93.7	104.2	101.9	97.8
pH 5, 100 °C, 60 min	93.0	100.0	107.5	104.3	103.1	98.8
pH 6, 120 °C, 20 min	102.7	95.8	93.3	103.3	102.3	99.0

[1] With respect to fortification level of 1 mg/L

III. Conclusion

Under hydrolysis conditions representative of pasteurisation (pH 4, 90 °C), baking, brewing and boiling (pH 5, 100 °C) and sterilisation (pH 6, 120 °C) there was no significant change in the concentration of test items or pH-values. It can be concluded that AMPA and *N*-Acetyl AMPA are stable under these test conditions or, if degradation products are formed, they altogether represent less than 10 % of the amount of test item prior to hydrolysis (maximum of 6.7 % estimated for AMPA under conditions representative of sterilisation).

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study assessing the high temperature hydrolysis of AMPA and *N*-Acetyl AMPA has been previously not evaluated at EU level. It was performed under GLP. The study was conducted with non-labelled material. This is a deviation to OECD Guideline for the Testing of Chemicals, 507 as the high temperature hydrolysis is usually conducted with radio labelled material. The result of the study shows, that there was no significant change in the concentration of test items. Therefore, the study is considered to be reliable. It complies with current requirements as laid down in Reg. (EU) No 283/2013.

Assessment and conclusion by RMS:
It is agreed with the applicant's assessment and conclusion of the study. The deviation from the OECD guideline 507 is considered acceptable for the reasons stated above. The RMS additionally notes that no studies on the stability of residues during storage are required since the experimental phase of the study took 10 days only. In conclusion, AMPA and *N*-Acetyl AMPA were shown stable during processing conditions simulating pasteurisation, baking/brewing/boiling, and sterilisation.

B.7.5.1.2. Study 2

1. Information on the study

Data point:	CA 6.5.1/002
Report author	[redacted]
Report year	2010
Report title	Nature of [^{14}C]Glyphosate Residues in Processed Commodities – High Temperature Hydrolysis
Report No	1925W-001
Document No	MSL0023072

Guidelines followed in study	OECD Guideline for the Testing of Chemicals, 507
Deviations from current test guideline	No deviation from OECD Guideline for the Testing of Chemicals, 507
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

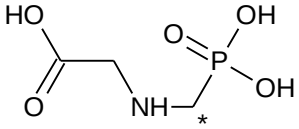
The purpose of this study was to investigate the hydrolytic transformation/degradation of *N*-phosphono-^{14}C-methyl)glycine (^{14}C-glyphosate). The experiments were carried out under laboratory conditions, which are representative for food processing operations of raw agricultural commodities (RACs). The following conditions were tested:

Pasteurisation:	90 °C at pH 4 for 20 min
Baking, brewing, boiling:	100 °C at pH 5 for 60 min
Sterilisation:	120 °C at pH 6 for 20 min

The recovery for the high temperature hydrolysis tests ranged from 95.6 % to 99.4 % of applied radioactivity. For all the tests conducted, no significant change in the concentration of glyphosate was detected in all samples at the end of the incubation period. Based on these results, glyphosate was found to be stable to hydrolysis in the pH range tested when subjected to high temperatures. Glyphosate is expected to be stable during common processing practices such as pasteurisation, sterilisation and baking/brewing/boiling.

I. Materials and Methods

A. Materials

Test material	N-(phosphono-^{14}C-methyl)glycine (= ^{14}C-glyphosate)
Chemical structure:	 * Position of the radio label
Radiochemical purity:	98.7 % (HPLC prior experimental start)
Specific activity:	10.28 MBq/mg (6.17 x 10^5 dpm/µg)

B. Study design

1. Preparation of stock and test solutions

The active substance *N*-(phosphono-^{14}C-methyl)glycine (^{14}C-glyphosate) was received as neat compound and was stored in a freezer (< 0 °C) when not in use. A stock solution of ^{14}C-glyphosate was prepared by dissolving the neat ^{14}C-glyphosate in 1 mL of HPLC grade water. HPLC analysis of this solution showed that the test substance was < 95 % pure and needed further purification. The test substance was purified by HPLC. The purified stock ^{14}C-glyphosate was dissolved in water. Radiochemical purity of the test substance determined was 98.7 % prior to use in the study. The concentration of the purified glyphosate stock solution was 7.498 x 10^4 dpm/µL.
All aqueous buffered solutions were prepared using sterile 0.1 M potassium biphthalate. Buffered solutions were prepared and adjusted to pH 4.0, 5.0 and 6.0 and solutions were sterilised by passing through sterile cellulose acetate membrane filter into previously autoclaved vials/bottles. Prior to application, nitrogen was bubbled for at least 5 minutes through each buffer via sterile bacterial air filter to avoid the effects of oxygen on the test systems. Aliquots of the test systems were taken at time 0 and final time point and were cultured on plates of Trypticase Soy Agar (TSA) in an incubator at 35 °C for sterility assay. After at least 48 hours of incubation, the cultures were evaluated for microbial growth.

Dose solutions were prepared by transferring aliquots of ^{14}C-glyphosate stock solution to sterile bottles containing buffer solution (pH 4.0, 5.0 or 6.0). Each dose solution was mixed well, and aliquots were radioassayed by liquid scintillation counting (LSC) to determine the concentration. The concentration of glyphosate in buffered solutions before hydrolysis ranged from 1.07 mg/L to 1.15 mg/L.
Aliquots of the dosing solutions were also taken after subsampling of each buffer solution to determine homogeneity of the solutions during the dosing process. Stability of the dosing solutions under conditions of administration was demonstrated by HPLC analysis after dosing and during analysis of time 0 samples.

2. Experimental conditions

The samples were prepared in duplicate for each buffer system using sterile amber glass vials. The test systems were dosed under aseptic conditions in a biological-hood flow cabinet.
Duplicate samples were analysed immediately for time zero where no heat was used. Two additional samples at pH 4 ± 0.1 were placed in an oven and maintained at 90 °C ± 5°C for 20 minutes. Two samples at pH 5 ± 0.1 were placed in an oven and maintained at 100 °C ± 5°C for 60 minutes. Two samples at pH 6 ± 0.1 were placed in an autoclave and maintained at sterilizing conditions (approximately 121 °C) for 20 minutes.

Table B.7.5.1.2-1: Parameters for hydrolysis conditions

pH	Temperature [°C]	Test period [min]	Representative Process
4.0 ± 0.1	90	20 min	Pasteurisation
5.0 ± 0.1	100	60 min	Baking, brewing and boiling
6.0 ± 0.1	~121	20 min	Sterilisation

3. Sampling

Duplicate samples were collected before (T_0) and after the respective hydrolytic condition. At sampling, duplicate samples were retrieved from the respective oven or autoclave. The pH of the solution was measured and recorded. Triplicate aliquots (3 x 0.1 mL) were taken for LSC analysis. All solutions were analysed by HPLC within two days of sampling.

4. Analytical phase

Each dose solution was mixed well, and radioactivity measurement was carried out by liquid scintillation counting (LSC) to determine the concentration.
^{14}C-glyphosate and its potential degradates were analysed and quantitated based on cation-exchange HPLC analysis with LSC analysis of the collected eluent fractions. The identity of ^{14}C-glyphosate was based on co-chromatography with glyphosate reference standard upon HPLC analysis. Reference standards were co-chromatographed with all samples. Confirmatory analysis was done by strong anion-exchange chromatography with LSC analysis of the collected eluent fractions.
Aliquots of the ^{14}C aqueous test samples were co-injected with glyphosate standard solution. HPLC radiochromatograms were produced from the fraction collection of the HPLC eluent (0.5 minutes fractions) employing a fraction collector with subsequent quantitation of the fractions by LSC.
For radiochemical purity checks, aliquots of the diluted solutions of test substance were co-injected with reference standard for analysis.

II. Results and Discussion

Aliquots of the dose solutions taken throughout the dosing processes showed that all dose solutions were homogeneous during the application processes. The pH of the samples was measured at each sampling time and demonstrated for all sets of samples that the buffering capacity was maintained in the solution during the study period. Sterility assay for pH 4, 5 and 6 samples showed no growth for any of the samples tested, indicating that sterility was preserved throughout the study. The hydrolysis of glyphosate test substance was examined at pH 4, pH 5 and pH 6 at 90 °C, 100 °C and approximately 121 °C, respectively. Aliquots of all test solutions were analysed in duplicate by HPLC before and after hydrolysis. Under the tested conditions representative for food processing, no hydrolysis of ^{14}C-glyphosate was observed. Radiocarbon recoveries for the high temperature hydrolysis tests ranged from 95.6 to 99.4 % of the applied dose for all solutions showing that glyphosate did not degrade at temperatures ranging from 90 °C to ~121 °C in any of the buffer systems tested. Detailed results are provided below.

Table B.7.5.1.2-2: Recovered radioactivity ^{14}C-glyphosate before and after hydrolysis in sterile buffer solutions

Hydro-lysis conditions	Sample	Re-plicate	Mass balance (% applied dose)	Glyphosate		Other peaks	
				% in HPLC chromatogram	% applied dose	% in HPLC chromatogram	% applied dose
pH 4, 90°C, 20 min	0 min	A	96.1	99.7	95.8	0.3	0.3
		B	95.7	99.6	95.3	0.4	0.4
	20 min	A	95.6	99.5	95.1	0.5	0.5
		B	95.9	99.4	95.3	0.6	0.6
pH 5, 100°C, 60 min	0 min	A	96.7	99.8	96.5	0.2	0.2
		B	96.6	99.7	96.3	0.3	0.3
	60 min	A	98.9	99.5	98.4	0.5	0.5
		B	99.3	99.5	98.8	0.5	0.5
pH 6, 120°C, 20 min	0 min	A	98.3	99.5	97.8	0.5	0.5
		B	97.6	99.7	97.3	0.3	0.3
	20 min	A	98.3	98.4	96.7	1.6	1.6
		B	99.4	98.5	97.9	1.5	1.5

C. Storage stability

All samples were analysed by HPLC within two days of sampling. All samples and standard solutions were stored frozen (< 0 °C) when not in use. Repeated injections of the glyphosate standard solution showed no degradation of the reference substance throughout the study.

III. Conclusion

The hydrolytic degradation behaviour of N-(phosphono-^{14}C-methyl)glycine (^{14}C-glyphosate) under conditions representative for food processing operations (pasteurisation, baking, brewing, boiling and sterilisation) was investigated. The recovery for the high temperature hydrolysis ranged from 95.6 % to 99.4 % of the applied dose for all solutions. The experiments showed that glyphosate did not degrade at temperatures ranging from 90°C to sterilizing conditions (~121°C) in any of the buffer systems tested, indicating that glyphosate should be stable in/on processed commodities during common processing practices.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study assessing the high temperature hydrolysis of glyphosate has been previously evaluated at EU level. It was performed under GLP. The study complies with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 507. Therefore the study is considered to be reliable.

Assessment and conclusion by RMS:
It is agreed with the applicant's assessment and conclusion of the study. Glyphosate was shown to be stable during processing conditions simulating pasteurisation, baking/brewing/boiling, and sterilisation.

B.7.5.1.3. Study 3

1. Information on the study

Data point:	CA 6.5.1/003
Report author	[redacted]
Report year	2006

Report title	High temperature hydrolysis of [^{14}C]IN-MCX20 in buffered aqueous solution at pH 4, 5, and 6
Report No	DuPont-19797
Document No	-
Guidelines followed in study	European Commission Working Document 1607/VI/97 Rev. 2, June 1999 (Appendix E, Processing Studies, 7035/VI/95 Rev. 5, July 1997)
Deviations from current test guideline	– It is reported that an oven (instead of an autoclave) was used to reach the temperature of 120 °C, simulating sterilisation, but the "oven" is not listed in the instrumental list. – Only representative, not-integrated HPLC chromatograms are shown in the study report. Co-chromatography (fortification of samples with radiolabelled reference standard) was only performed on selected samples (only one is shown). – Minor peaks (other peaks) were < 10 % AR, but > 0.01-0.05 mg/kg and > 0.05 mg/kg, respectively. The peaks were detected both in control samples (taken before hydrolysis, at 0 min) as after hydrolysis were comparable. Further characterisation of these minor peaks was not attempted. – The average material balance of control (T_0) samples at pH 5 are slightly below 90 %. – Sterility of the buffers was not determined in this study. However, *N*-acetyl-glyphosate remained stable throughout the study in all test systems and no difference was observed between heated and control samples. – Purity of the radiolabelled test substance was not determined as part of this study prior to day 0. – Samples were stored at -10 °C.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable for evaluation.

2. Full summary of the study according to OECD format

The purpose of this study was to investigate the hydrolytic transformation/degradation of [^{14}C]-*N*-acetyl-glyphosate. The experiments were carried out under laboratory conditions, which are representative for food processing operations of raw agricultural commodities (RACs). The following conditions were tested:

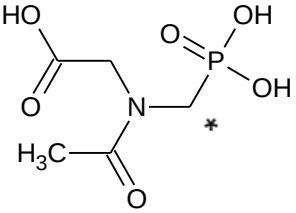
Pasteurisation:	90 °C at pH 4 for 20 min
Baking, brewing, boiling:	100 °C at pH 5 for 60 min
Sterilisation:	120 °C at pH 6 for 20 min

Solutions of [^{14}C]-*N*-acetyl-glyphosate were prepared in 0.01 M citrate buffer (pH 4, 5, and 6) at a nominal test concentration of 1.0 mg/L. At the end of the incubation period, samples were analysed by LSC to determine the quantity of radioactivity present in each sample. Radioactivity was quantitatively recovered from each test solution. Average radiocarbon recoveries for the high temperature hydrolysis tests generally ranged from 95.3 to 98.6 % of the applied radioactivity (AR) for all solutions, except for the pH 5 T_0-sample that accounted for 87.7 % AR, likely due to cracking of the two vials during freezing.
Test solutions were subject to chromatographic analysis (HPLC) to investigate the nature of any hydrolysis products formed. In all samples, the majority of applied radioactivity was recovered as *N*-acetyl-glyphosate. In all samples, no significant degradation occurred during incubation. LC-MS analysis was performed on selected samples to confirm identifications of *N*-acetyl-glyphosate made using HPLC.
Based on these results, *N*-acetyl-glyphosate was found to be stable to hydrolysis in the pH range tested when

subjected to high temperatures. *N*-acetyl-glyphosate is expected to be stable during common processing practices such as pasteurisation, sterilisation and baking/brewing/boiling.

I. Materials and Methods

A. Materials

Test material	N-acetyl-[phosphonomethylene-^{14}C-]glycine [^{14}C]-*N*-acetyl-glyphosate
Chemical structure:	 * position of radiolabel
Radiochemical purity:	>96 % (97.2 %, HPLC; assay conducted by Perkin Elmer Life and Analytical Sciences)
Specific activity:	0.51 MBq/mg (13.83 µCi/mg)

B. Study design

1. Preparation of stock and test solutions

The active substance *N*-acetyl-[phosphonomethylene-^{14}C]glycine ([^{14}C]-*N*-acetyl-glyphosate) was received as neat compound and was stored in a freezer (< -10 °C) when not in use. A stock solution of ^{14}C-*N*-acetyl-glyphosate was prepared by dissolving 0.160 mL of the radioactive test substance in 5 mL of HPLC grade water. Radiochemical purity of the test substance determined was > 96 %. The concentration of the purified glyphosate stock solution was 1708.8 µg/mL.
A buffer concentration of 0.01 M was selected to minimize possible catalytic effects. All solutions were prepared by combining 0.1 M citric acid solution and 0.1 M trisodium citrate solution with distilled grade water. Buffered solutions were adjusted to pH 4.0, 5.0 and 6.0.
Since *N*-acetyl-glyphosate remained stable throughout the study in all test systems and no difference was observed between heated and control samples, sterility of the buffers was not determined.
Test solutions were prepared by transferring aliquots of the radiolabelled stock solution to buffer solution (pH 4, 5 or 6) to obtain a nominal concentration of approximately 1.0 mg/L ^{14}C- *N*-acetyl-glyphosate. The pH of each buffer solution was measured at the time of preparation, after addition of the test item and after sampling and was deemed acceptable.
Each test solution was mixed well and aliquots were radioassayed by liquid scintillation counting (LSC) to determine the concentration. The concentration of *N*-acetyl-glyphosate in buffered solutions before hydrolysis ranged from 1.176 mg/L to 1.184 mg/L.

2. Experimental conditions

The samples were prepared in triplicate for each buffer system using sterile glass vials. Triplicate samples were analysed immediately for time zero (control samples). Three additional samples at pH 4 ± 0.1 were placed in an oven and maintained at 90 °C ± 5 °C for 20 minutes. Three samples at pH 5 ± 0.1 were placed in an oven and maintained at 100 °C ± 5 °C for 60 minutes. Three samples at pH 6 ± 0.1 were placed in an oven and maintained at sterilizing conditions (approximately 121 °C) for 20 minutes.

Table B.7.5.1.3-1: Parameters for hydrolysis conditions

pH	Temperature [°C]	Test period [min]	Representative Process
4.0 ± 0.1	90 ± 5	20 min	Pasteurisation
5.0 ± 0.1	100 ± 5	60 min	Baking, brewing and boiling
6.0 ± 0.1	121 ± 5	20 min	Sterilisation

Temperatures were recorded before and after exposure to the test conditions. Control samples were placed at room temperature for the same time period as the corresponding heat-treated samples. At the end of the incubation period, the vessels containing samples were allowed to cool to room temperature before being analysed.

3. Sampling

Triplicate samples were collected before (T_0) and after the respective hydrolytic condition. At sampling, triplicate samples were retrieved from the respective oven. The pH of the solution was measured and recorded. Triplicate aliquots (3 x 1 mL) were taken for LSC analysis. All samples were initially analyzed via HPLC on the sampling day. Samples were stored frozen, after the initial analysis, at less than *ca* -10 °C. LSC analyses were conducted on thawed samples that had been frozen overnight.

4. Analytical phase

Each dose solution was mixed well, and radioactivity measurement was carried out by liquid scintillation counting (LSC) to determine the concentration. ^{14}C-*N*-acetyl-glyphosate and its potential degradates were analysed and quantitated based on reverse phase HPLC analysis with LSC analysis of the collected eluent fractions. The identity of ^{14}C-*N*-acetyl-glyphosate was based on co-chromatography with *N*-acetyl-glyphosate reference standard upon HPLC analysis: Unchanged *N*-acetyl-glyphosate in samples was identified using HPLC by comparing the retention time of the radioactive peak with that of an authentic standard. Representative samples were fortified with the radiolabelled reference standard and analysed using HPLC. The limit of quantification was 0.4 % AR. The identification of *N*-acetyl-glyphosate was confirmed by the analysis of selected samples using a second analytical method (LC-MS analysis). *N*-acetyl-glyphosate was identified by comparing LC/MS profiles to that of an authentic unlabelled reference standard under the same conditions.

II. Results and Discussion

The pH of the samples was measured at each sampling time. The pH results for all sets of samples indicated that the buffering capacity was maintained in the solution during the study period.
The hydrolysis of *N*-acetyl-glyphosate test substance was examined at pH 4, pH 5 and pH 6 at 90 °C, 100 °C and approximately 120 °C, respectively. Aliquots of all test solutions were analysed in triplicate by HPLC before and after hydrolysis. Under the tested conditions representative for food processing, no hydrolysis of ^{14}C-*N*-acetyl-glyphosate was observed.
Average radiocarbon recoveries for the high temperature hydrolysis tests generally ranged from 95.3 to 98.6 % of the applied radioactivity (AR) for all solutions, except for pH 5 control samples that accounted for 87.7 % AR. The lower recoveries observed in pH 5 control samples likely resulted from the cracking of two out of three vials during the overnight freezing prior to LSC analysis. The replicate sample that did not crack had a material balance of 98.8 % AR. Since *N*-acetyl-glyphosate remained stable throughout the study and the pH 5 heated samples showed acceptable recoveries, the pH 5 control samples were not retested. It was shown that *N*-acetyl-glyphosate did not degrade at temperatures ranging from 90 °C to sterilizing conditions (120 °C) in any of the buffer systems tested.
The amount of *N*-acetyl-glyphosate in the pH 4 samples incubated at 90 °C was 93.2 % AR after 20 min. Other minor radiolabelled components were detected, which collectively accounted for less than 4 % AR. The amount of *N*-acetyl-glyphosate in the corresponding control samples was 91.4 % AR. Other minor components were detected, which collectively accounted for less than 4 % AR.
The amount of *N*-acetyl-glyphosate in the pH 5 samples incubated at 100 °C was 92.1 % AR after 60 min. Other minor radiolabelled components were detected, which collectively accounted for less than 5 % AR. The amount of *N*-acetyl-glyphosate in the corresponding control samples was 84.0 % AR. Other minor components were detected, which collectively accounted for less than 5 % AR.
The amount of *N*-acetyl-glyphosate in the pH 6 samples incubated at 120 °C was 93.9 % AR after 20 min. Other minor radiolabelled components were detected, which collectively accounted for less than 5 % AR. The amount of *N*-acetyl-glyphosate in the corresponding control samples was 93.1 % AR. Other minor components were detected, which collectively accounted for maximally 5.5 % AR.
No hydrolysis products formed in concentrations of $\geq$ 5 % of the initial applied radioactivity. Therefore, these minor products were not further identified. Detailed results are provided in the table below.

Table B.7.5.1.3-2: Recovered radioactivity ^{14}C- *N*-acetyl-glyphosate before and after hydrolysis in sterile buffer solutions

Hydrolysis conditions	Sample	Replicate	Mass balance (% applied dose)	*N*-acetyl-glyphosate	Sum of other peaks
				% applied dose	% applied dose
pH 4, 90 °C, 20 min	0 min	1	99.7	96.8	2.9
		2	87.0	83.6	3.4
		3	99.3	93.8	5.5
		Mean	**95.3**	**91.4**	**3.9**
	20 min	1	92.0	89.5	2.5
		2	99.9	96.9	2.9
		3	98.1	93.2	4.9
		Mean	**96.7**	**93.2**	**3.4**
pH 5, 100 °C, 60 min	0 min	1	86.5	80.9	5.6
		2	77.7	74.8	2.9
		3	98.8	96.3	2.5
		Mean	**87.7**	**84.0**	**3.7**
	60 min	1	98.7	94.5	4.2
		2	99.3	91.7	7.5
		3	92.5	90.2	2.2
		Mean	**96.8**	**92.1**	**4.7**
pH 6, 120 °C, 20 min	0 min	1	98.4	91.7	6.7
		2	99.1	92.6	6.6
		3	98.4	95.0	3.4
		Mean	**98.6**	**93.1**	**5.5**
	20 min	1	97.8	94.2	3.7
		2	98.0	91.2	6.8
		3	98.2	96.3	1.9
		Mean	**98.0**	**93.9**	**4.1**

C. Storage stability

All samples were analysed by HPLC within one day of sampling. A reference standard was analysed with each HPLC run, which verified proper column and instrument operation and degradation of the reference substance throughout the study was not reported. All samples and standard solutions were stored frozen (< -10 °C) when not in use. Samples were analysed by LSC after being frozen overnight and allowed to thaw.

III. Conclusion

This study demonstrated that *N*-acetyl-glyphosate remained stable under simulated pasteurisation (pH 4, 90 °C, 20 minutes), baking, brewing or boiling (pH 5, 100 °C, 60 minutes), and sterilisation (pH 6, 120 °C, 20 minutes) conditions.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study assessing the high temperature hydrolysis of *N*-acetyl-glyphosate has been previously evaluated at EU level. It was performed under GLP. The study complies with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 507, with minor deficits: It is reported that an oven (instead of an autoclave) was used to reach the temperature of 120 °C, simulating sterilisation, but the "oven" is not listed in the instrumental list; Only representative, not-integrated HPLC chromatograms are

shown in the study report. Co-chromatography (fortification of samples with radiolabelled reference standard) was only performed on selected samples (only one is shown). Minor peaks (other peaks) were < 10 % AR, but >0.01-0.05 mg/kg and > 0.05 mg/kg, respectively. However, the other peaks were detected both in control samples (taken before hydrolysis, at 0 min) as after hydrolysis and were comparable (3.7-5.5 % AR before hydrolysis and 3.4 – 4.7 % AR after hydrolysis). Further characterisation of these minor peaks was therefore not attempted; The average material balance of control samples at pH 5 are slightly below 90 %. However, the lower recoveries were only observed in two of the three vials, because they had cracked during the overnight freezing prior to LSC analysis. The replicate sample that did not crack had a material balance of 99 % AR. Since *N*-acetyl-glyphosate remained stable throughout the study and the pH 5 heated samples showed acceptable recoveries, the pH 5 control samples were not retested; Sterility of the buffers was not determined in this study. However, *N*-acetyl-glyphosate remained stable throughout the study in all test systems and no difference was observed between heated and control samples; Purity of the radiolabeled test substance was not determined as part of this study prior to day 0. It was however determined after sample analysis and found to be >96 %. There was no impact on the study; Samples were stored at -10 °C, but analysed by HPLC on the day of sampling and by LSC after being frozen overnight and allowed to thaw.
The study is considered to be reliable.

Assessment and conclusion by RMS:
Several deviations from OECD Guideline 507 are noticed by the applicant (see 'Deviations from the current test guideline'), however, the RMS does not consider these deviations to have a significant impact on the study outcome. Overall, the study is considered to be acceptable for evaluation. *N*-Acetyl glyphosate was shown stable during processing conditions simulating pasteurisation, baking/brewing/boiling, and sterilisation.

B.7.5.2. Distribution of the residue in peel and pulp

Results of available and relevant processing studies are presented in Section B.7.5.3. of this Volume 3.

B.7.5.3. Magnitude of residues in processed commodities

A total of six studies were submitted to address the magnitude of residues during processing: in citrus fruits (CA 6.5.3/001 and CA 6.5.3/002), potatoes (CA 6.5.3/003), and olives (CA 6.5.3/004, CA 6.5.3/005, and CA 6.5.3/006). These studies were already submitted in the frame of the previous evaluation of glyphosate (RAR, 2015), however, the studies were re-assessed according to current guidelines and standards.
It is noted that additional studies were submitted for the previous evaluation which the applicant did not include in this dossier anymore. These studies address the magnitude of residues during processing of linseed, oilseed rape, soya bean, barley, maize, oat, rye, and wheat following a pre-harvest treatment. Since neither a pre-harvest use, nor a use in cereals or oilseeds is defended anymore, it is considered acceptable that these studies are not submitted for the current evaluation.

B.7.5.3.1. Study 1 and 2

1. Information on the study

Data point:	CA 6.5.3/001
Report author	[redacted]
Report year	1986
Report title	Determination of Glyphosate and Aminomethylphosphonic acid residues in citrus fruit and process fractions following post-directed treatment with Roundup herbicide
Report No	MSL-6194
Document No	-
Guidelines followed in study	According to the GRG, OECD GLP and FAO Guidelines were followed. The AGG, however, was not able to verify this based on the available study report since no statement about guidelines is given. Besides, it is questionable whether any guidelines are applicable to a study that only re-calculates data

	that were generated in a study not conducted according to any guideline (i.e. CA 6.5.3/002).
Deviations from current test guideline	– The test formulation used in the trials is not described. – A description of the test facility is not provided.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory at the time the study was performed.
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered not acceptable for evaluation.

1. Information on the study

Data point:	CA 6.5.3/002
Report author	[redacted]
Report year	1975
Report title	CP 57573, Residue and metabolism part 27: Determination of CP 67573 and CP 50435 residues in citrus process fractions
Report No	377
Document No	-
Guidelines followed in study	No test guidelines cited in the report.
Deviations from current test guideline	– The test formulation used in the trials is not described. – A description of the test facility is not provided. – Multiple recoveries are below 70 %. – Residues were not measured in the RAC.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory at the time the study was performed.
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered not acceptable for evaluation.

2. Full summary of the study according to OECD format

The objective of the study was to determine the magnitude of the residues of glyphosate and AMPA in citrus (fruit) and processed fraction juice, peel, press liquor and feed meal after three applications of Roundup. The study included 6 field trials with processing in the USA. The citrus trees were treated to the soil under the trees, at rates of 3x 4.48 kg glyphosate per hectare or 3x 8.97 kg glyphosate per hectare. Citrus fruit samples for processing were taken 1, 7 and 21 days after the last application. Residues of glyphosate in whole fruit sampled 1-21 days after the last application ranged from <0.05 mg/kg to 0.22 mg/kg. No residues of AMPA above the LOQ were found in any whole fruit taken 1, 7 and 21 days after the last application or in the processed commodities.
For juice the mean processing factor for glyphosate was 0.78, indicating that there was no concentration of glyphosate residue into juice relative to the raw commodity whole fruit. For citrus peel, feed meal and press liquor the mean processing factors for glyphosate were 2.43, 3.08 and 1.92, respectively.

I. Materials and Methods

A. Materials

1. Test material	
Description:	Roundup
Batch number:	Not reported
Active ingredient(s):	Glyphosate
CAS number:	1071-83-6
Content of a.s. nominal:	Not reported
Content of a.s. analysed:	Not reported
Formulation type:	Not reported

B. Methods

1. Field phase

Six residue trials were conducted on different citrus fruits (orange, lemon, grapefruit) during 1972 in the USA. Three applications of Roundup were performed onto the soil under the citrus trees at 4.48 kg a.s./ha and/or 8.97 kg a.s./ha per application. The main application parameters are outlined in the table below. Regions, varieties and cultivation were typical for the cultivation of citrus fruit.

Table B.7.5.3.1-1: Application information

Trial no.	Crop	Scientific names	Variety	Application rate (kg a.s./ha)
California [redacted] / USA / 1973	Orange	*Citrus paradisi*	Navel	3 x 4.48
				3 x 8.97
Florida [redacted] USA / 1973	Orange	*Citrus paradisi*	Pineapple	3 x 8.97
Florida, [redacted] USA / 1973	Orange	*Citrus paradisi*	Valencia	3 x 8.97
California, [redacted] USA / 1973	Lemon	*Citrus limon*	not specified	3 x 4.48
				3 x 9.97
Texas [redacted] USA / 1973	Grapefruit	*Citrus paradisi*	Ruby Red	3 x 8.97
Florida, [redacted] USA / 1973	Grapefruit	*Citrus paradisi*	Marsh	3 x 8.97

2. Sampling

Specimens of citrus fruit were taken by hand from treated and untreated plots at 1 and 21 days after the last application. In some trials, an additional sample was taken at 7 days after the last application. Control specimens were taken before treated specimens. In the trial in [redacted] (California), oranges were sampled from the lower (0-90 cm) and upper (> 90 cm) tree branches. This distinction is not made for the other trials. Samples were frozen shortly after sampling and kept frozen until analysis.

3. Processing

Processing was performed to obtain the processed fractions of juice, peel, feed meal and press liquor. The technology used was a lab-scale process similar to the industrial process. The applicant did not include any processing schemes in its summary, therefore, the RMS included a screenshot from the study report at the end of the study summary. It is noted that various different fractions were generated during processing, but not all fractions were sampled for each RAC or trial.
The citrus fruit was defrosted and washed. Samples of pre-wash water and after wash water were taken. Next, fruits were juiced in an in-line press ("F.M.C. extractor" in Figure B.7.5.3.1-1) and the juice was passed through

a finisher. Samples of juice and finisher pulp (extracted juice sacs) were taken. Besides, the oil/water/peel fragment emulsion from the F.M.C. in-line extractor was passed through a finisher to generate peel bits which were combined with the peel, rag, seeds resulting from the F.M.C. extractor; and an emulsion for the generation of citrus oil. The peel, rag, seeds, and bits fraction was chopped in a peel hopper and calcium hydroxide (lime) was added to obtain a liquid slurry. The slurry is then passed through a press to obtain press liquor and citrus pulp. Part of the press liquor is vacuum-concentrated to obtain molasses. The citrus pulp is passed through a triple-pass-drier to produce citrus dried pulp, (dried) meal, and (dried) fines.

4. Analytical phase

Glyphosate and AMPA were isolated from citrus specimens by aqueous extraction followed by ion exchange chromatography. After derivatisation to the N-trifluoroacetyl methyl esters the samples were subjected to GLC using a phosphorus specific flame photometric detector. For both analytes the limit of detection was 0.05 mg/kg in most fractions and 0.025 mg/kg in pre-wash and wash water samples.
The method was validated within this study: fortification experiments with glyphosate and AMPA at fortification levels of 0.05 mg/kg (LOQ), 0.025 mg/kg for prewash water and after wash water, and higher levels were performed. Some of the mean recoveries are below 70 % and therefore outside the guidance requirements. These low mean recovery values are mainly found for the higher fortification levels. The lower fortification levels are sufficiently validated. The results are summarised in the table below.

Table B.7.5.3.1-2: Recovery results

Matrix[1]	Analyte	Fortification level (mg/kg)	Recovery[2]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Juice	Glyphosate	0.1	55-104	71	14	19	22
		0.2	50-89	64	9.9	16	21
		Overall	**50-104**	**68**	**12**	**18**	**43**
	AMPA	0.1	53-106	75	14	19	24
		0.2	53-99	72	16	22	24
		Overall	**53-106**	**73**	**15**	**20**	**48**
Peel	Glyphosate	0.05	59-98	80	20	25	4
		0.1	52-95	71	11	16	22
		0.2	53-80	66	8.4	13	21
		Overall	**52-98**	**70**	**11**	**16**	**47**
	AMPA	0.05	55-95	66	19	29	4
		0.1	41-110	67	16	24	21
		0.2	44-99	66	14	21	19
		Overall	**41-110**	**67**	**15**	**23**	**44**
Press liquor	Glyphosate	0.05	106	106	N/A	N/A	1
		0.1	71-92	80	8.3	10	6
		0.2	56-85	74	10	14	8
		0.4	73	73	N/A	N/A	1
		Overall	**56-106**	**78**	**12**	**15**	**16**
	AMPA	0.05	108	108	N/A	N/A	1
		0.1	80-106	93	9.2	9.9	7
		0.2	57-102	81	16	19	8
		0.4	85	85	N/A	N/A	1
		Overall	**57-108**	**88**	**14**	**16**	**17**

Table B.7.5.3.1-2: Recovery results

Matrix[1]	Analyte	Fortification level (mg/kg)	Recovery[2]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Citrus meal	Glyphosate	0.05	106	106	N/A	N/A	1
		0.1	59-93	76	13	18	10
		0.2	58-86	72	10	14	7
		Overall	**58-106**	**76**	**14**	**18**	**18**
	AMPA	0.1	48-98	63	15	23	10
		0.2	43-65	56	7	13	8
		Overall	**43-98**	**60**	**12**	**20**	**18**
Oil	Glyphosate	0.05	77-87	82	6.7	8.2	2
		0.1	56-80	67	8.2	12	8
		0.2	66	66	N/A	N/A	1
		Overall	**56-87**	**70**	**9.4**	**14**	**11**
	AMPA	0.05	77-80	78	2.3	2.9	2
		0.1	76-95	83	6.8	8.1	8
		0.2	96	96	N/A	N/A	1
		Overall	**76-96**	**84**	**7.2**	**8.7**	**11**
Molasses	Glyphosate	0.1	58-81	71	9.7	13.7	5
		0.2	58-70	66	5.2	7.8	5
		Overall	**58-81**	**69**	**7.7**	**11**	**10**
	AMPA	0.1	71-96	87	9.8	11	5
		0.2	61-86	77	12	15	5
		Overall	**61-96**	**82**	**12**	**14**	**10**
Grapefruit pulp, rag, seed	Glyphosate	0.1	83-95	89	8.3	9.4	2
		0.2	80-94	87	10	12	2
		Overall	**80-95**	**88**	**7.8**	**8.8**	**4**
	AMPA	0.1	91-97	94	4.2	4.5	2
		0.2	76-85	81	6.3	7.8	2
		Overall	**76-97**	**87**	**8.8**	**10**	**4**
Finisher pulp	Glyphosate	0.1	81-115	95	18	19	3
		0.2	72-93	81	11	13	3
		Overall	**72-115**	**88**	**15**	**17**	**6**
	AMPA	0.1	96-112	103	8.4	8.1	3
		0.2	87-88	88	0.6	0.7	3
		Overall	**87-112**	**95**	**9.9**	**10**	**6**
Peel bits	Glyphosate	0.1	57-71	62	8.0	13	3
		0.2	56-63	59	3.6	6.0	3
		Overall	**56-71**	**61**	**5.7**	**9.5**	**6**
	AMPA	0.1	62-69	64	3.7	5.8	3
		0.2	68-90	80	11	14	3
		Overall	**62-90**	**72**	**11**	**16**	**6**

Table B.7.5.3.1-2: Recovery results

Matrix[1]	Analyte	Fortification level (mg/kg)	Recovery[2]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Citrus pulp	Glyphosate	0.1	86-89	88	2.1	2.4	3
		0.2	73-83	78	5.0	6.4	3
		Overall	**73-89**	**83**	**6.6**	**8.0**	**6**
	AMPA	0.1	79-83	80	2.6	3.2	3
		0.2	79-86	81	3.8	4.7	3
		Overall	**79-86**	**81**	**3.0**	**3.7**	**6**
Oil/water/ peel fragment emulsion	Glyphosate	0.1	93-96	94	1.8	1.9	3
		0.2	69-90	78	11	14	3
		Overall	**69-96**	**86**	**11**	**13**	**6**
	AMPA	0.1	77-97	88	10	11	3
		0.2	75-89	82	7.2	8.8	3
		Overall	**75-97**	**85**	**8.4**	**9.9**	**6**
Pre-wash water	Glyphosate	0.03	74-97	88	12	14	3
		0.05	82-93	88	5.9	6.6	3
		Overall	**74-97**	**88**	**8.5**	**9.6**	**6**
	AMPA	0.03	63-93	79	16	20	3
		0.05	73-84	77	5.6	7.2	3
		Overall	**62-93**	**78**	**11**	**14**	**6**
After wash water	Glyphosate	0.03	91-96	94	3.1	3.3	3
		0.05	96	96	N/A	N/A	1
		0.1	82-96	89	10	11	2
		Overall	**82-96**	**93**	**5.9**	**6.3**	**6**
	AMPA	0.03	84-90	88	3.5	3.9	3
		0.05	90	90	N/A	N/A	1
		0.1	84-90	87	4.2	4.9	2
		Overall	**84-90**	**88**	**3.0**	**3.5**	**6**

1 Values for matrices from orange, lemon and grapefruit were combined.
2 Calculations of mean, SDs, RSDs and overall values were performed using excel with individual recovery values as given in the report.
N/A Not applicable

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA in processed commodities after usage of Roundup when applied as per the study. Residues of glyphosate corrected for recoveries in whole fruit sampled 1-21 days after the last application ranged from <0.05 mg/kg to 0.22 mg/kg. No (corrected) residues of AMPA above the LOQ were found in any whole fruit taken 1, 7 and 21 days after the last application or in the processed commodities.

For juice, corrected residues of glyphosate raged from <0.05 mg/kg to 0.10 mg/kg resulting in processing factors from 0.45 to <1, indicating that there was no concentration of glyphosate residue into juice relative to the raw commodity whole fruit. For peel, feed meal and press liquor, corrected residues of glyphosate raged from <0.05 mg/kg to 0.69 mg/kg, from <0.05 mg/kg to 0.39 mg/kg and from <0.05 mg/kg to 0.37 mg/kg, respectively. The resulting processing factors for peel, feed meal and press liquor ranged from 1.1 to 3.1, from 1.4 to 5.3 and from <0.83 to 2.7, respectively. In the cases where no (corrected) residues of glyphosate and AMPA in the whole

fruit above the LOQ of 0.05 mg/kg were found, the calculation of a processing factor was not possible. Detailed residue levels are shown in the table below.

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
California, [redacted]/ USA / 1973	Orange / Navel upper level	3 x 4.48	1	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			7	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	0.06, 0.05 Mean=0.06	>1.2	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Press liquor	<0.05	-	<0.05	-
		3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05, <0.05, 0.06, <0.05 Mean=0.05	>1.0	<0.05	-
				Dried meal	0.06, 0.08 Mean=0.07	>1.4	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			7	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Press liquor	<0.05	-	<0.05	-
California, [redacted] / USA / 1973	Orange / Navel lower level	3 x 4.48	1	Whole fruit[4]	0.05, <0.05, 0.06, 0.05 Mean=0.05	-	<0.05	-
				Juice	<0.05	<1.0	<0.05	-
				Peel	0.16, <0.05, 0.18, 0.16 Mean=0.14	2.8	<0.05	-
				Dried meal	0.27, 0.26 Mean=0.27	5.3	<0.05	-
				Press liquor	0.12, 0.11 Mean=0.12	2.3	<0.05	-
			7	Whole fruit[4]	0.05, 0.05, 0.08, 0.06 Mean=0.06	-	<0.05	-
				Juice	<0.05	<0.83	<0.05	-
				Peel	<0.05, 0.11, 0.33, 0.25 Mean=0.19	3.1	<0.05	-
				Dried meal	0.08, 0.13 Mean=0.11	1.8	<0.05	-
				Press liquor	0.11, 0.12 Mean=0.12	1.9	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Juice	<0.05	-	<0.05	-
				Peel	0.06, <0.05, 0.08, 0.08 Mean=0.07	>1.4	<0.05	-
		3 x 8.97	1	Whole fruit[4]	0.05, 0.17, 0.50, 0.17 Mean=0.22	-	<0.05	-
				Juice	<0.05, <0.05, 0.23, <0.05 Mean=0.10	0.45	<0.05	-
				Peel	0.13, 0.64, 1.31, 0.69 Mean=0.69	3.1	<0.05	-
				Dried meal	0.38, 0.39 Mean=0.39	1.8	<0.05	-
				Press liquor	0.36, 0.37 Mean=0.37	1.7	<0.05	-
			7	Whole fruit[4]	<0.05, 0.11, <0.05, <0.05 Mean=0.07	-	<0.05	-
				Juice	<0.05	<0.71	<0.05	-
				Peel	0.09, 0.45, 0.05, <0.05 Mean=0.16	2.3	<0.05	-
				Dried meal	0.35, 0.34 Mean=0.35	4.9	<0.05	-
				Press liquor	0.13, 0.17 Mean=0.15	2.1	<0.05	-
			21	Whole fruit[4]	0.10, 0.17, <0.05, <0.05 Mean=0.09	-	<0.05	-
				Juice	0.09, 0.11, <0.05, <0.05 Mean=0.08	0.83	<0.05	-
				Peel	0.14, 0.38, 0.15, 0.12 Mean=0.20	2.2	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Dried meal	0.24, 0.35 Mean=0.30	3.3	<0.05	-
				Press liquor	0.25, 0.24 Mean=0.25	2.7	<0.05	-
Florida, [redacted] USA / 1973	Orange/ Pineapple	3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Pulp	<0.05	-	<0.05	-
				Dried pulp	<0.05, 0.05 Mean=0.05	>1.0	<0.05	-
				Oil	<0.05	-	<0.05	-
				Molasses	0.05, <0.05 Mean=0.05	>1.0	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
Florida, [redacted] USA / 1973	Orange/ Valencia	3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Pulp	<0.05	-	<0.05	-
				Dried pulp	<0.05	-	<0.05	-
				Oil	<0.05	-	<0.05	-
				Molasses	<0.05	-	<0.05	-
California, [redacted] USA / 1973	Lemon/ not specified	3 x 4.48	1	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05, <0.05, 0.09, <0.05 Mean=0.06	>1.2	<0.05	-
				Dried meal	0.18, 0.12 Mean=0.15	>3.0	<0.05	-
				Press liquor	0.05, 0.06 Mean=0.06	>1.2	<0.05	-
			7	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	0.05, <0.05 Mean=0.05	>1.0	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Press liquor	<0.05	-	<0.05	-
		3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Peel	0.06, <0.05, 0.09, <0.05 Mean=0.06	>1.2	<0.05	-
				Dried meal	0.09, 0.07 Mean=0.08	>1.6	<0.05	-
				Press liquor	<0.05	-	<0.05	-
			7	Whole fruit[4]	<0.05, <0.05, 0.08, <0.05 Mean=0.06	-	<0.05	-
				Juice	<0.05	<0.83	<0.05	-
				Peel	<0.05, <0.05, 0.11, <0.05 Mean=0.07	1.1	<0.05	-
				Dried meal	0.09, 0.08 Mean=0.09	1.4	<0.05	-
				Press liquor	<0.05	<0.83	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	0.07, 0.05 Mean=0.06	>1.2	<0.05	-
				Press liquor	<0.05	-	<0.05	-
Texas, [redacted] USA / 1973	Grapefruit, Ruby Red	3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Pulp, rag, seeds	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-

Table B.7.5.3.1-3: Residues of glyphosate and AMPA in citrus processed fractions, corrected for procedural recoveries

Location/ Country/Year	Crop/Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate[#]		AMPA[#]	
					Residues[2] (mg/kg)	Processing factors[3]	Residues (mg/kg)	Processing factors
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Dried meal	<0.05	-	<0.05	-
				Pulp, rag, seeds	<0.05	-	<0.05	-
Florida, [redacted] USA / 1973	Grapefruit/ Marsh	3 x 8.97	1	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-
				Pulp	<0.05	-	<0.05	-
				Dried pulp	<0.05	-	<0.05	-
				Oil	<0.05	-	<0.05	-
			21	Whole fruit[4]	<0.05	-	<0.05	-
				Washed	<0.05	-	<0.05	-
				Juice	<0.05	-	<0.05	-
				Peel	<0.05	-	<0.05	-

1 Days after last application
2 In the case all replicates were <0.05 mg/kg only the mean value of < 0.05 mg/kg is given
3 The processing factor is calculated by dividing the residue in the processed fraction by the residue in the RAC sample
4 Calculated value based on juice yield x residue in juice + peel yield x residue in peel
Residue levels presented in this table are corrected for procedural recoveries.

III. Conclusion

The median calculated processing factors of glyphosate for citrus juice, peel, feed meal and press liquor were 0.83, 1.8, 1.8 and 1.9, respectively. Glyphosate does not concentrate in matrices destined for human consumption. Processing factors could be calculated for oranges and lemon processed commodities. For grapefruit, the residues of glyphosate and AMPA in whole fruit and all processed commodities were always below the LOQ and no processing factors could be calculated. For AMPA no residues above the LOQ were present in the raw agricultural commodity. Therefore, a calculation of processing factors was not possible.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study was previously evaluated at EU level. It was not performed under GLP. Some of the mean recoveries for AMPA are below 70 % and therefore outside the guidance requirements. These low mean recovery values are mainly found for the higher fortification level. The lower fortification levels are sufficiently validated. The residue studies in orchards show that the residues of glyphosate and AMPA are both below the LOQ of 0.05 mg/kg. Since this low residue levels are sufficiently validated the deviation from the guideline can regarded as minor. Even though there are some minor deviations from the current test guideline (test formulation used in the trials is not described; description of the test facility is not provided) the study is considered to be reliable and acceptable. The study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 508. It adequately supports the representative processing processes for glyphosate and AMPA in citrus fruit.

Summary of citrus processing factors

Source: DocID (trial reference)	**PHI**	**Citrus juice**	**Citrus peel**	**Feed meal**	**Press liquor**
Orange / Navel (lower level) 3 x 4.48 kg a.s./ha	1	<1.0	2.8	5.3	2.3
	7	<0.83	3.1	1.8	1.9
	21	-	>1.4		
Orange / Navel (upper level) 3 x 8.97 kg a.s./ha	1	-	>1.0	-	-
Orange / Navel (lower level) 3 x 8.97 kg a.s./ha	1	0.45	3.1	1.8	1.7
	7	<0.71	2.3	4.9	2.1
	21	0.83	2.2	3.3	2.7
Lemon 3 x 4.48 kg a.s./ha	1	-	>1.2	>3.0	>1.2
	7	-	-	>1.0	-
Lemon 3 x 8.97 kg a.s./ha	1	-	>1.2	>1.6	-
	7	<0.83	1.1	1.4	<0.83
	21	-	-	>1.2	-
Median	1	0.725	1.2	2.4	1.7
	7	0.83	2.3	1.6	1.9
	21	0.83	1.8	2.25	2.7
	Overall	**0.83**	**1.8**	**1.8**	**1.9**
Mean	*1*	*0.725*	*1.86*	*2.93*	*1.73*
	7	*0.79*	*2.17*	*2.28*	*1.61*
	21	*0.83*	*1.8*	*2.25*	*2.7*
	Overall	***0.78***	***1.94***	***2.53***	***1.82***

Assessment and conclusion by RMS:

The processing of different citrus fruits was investigated in the study by [redacted] (1975; CA 6.5.3/002) and processing factors were derived. According to [redacted] (1986; CA 6.5.3/001), calculation procedures on processing factors have evolved, and therefore a study report was written in which processing factors were re-calculated using more up-to-date calculation methods ([redacted] 1986; CA 6.5.3/001). [redacted] states in the study report that the following two points differ in the way processing factors were calculated: (i) instead of using the highest replicate residue for a sample, all replicates were averaged; and (ii) all residue values have been corrected for the average recovery. Since no guidelines are specified in the study reports, the RMS is not able to state which guidelines and calculation rules were exactly followed. According to current EU legislation, however, it is indeed acceptable to average residue levels from field replicate samples. In contrast to this, residue values should not be corrected for procedural recoveries.

The RMS notes that some trials are not considered independent. In the trials conducted on oranges and lemons in [redacted] (California), two application rates were tested which is rather considered a different experimental condition than an independent trial. The same accounts for the trials on orange in [redacted] (Florida) in which only two different varieties were tested. Since the study is not considered acceptable for evaluation anyway, this has no further impact on the risk assessment.

The RMS also notes that different processed commodities were generated, depending on the RAC to be processed. The terms used by the applicant in its summary were not always consistent with those from the study report, therefore, the RMS made some amendments for better understanding.

The study summary presented above was submitted by the applicant and it is based on the study report by [redacted] (CA 6.5.3/001), i.e. residue values are averages of replicates, but corrected for procedural recoveries. In order to have a summary that is in line with current EU regulations, the results need to be amended so that uncorrected residue values are shown. For the sake of convenience, however, the RMS did not amend the study summary since the study is not considered reliable for evaluation anyway. The main reasons are presented in the following:

(i) The study was not conducted according to GLP, although the RMS recognises that this was not compulsory at the time the study was performed

(ii) The study was not conducted according to any guideline, although the RMS recognised that the study setup is similar to OECD Guideline 508.

(iii) Many procedural recoveries were outside the acceptable guidance requirements (70-120% and/or RSD < 20%). According to the applicant, these low mean recovery values were mainly found for the higher fortification levels, but not at lower fortification levels. Since these low residue levels were sufficiently validated, the deviation is regarded as minor by the applicant. The RMS notes, however, that some procedural recoveries were generated at fortification levels significantly above the measured residue level. For instance, recoveries in citrus juice were determined at 0.1 and 0.2 mg/kg only, whereas the LOQ was 0.05 mg/kg and residue levels were determined below the LOQ. In conclusion, recovery data are not considered reliable and the performance of the analytical method was not sufficiently demonstrated. Besides, the method is not considered validated either (see Vol. 3, B.5).

(iv) It seems that storage stability was investigated in the frame of this study, however, the information provided in the study report is very limited and no conclusions regarding the integrity of residues during storage can be made.

(v) One major limitation of the study is the fact that residues were not measured in the RAC to be processed, i.e. in oranges, lemons, or grapefruits. Instead, residues in whole fruits were calculated backwards based on residue levels in juice and peel and the weight of the respective fractions, which is not considered acceptable by the RMS. The residue concentration should have been determined in the RAC as well.

(vi) In nearly all experiments, calculated levels of glyphosate and AMPA were below the LOQ in the RACs. Therefore, it is not possible to calculate processing factors with these trials. In one trial on lemons ([redacted], California), one single calculated residue value of glyphosate was above the LOQ (0.06 mg/kg) in the RAC, whereas all other values were below the LOQ. Next to the trial on lemon, calculated levels of glyphosate were above the LOQ in the RAC in one trial on oranges ([redacted], California). In the study report, however, the author already stated that these residues are likely caused by drift and accidental spraying of lower tree branches and their fruits rather than uptake of residues via soil. This is further supported by the fact that this was the only trial in which treatment was performed by hand sprayers instead of covered boom equipment, and by the fact that the available metabolism studies indeed indicate that residues uptake via the roots is limited. Therefore it is questionable whether it is possible to derive a valid processing factor from this trial.

Taken all points together and as stated before, the study is not considered reliable for evaluation. The RMS again notes that the results in the study summary are residue levels corrected for recoveries, which is not in line with EU legislation. At best, the study results give some qualitative information on the distribution of the residue within the RAC after processing, however, no quantitatively reliable processing factors can be derived.

It is worthwhile to mention that, although residues levels of glyphosate were mostly below the LOQ in whole fruits, residue levels seemed to concentrate in certain fractions. This indicates that certain amounts of residues of glyphosate may indeed be present in the RACs. It is therefore unlikely that the ground-directed use in orchard crops indeed represents a zero-residue situation. The RMS, however, recognises that trials were all overdosed compared to the representative GAP.

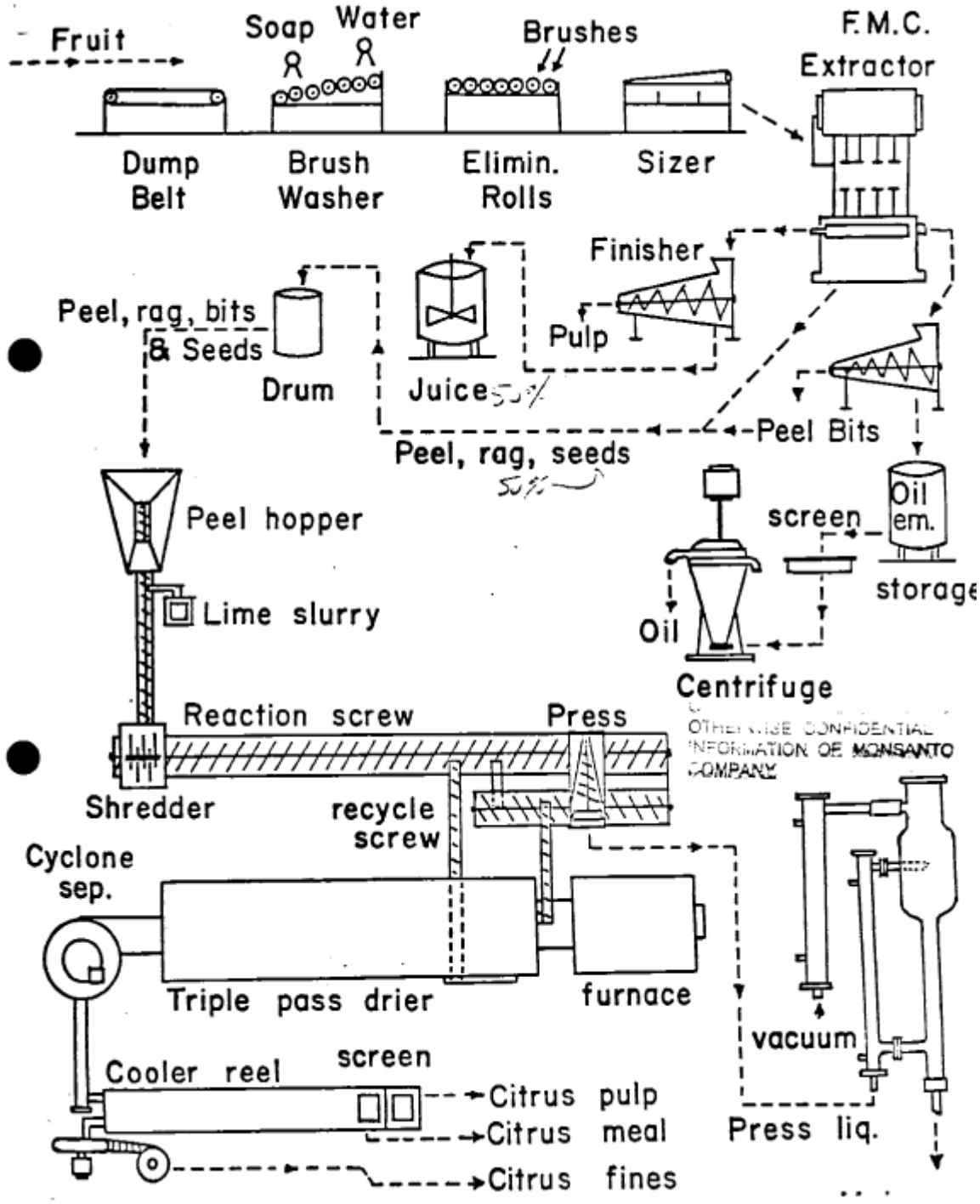


Figure B.7.5.3.1-1: Schematic overview of citrus fruits processing steps

B.7.5.3.2. **Study 3**

1. Information on the study

Data point:	CA 6.5.3/003
Report author	[redacted]
Report year	1988

Report title	Glyphosate residues in potatoes and processed fractions of potatoes after treatment with Roundup herbicide
Report No	MSL-7877
Document No	-
Guidelines followed in study	EPA Guideline 171-4: Magnitude of Residue-Crop Field Trials
Deviations from current test guideline	See "assessment and conclusion RMS" below
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No (it is noted by the RMS that a GLP compliance statement is included in the study report, however, it states that the study was "*to the best of our knowledge, […] conducted in accordance with the principles of 40 CFR 160*" but it is also stated that the study "*is not subject to the requirements of that Rule for regulatory purposes*"). The RMS notes, however, that GLP was not compulsory at the time of study conduct.
Acceptability/Reliability:	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is not considered to be acceptable (see 'assessment and conclusion by RMS').

2. Full summary of the study according to OECD format

The objective of the study was to determine the magnitude of the residues of glyphosate and AMPA in potato (tuber) and processed fraction chips, wet peel (chips), flakes, wet peel (flakes), dry peel (flakes) and granules after one application of Roundup, an EC formulation.
The study included 2 processing trials in the USA. There was one soil treatment before emergence of the potatoes at rates of either 4.2, 8.4, 21 or 42 kg glyphosate per hectare. Potato samples for processing were collected at 97-104 days after the application. The residues of glyphosate in potato tubers were always below the LOQ of 0.05 mg/kg. The residues of AMPA in potato tubers were <0.05 mg/kg for the lower application rates of 4.2 and 8.4 kg a.s./ha and for the higher application rates of 21 and 42 kg a.s./ha between <0.05 and 0.23 mg/kg.
The mean processing factors for AMPA were 1.7 in chips, 0.45 in wet peel (from chips processing), 1.4 in flakes, 0.47 in wet peel (from flakes processing), 1.5 in dry peel (from flakes processing), and 1.7 in granules.

I. Materials and Methods

A. Materials

1. Test material	
Description:	Roundup
Active ingredient(s):	Glyphosate
CAS number:	1071-83-6
Content of a.s. analysed:	41.36 %
Formulation type:	EC

B. Methods

1. Field phase

Two residue trials were conducted on potatoes (*Solanum tuberosum*) during 1987 in the USA. There was one application of Roundup to the soil before emergence of the potatoes at rates of either 4.2, 8.4, 21 or 42 kg glyphosate per hectare. The volume of water used to prepare the spray solution was 374 L/ha. Care was taken that the spray solution was properly homogenised by mixing before application. Ground spray applications were made via backpack sprayer with a boom equipped with flat fan nozzles.

2. Sampling

Specimens of potato were taken by hand from treated and untreated plots 97 or 104 days after the application. Treated and specimens were maintained in a deep frozen condition and adequately separated during storage and shipment to the processing facility.

Table B.7.5.3.2-1: Crop sampling information

Trial	Crop	Commodity	DALA[1]	Quantity	Date of sampling
California, [redacted]	Potato	Tuber	97	4.5 kg	23.06.1987
		Tuber for processing		113-159 kg	
California, [redacted]	Potato	Tuber	104	4.5 kg	30.06.1987
		Tuber for processing		113-159 kg	

1 Days after last application

3. Processing

Processing was performed to obtain the processed fractions of chips, wet peel (from chips processing), flakes, wet and dry peel (from flakes processing) and granules. The technology used was a lab-scale process similar to the industrial process. The applicant did not include any processing schemes in its summary, therefore, the RMS included screenshots from the study report at the end of the study summary. These also include further details on the process steps.

For potato chips, the raw potatoes were washed and destoned. After peeling (wet peel = stock feed) and slicing, the starch on the surface of the slices was washed away. Then the slices were fried for 60-70 seconds at approximately 185 °C.

For potato flakes the raw potatoes were washed and destoned. After peeling the potatoes were sliced, washed, cooked and mashed, then crushed by rollers on the surface of a drum. The mashed potatoes were dried by heating to temperatures from 162 to 170 °C, and then the layer of dried, mashed potatoes is scraped from the drum. The flakes were milled and frozen. The peels resulting from peeling were either sampled directly (wet peel = raw stock feed) or dried (dry peel = dried stock feed)

For potato granules the raw potatoes were washed and destoned. After peeling the potatoes were sliced, washed, cooked and mashed. Subsamples were frozen and after thawing and drying of one subsample, this subsample was mixed with a freshly thawed subsample. This process was repeated six times. The final mixture was dried to a water content of 8-10 %.

4. Analytical phase

All samples were analysed using the analytical method XA001, which based on the well-established method DFG 405 (refer to CA 4.1.2). Potato samples were maintained deep frozen until analysis, except when being processed. For the determination of glyphosate and AMPA the samples were extracted with water and dichloromethane and cleaned-up by elution through Chelex 100 resin followed by anion exchange chromatography. Glyphosate and AMPA were quantified by HPLC after post-column derivatisation with o-phthaldialdehyde with a fluorescence detector. For glyphosate and AMPA, the limit of quantitation (LOQ) was 0.05 mg/kg.

During analysis, fortification experiments with glyphosate and AMPA at fortification levels of 0.05 mg/kg (LOQ) and higher were performed. The results are summarised in the table below. Some individual recoveries are below or above the acceptable range, but most mean recoveries per fortification level are in the range from 70 to 110 % the results can be regarded as valid. Some exceptions were noted, i.e. mean procedural recoveries were outside the acceptable range, however, this was observed for recoveries where only one sample was measured. When looking at the overall data set, the adequacy of the analytical method is considered sufficiently demonstrated.

Table B.7.5.3.2-2: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery				
			Range (%)	Mean (%)	Standard deviation (%)[1]	Relative standard deviation (%)[1]	Number analyses (n)
Potato whole	Glyphosate	0.05	94-109	101	10.1	10.0	2
		0.1	99-101	100	1.6	1.6	2
		0.2	100	100	N/A	N/A	1
		0.5	97-104	100	3.8	3.8	3
		1.0	92-105	97	6.4	6.6	3
		2.0	103	103	N/A	N/A	1

Table B.7.5.3.2-2: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery				
			Range (%)	Mean (%)	Standard deviation (%)[1]	Relative standard deviation (%)[1]	Number analyses (n)
		Overall	**92-109**	**100**	**4.7**	**4.8**	**12**
	AMPA	0.05	82-110	96	20.2	21.1	2
		0.1	81-96	89	10.6	12.0	2
		0.2	80	80	N/A	N/A	1
		0.5	82-91	88	5.5	6.3	3
		1.0	91-98	95	3.5	3.7	3
		2.0	91	91	N/A	N/A	1
		Overall	**80-110**	**91**	**8.8**	**9.7**	**12**
Potato chips	Glyphosate	0.05	67	67	N/A	N/A	1
		0.2	100	100	N/A	N/A	1
		0.5	102	102	N/A	N/A	1
		2.0	96	96	N/A	N/A	1
		Overall	**67-102**	**91**	**16.6**	**18.1**	**4**
	AMPA	0.05	135	135	N/A	N/A	1
		0.2	111	111	N/A	N/A	1
		0.5	102	102	N/A	N/A	1
		2.0	86	86	N/A	N/A	1
		Overall	**86-135**	**108**	**20.5**	**18.9**	**4**
Potato chips, wet peel/stock feed	Glyphosate	0.05	96	96	N/A	N/A	1
		0.2	88	88	N/A	N/A	1
		0.5	92	92	N/A	N/A	1
		2.0	91	91	N/A	N/A	1
		Overall	**88-96**	**92**	**3.4**	**3.7**	**4**
	AMPA	0.05	103	103	N/A	N/A	1
		0.2	85	85	N/A	N/A	1
		0.5	91	91	N/A	N/A	1
		2.0	85	85	N/A	N/A	1
		Overall	**85-103**	**91**	**8.3**	**9.2**	**4**
Potato flakes	Glyphosate	0.05	80-91	86	7.9	9.2	2
		0.1	78	78	N/A	N/A	1
		0.2	85	85	N/A	N/A	1
		Overall	**78-91**	**83**	**5.8**	**7.0**	**4**
	AMPA	0.05	91-109	100	12.6	12.5	2
		0.1	74	74	N/A	N/A	1
		0.2	82	82	N/A	N/A	1
		Overall	**74-109**	**89**	**15.2**	**17.1**	**4**
Potato flakes, wet peel/raw	Glyphosate	0.1	85-92	89	4.8	5.4	2
		1.0	92-95	93	2.5	2.6	2
		Overall	**85-95**	**91**	**4.1**	**4.5**	**4**

Table B.7.5.3.2-2: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery				
			Range (%)	Mean (%)	Standard deviation (%)[1]	Relative standard deviation (%)[1]	Number analyses (n)
stock feed	AMPA	0.1	94-92	88	5.3	6.0	2
		1.0	82-87	85	3.2	3.8	2
		Overall	**82-92**	**86**	**4.1**	**4.7**	**4**
Potato flakes, dry peel/dry stock feed	Glyphosate	0.05	90-93	92	2.3	2.5	2
		0.1	64	64	N/A	N/A	1
		0.2	82-95	88	8.7	9.9	2
		Overall	**64-95**	**85**	**12.5**	**14.7**	**5**
	AMPA	0.05	60-87	78	14.9	19.2	3
		0.1	91	91	N/A	N/A	1
		0.2	70-78	74	5.4	7.4	2
		0.5	69	69	N/A	N/A	1
		Overall	**60-91**	**77**	**11.2**	**14.5**	**7**
Potato granules	Glyphosate	0.05	84-92	88	3.8	4.3	3
		0.2	80	80	N/A	N/A	1
		0.5	80-85	83	3.7	4.5	2
		Overall	**80-92**	**85**	**4.7**	**5.5**	**6**
	AMPA	0.05	72-94	83	10.8	13.0	3
		0.2	69	69	N/A	N/A	1
		0.5	68-71	69	2.1	3.1	2
		Overall	**68-94**	**76**	**10.4**	**13.6**	**6**

1 Calculations of mean, SDs, RSDs and overall values were performed using excel with individual recovery values as given in the report.
N/A Not applicable

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA in processed commodities of potatoes after usage of Roundup when applied as per the study. The residues of glyphosate in the RAC potato tubers were always below the LOQ of 0.05 mg/kg, i.e. no processing factors could be calculated. In one sample of wet peel (flakes) and for dry peel (flakes), residues of glyphosate were 0.06 mg/kg and 0.28 mg/kg, respectively.
The residues of AMPA in potato tubers were <0.05 mg/kg for the lower application rates of 4.2 and 8.4 kg a.s./ha and between <0.05 and 0.23 mg/kg for the higher application rates of 21 and 42 kg a.s./ha, i.e. processing factors could be determined in case residues were above the LOQ in the RAC. Residue levels of AMPA and calculated processing factors are shown in the table below.

Table B.7.5.3.2-3: Residues of glyphosate and AMPA in potato processed fractions

Location / Country / Year	Crop/ Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found[2] (mg/kg)	Processing factors	Residues found (mg/kg)	Processing factors[3]
California, [redacted]/ USA / 1987	Potato / Kennebeck	1 x 4.2	97	Tuber	<0.05	-	<0.05	-
				Chips	N/A	-	N/A	-
				Wet peel (chips)	N/A	-	N/A	-
				Flakes	N/A	-	N/A	-
				Wet peel (flakes)	N/A	-	N/A	-
				Dry peel (flakes)	N/A	-	N/A	-
				Granules	N/A	-	N/A	-
		1 x 8.4	97	Tuber	<0.05	-	<0.05	-
				Chips	N/A	-	N/A	
				Wet peel (chips)	N/A	-	N/A	
				Flakes	N/A	-	N/A	
				Wet peel (flakes)	N/A	-	N/A	
				Dry peel (flakes)	N/A	-	N/A	
				Granules	N/A	-	N/A	
		1 x 21	97	Tuber	<0.05	-	0.062, 0.065 Mean=0.063	-
				Chips	<0.05	-	0.120, 0.124 Mean=0.122	1.9
				Wet peel (chips)	<0.05	-	<0.05	<0.79
				Flakes	<0.05	-	0.107, 0.107 Mean=0.107	1.7
				Wet peel (flakes)	<0.05	-	<0.05	<0.79
				Dry peel (flakes)	<0.05	-	0.141, 0.157 Mean=0.149	2.4
				Granules	<0.05	-	0.121, 0.140 Mean=0.131	2.1

Table B.7.5.3.2-3: Residues of glyphosate and AMPA in potato processed fractions

Location / Country / Year	Crop/ Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found[2] (mg/kg)	Processing factors	Residues found (mg/kg)	Processing factors[3]
		1 x 42	97	Tuber	<0.05	-	<0.05	-
				Chips	<0.05	-	0.129, 0.133 Mean=0.131	-
				Wet peel (chips)	<0.05	-	<0.05	-
				Flakes	<0.05	-	0.076, 0.091 Mean=0.084	-
				Wet peel (flakes)	0.06, 0.06 Mean=0.06	-	<0.05	-
				Dry peel (flakes)	0.07, 0.08 Mean=0.08	-	0.114, 0.123 Mean=0.119	-
				Granules	<0.05	-	0.145, 0.152 Mean=0.149	-
California, [redacted] USA / 1987	Potato / Kennebeck	1 x 4.2	104	Tuber	<0.05	-	<0.05	-
				Chips	<0.05	-	N/A	-
				Wet peel (chips)	<0.05	-	N/A	-
				Flakes	<0.05	-	N/A	-
				Wet peel (flakes)	<0.05	-	N/A	-
				Dry peel (flakes)	<0.05	-	N/A	-
				Granules	<0.05	-	N/A	-
		1 x 8.4	104	Tuber	<0.05	-	<0.05	-
				Chips	<0.05	-	N/A	-
				Wet peel (chips)	<0.05	-	N/A	-
				Flakes	<0.05	-	N/A	-
				Wet peel (flakes)	<0.05	-	N/A	-
				Dry peel (flakes)	<0.05	-	N/A	-

Table B.7.5.3.2-3: Residues of glyphosate and AMPA in potato processed fractions

Location / Country / Year	Crop/ Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found[2] (mg/kg)	Processing factors	Residues found (mg/kg)	Processing factors[3]
				Granules	<0.05	-	N/A	-
		1 x 21	104	Tuber	<0.05	-	0.147, 0.150 Mean=0.149	-
				Chips	<0.05	-	0.205, 0.218 Mean=0.212	1.4
				Wet peel (chips)	<0.05	-	<0.05	<0.34
				Flakes	<0.05	-	0.153, 0.187 Mean=0.170	1.1
				Wet peel (flakes)	<0.05	-	<0.05	<0.34
				Dry peel (flakes)	<0.05	-	0.175, 0.191 Mean=0.183	1.2
				Granules	<0.05	-	0.162, 0.183 Mean=0.183	1.2
		1 x 42	104	Tuber	<0.05	-	0.233, 0.235 Mean=0.234	-
				Chips	<0.05	-	0.365, 0.398 Mean=0.382	1.6

Table B.7.5.3.2-3: Residues of glyphosate and AMPA in potato processed fractions

Location / Country / Year	Crop/ Variety	Application rate (kg a.s./ha)	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found[2] (mg/kg)	Processing factors	Residues found (mg/kg)	Processing factors[3]
				Wet peel (chips)	<0.05	-	0.049, 0.052 Mean=0.051	0.22
				Flakes	<0.05	-	0.299, 0.422 Mean=0.361	1.5
				Wet peel (flakes)	<0.05	-	0.065, 0.067 Mean=0.066	0.28
				Dry peel (flakes)	<0.05	-	0.242, 0.259 Mean=0.251	1.1
				Granules	<0.05	-	0.365, 0.529 Mean=0.447	1.9

1 Days after last application
2 In the case all replicates were <0.05 mg/kg only the mean value of < 0.05 mg/kg is given
3 The processing factor is calculated by dividing the residue in the processed fraction by the residue in the RAC sample
N/A Not analysed, due to residues <0.05 mg/kg in the potato tuber (RAC).

III. Conclusion

No residues above the LOQ of 0.05 mg/kg were found for glyphosate in potato tubers (RAC). Therefore, no processing factors could be derived for glyphosate. Only in one sample of wet peel (flakes) and dry peel (flakes) residues of glyphosate of 0.06 and 0.08 mg/kg, respectively, were found, indicating that residues of glyphosate might concentrate in these processed commodities.
In contrast to glyphosate, residues of AMPA above the LOQ were determined in potato tubers from three trials and processing factors were calculated.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study was previously evaluated at EU level. It was performed under GLP and is considered to be reliable and acceptable. Processing factors could only be derived in the trials where the application rate exceeded by far the supported application rate in the EU. Nevertheless, the study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 508. It adequately supports the representative processing processes for glyphosate and AMPA in potato.

Summary of potato processing factors

Source: DocID (trial reference)	**Chips**	**Wet peel (chips)**	**Flakes**	**Wet peel (flakes)**	**Dry peel (flakes)**	**Granules**
Glyphosate						
California, [redacted] 42 kg a.s./ha	-	-	-	1.2	1.6	-
AMPA						
California, [redacted] 21 kg a.s./ha	1.9	<0.79	1.7	<0.79	2.3	2.1
California [redacted] 42 kg a.s./ha	>2.62	-	>1.68	-	>2.38	>2.98
California, [redacted] 21 kg a.s./ha	1.4	<0.34	1.1	<0.34	1.2	1.2
California, [redacted] 42 kg a.s./ha	1.6	0.22	1.5	0.28	1.1	1.9
Median	**1.8**	**0.34**	**1.6**	**0.34**	**1.8**	**2.0**
Mean	**1.9**	**0.45**	**1.5**	**0.47**	**1.7**	**2.0**

Assessment and conclusion by RMS:

In the current study, the magnitude of residues during processing of potatoes was investigated in two trials following pre-emergence treatment with glyphosate. Per trial, one untreated and four treated plots (4.2, 8.4, 21, and 42 kg/ha) were installed. Since the trial locations are only 10-15 km apart from each other, the treated crop varieties are identical, and since the trials were performed less than one month apart from each other, the trials cannot be considered independent.

Potatoes were sampled 97-104 days after application and stored frozen until analysis, except when samples were processed. Samples were stored for maximally 296 days between harvest and analysis, i.e. a period for which sample integrity was demonstrated. The RMS notes that all processed fractions are considered to belong to the high starch matrices.

The applicant did not include any processing schemes in its summary, therefore, the RMS included screenshots from the study report (see below).

Mean procedural recoveries were within the acceptable ranges, although it is noted that some individual recoveries were outside these ranges. The analytical method, however, was only validated for the determination of glyphosate and AMPA in potato whole tuber with a LOQ of 0.1 mg/kg for glyphosate and 0.5 mg/kg for AMPA. The method is not considered validated for other processed matrices. Furthermore, linearity data are missing. Lastly it is noted that additional information regarding the extraction efficiency would be needed for confirmation.

With regard to the reported residue levels, the RMS also notes the following: values shown in Table B.7.3.5.2-3 are residue levels not corrected for procedural recoveries. In the study report itself, however, the main body text describes the results of corrected residue levels. Only in the appendices, where raw data are included, uncorrected residue levels are available. The RMS used these data to verify the results shown in Table B.7.3.5.2-3.

No residues were detected in the control specimens. Residues of glyphosate were below the LOQ at harvest (97-104 days after application) in the RAC to be processed (potato tubers), even at application rates up to 42 kg/ha. In one sample of wet peel (flakes) and for dry peel (flakes), residues of glyphosate were 0.06 mg/kg and 0.28 mg/kg, respectively. Although no processing factors can be calculated, these results give indications that glyphosate was indeed present in the RAC, but at concentrations below the LOQ.

Residues of AMPA above the LOQ were determined in potato tubers in three trials. Consequently, processing factors can be calculated. It is worthwhile to note that no residues of glyphosate were determined above the LOQ, whereas residues of AMPA were indeed determined above the LOQ. This stands in contrast to the observations made in the supervised residue trials.

As stated before, the trials and different plots are not considered independent and therefore, only one processing factor can be calculated. It is debatable whether the processing factor should be calculated on one trial only, i.e. the trial that would yield the highest/worst-case processing factor, or whether a mean processing factor should be calculated based on all trials. The RMS considers it adequate to calculate the processing factor on all available trials/plots so that slight variations during processing are accounted for. Nevertheless, to obtain a fully reliable processing factor, a second independent trial would be required.

It is noted that the applicant considered all trials acceptable for the calculation of processing factors, also the trial conducted in [redacted] (California) where potatoes were treated at 1 x 42 kg/ha. However, residues of AMPA were below the LOQ in the RAC and therefore it is not appropriate to use this trial for the calculations.

In conclusion, the study was largely conducted according to OECD Guideline 508, however, some deviations were noted. No processing factors can be derived for glyphosate since residue levels were below the LOQ in the RAC. For AMPA, processing factors can be calculated based on one trial since none of the trials and plots are considered independent. However, since the analytical method was not successfully validated, the study and the calculated processing factors are not taken into account for evaluation. For the sake of completeness, the RMS nevertheless calculated the processing factors for information purposes:

	Residues [mg/kg]			**Processing factors (PFs)**			
	Trial 1[1]	**Trial 2[2]**	**Trial 3[3]**	**PF1**	**PF2**	**PF3**	**Mean PF**
Glyphosate							
No processing factors calculated							
AMPA							
Potato tubers (RAC)	0.063	0.149	0.234	-	-	-	-
Chips	0.122	0.212	0.382	1.9	1.4	1.6	**1.6**
Wet peel (chips)	<0.05	<0.05	0.051	<0.79	<0.34	0.22	**<0.45**
Flakes	0.107	0.170	0.361	1.7	1.1	1.5	**1.4**
Wet peel (flakes)	<0.05	<0.05	0.066	<0.79	<0.34	0.28	**<0.47**
Dry peel (flakes)	0.149	0.183	0.251	2.4	1.2	1.1	**1.6**

Granules	0.131	0.183	0.447	2.1	1.2	1.9	**1.7**

[1] [redacted] (California), 1 x 21 kg/ha
[2] [redacted]t (California), 1 x 21 kg/ha
[3] [redacted] (California), 1 x 42 kg/ha

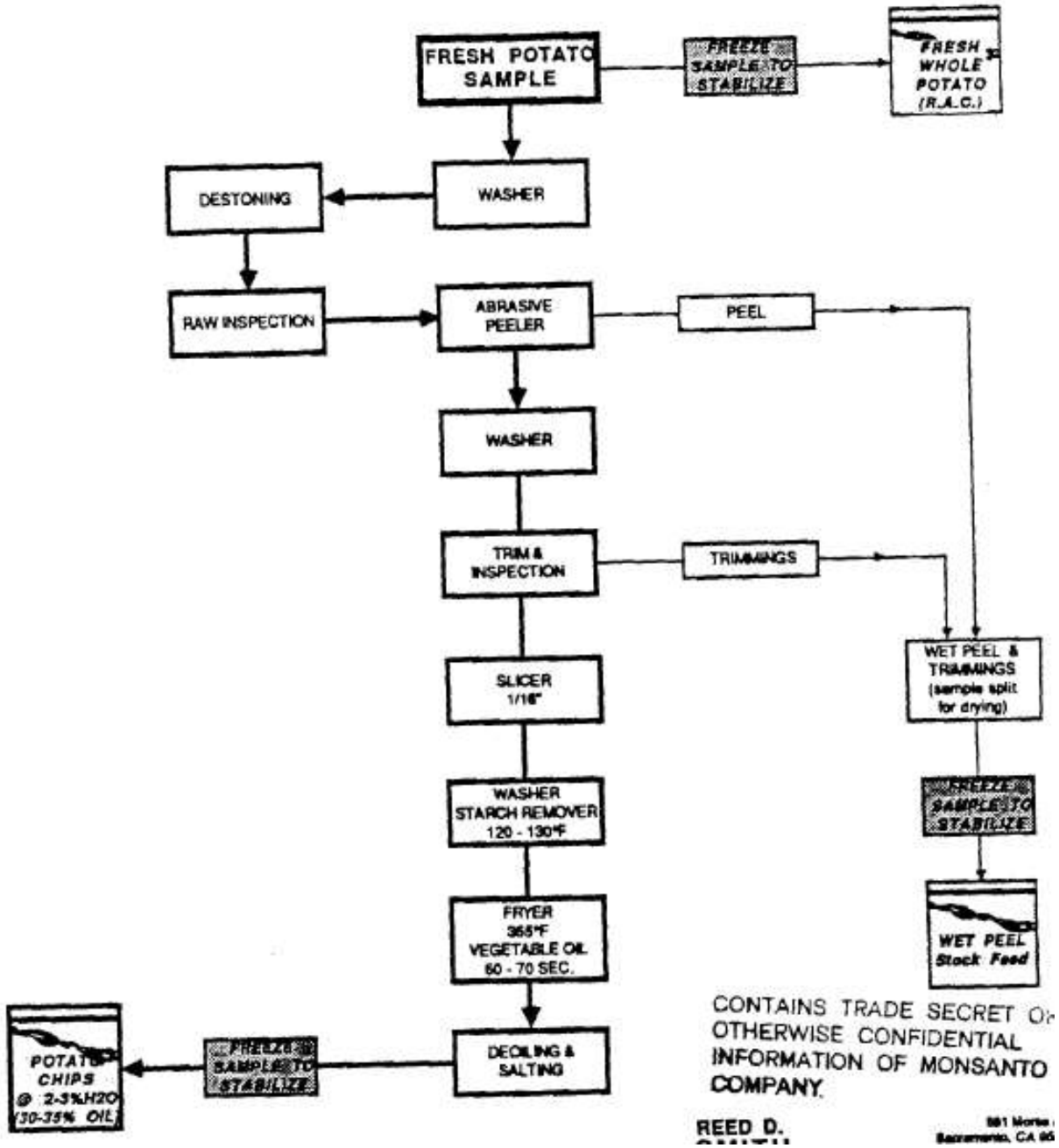


Figure B.7.5.3.2-1: Potato chip processing protocol

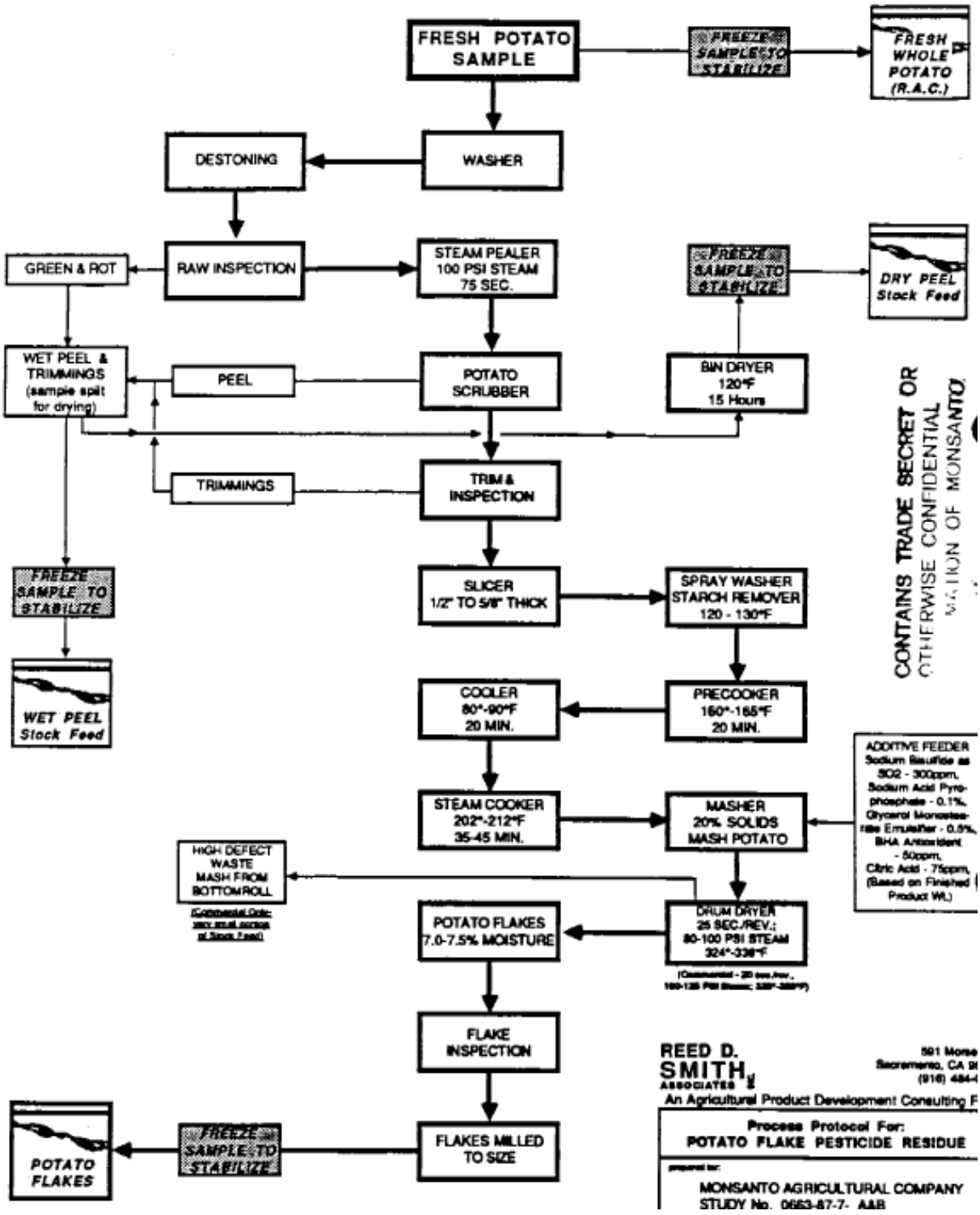


Figure B.7.5.3.2-2: Potato flake processing protocol

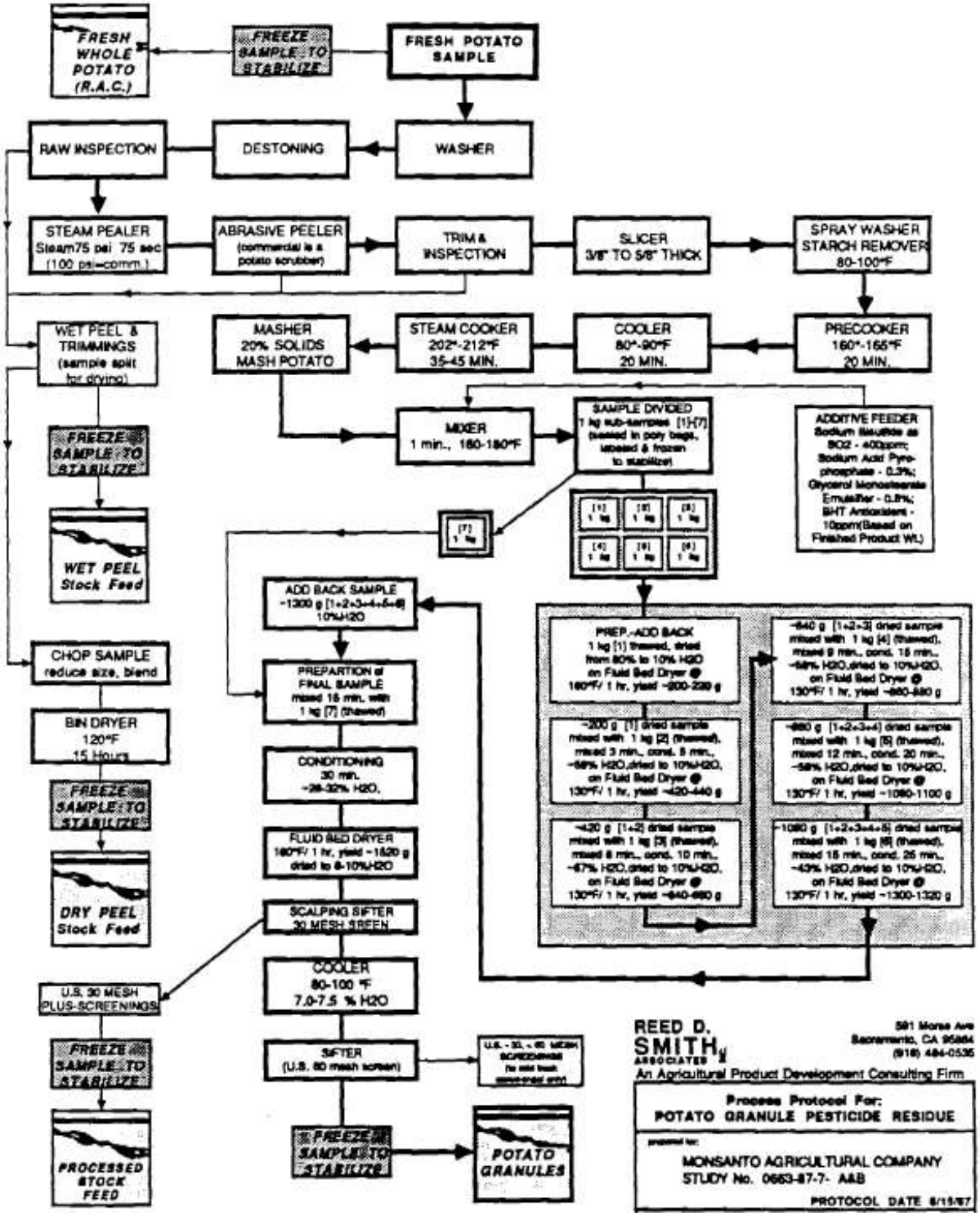


Figure B.7.5.3.2-3: Potato granule processing protocol

For olives, the essential processing product is oil. In the following three studies on the processing of olives to olive oil are presented.

B.7.5.3.3. Study 4

1. Information on the study

Data point:	CA 6.5.3/04
Report author	[redacted]
Report year	1996
Report title	Residues of glyphosate and AMPA in olives and olive oil, following a soil treatment with Roundup® herbicide. Spanish field trials, 1995
Report No	MLL 30469
Document No	95-GLY-20 Sp
Guidelines followed in study	OECD GLP FAO Guidelines
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Testing of Chemicals, 508: • Number of trees sampled were not provided. • The oil samples were stored at room temperature (after processing and until shipment to the analytical facility) and < 5 °C (in the analytical facility). • The study was not conducted at exaggerated application rates.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

The objective of the study was to determine the magnitude of the residues of glyphosate and AMPA in olive (fruit) and the processed fraction olive oil (raw and refined) after one application of Roundup, an SL formulation containing 360 g/L of glyphosate.
The study included 4 field trials with processing in the southern European zone. There was one application to the soil under the olive trees at a target rate of 2.16 kg glyphosate per hectare either 28, 14, or 7 days before commercial harvest (each trial included 3 treated plots, one per pre-harvest interval). Olive samples for oil production were collected at commercial harvest from the soil (ground fallen). The residues of glyphosate in ground fallen olives harvested 7 or 14 days after application ranged from 0.11 mg/kg to 0.93 mg/kg. The residues of glyphosate in ground fallen olives harvested 28 days after application were below the limit of quantification (LOQ) of 0.05 mg/kg. No residues of AMPA above the LOQ were found in ground fallen olives harvested 7, 14, or 28 days after application.
The processing factors for glyphosate and AMPA in all trials were < 1, indicating that there was no concentration of glyphosate or AMPA residue in raw or refined olive oil relative to the raw commodity, whole olive fruit collected from the ground.

The RMS notes that the study report contains trials addressing the magnitude of residues (supervised residue trials) and the magnitude of residues during processing. The former data point is addressed in Section B.7.3.1.15 of this Volume 3, whereas the latter data point will be addressed in this section of Volume 3.

I. Materials and Methods

A. Materials

1. Test material	
Description:	Roundup
Active ingredient(s):	Glyphosate

CAS number:	1071-83-6
Content of a.s. analysed:	31.2 %
Formulation type:	SL

B. Methods

1. Field phase

Four residue trials were conducted on olives (*Olea europaea*) during 1995 in Spain (AP/3065/ME/1, AP/3065/ME/2, AP/3065/ME/3, and AP/3065/ME/4). One pre-harvest application of Roundup (360 g/L glyphosate) was performed onto the soil under the olive trees (6-10 plants per plot) at 6.0 L product/ha (2.16 kg a.s./ha) either 28, 14, or 7 days before harvest. The volume of water used to prepare the spray solution was in the range of 381-440 L/ha. The main application parameters are outlined in the table below.

Table B.7.5.3.3-1: Application information

Trial no.	Application code	Timing	Application rate kg a.s./ha	Water volume L/ha
AP/3065/ME/1	T3	7 days before harvest	2.141	396
	T2	14 days before harvest	2.188	405
	T1	28 days before harvest	2.147	398
AP/3065/ME/2	T3	7 days before harvest	2.341	433
	T2	14 days before harvest	2.374	440
	T1	28 days before harvest	2.160	400
AP/3065/ME/3	T3	7 days before harvest	2.281	422
	T2	14 days before harvest	2.143	397
	T1	28 days before harvest	2.056	381
AP/3065/ME/4	T3	7 days before harvest	2.279	422
	T2	14 days before harvest	2.132	395
	T1	28 days before harvest	2.151	398

Regions, varieties and cultivation were typical for the cultivation of olives. Care was taken that the spray solution was properly homogenised by mixing before application. Ground spray applications were made via plot sprayer according to the label directions. The actual applied amount was calculated by measuring the remaining spray solution after application.

2. Sampling

Specimens of olive were taken by hand from treated and untreated plots at 7, 14, and 28 days after treatment (commercial harvest). Specimens intended for processing were taken from the ground underneath the trees. The stones were not removed. Specimens were stored frozen until processing.

Table B.7.5.3.3-2: Crop sampling information

Trial	Crop	Commodity	DALA[1]	Quantity	Date of sampling
AP/3065/ME/1	Olive	Fruit, from ground (for processing)	7 14 28	≥ 10.0 kg	05.12.1995
AP/3065/ME/2	Olive	Fruit, from ground (for processing)	7 14 28	≥ 10.0 kg	07.12.1995, 08.12.1995
AP/3065/ME/3	Olive	Fruit, from ground (for processing)	7 14 28	≥ 10.0 kg	04.12.1995

Table B.7.5.3.3-2: Crop sampling information

Trial	Crop	Commodity	DALA[1]	Quantity	Date of sampling
AP/3065/ME/4	Olive	Fruit, from ground (for processing)	7 14 28	≥ 10.0 kg	06.12.1995

1 Days after last application

3. Processing

Processing was performed to obtain the processed fractions of raw and refined olive oil. The technology used was a lab-scale process similar to the industrial process. The applicant did not include any processing schemes in its summary, therefore, the RMS included a screenshot from the study report (see at the end of the study summary).
The olives were defrosted and placed in a shallow layer in a grinder. It is not stated in the study report that olives were destoned, i.e. the RMS assumes that olives including stone were processed. In cases where the olives were small or not ripe, they were initially crushed with an electric fruit crusher prior to grinding. Olive pulp was recovered and mixed at 25 to 30 °C. The pulp was recovered in nylon cloths, which were then pressed, and the vegetable water and oil were collected. The water and oil mixture was heated at approx. 30 °C and the raw oil was recovered. A 2.9 M NaOH solution was added to the raw oil and the mixture heated in an oven at 60 to 70 °C. Refined olive oil was decanted off and filtered prior to use. The raw and refined oil were stored at 20 °C until shipment to the analytical facility.

4. Analytical phase

Residue analysis was conducted according to Monsanto method XA001. The residues of glyphosate and AMPA were extracted from the samples by water/dichloromethane partitioning/extraction followed by Chelex 100 resin isolation and anion exchange chromatographic clean-up. Quantification was based on a HPLC post column O-phthalaldehyde reaction system and comparison of peak area/height with known standards. For glyphosate and AMPA in olives (fruit) and olive oil, the limit of quantitation (LOQ) was 0.05 mg/kg with a limit of detection (LOD) of 0.02 mg/kg each.
Treated and untreated RAC specimens were maintained deep frozen until analysis. Raw and refined olive oil were stored at room temperature after processing until they were shipped to the analytical facility, where they were stored in cold storage (< 5 °C) until analysis. The maximum sample storage interval from harvest to extraction was 207 days.
During analysis of olive (fruit) specimens, fortification experiments were performed with glyphosate and AMPA at fortification levels of 0.05, 0.1, 0.5, and 1.0 mg/kg, with additional fortifications at 10 and 20 mg/kg for glyphosate alone. Concurrent recoveries for glyphosate and AMPA in olive oil were determined at fortification levels of 0.05 and 0.1 mg/kg. The results are summarised in the table below.

Table B.7.5.3.3-3: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)[2]	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Olives, fruit	Glyphosate	0.05	63-110	86	21	24	5
		0.1	100-109	105	3.6	3.4	5
		0.5	94-100	98	2.3	2.3	5
		1.0	97-108	103	4.2	4.1	6
		10	79	-	-	-	1
		20	85	-	-	-	1
		Overall	**63–110**	**97**	**12**	**13**	**23**
	AMPA	0.05	67-90	74	8.2	11	7
		0.1	61-96	74	11	15	8
		0.5	77-80	79	1.7	2.2	4
		1.0	82	-	-	-	1
		Overall	**61–96**	**76**	**8.4**	**11**	**20**

Table B.7.5.3.3-3: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)[2]	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Olive, oil	Glyphosate	0.05	71-91	81	7.2	8.9	5
		0.1	68-99	87	14	16	5
		Overall	**68-99**	**84**	**11**	**13**	**10**
	AMPA	0.05	68-101	83	14	17	5
		0.1	64-106	80	18	23	6
		Overall	**64-106**	**81**	**16**	**20**	**11**

1 Residues of glyphosate and AMPA in blank matrix were below the limit of detection (< 0.02 mg/kg).
2 Mean and standard deviation values at each individual fortification level, as well as all relative standard deviation values, were calculated for this summary.

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA in processed commodities after usage of Roundup when applied as per the study. Residues of glyphosate in ground fallen olives harvested 7, 14 or 28 days after application ranged from 0.11 mg/kg to 0.93 mg/kg. Residues of glyphosate in ground fallen olives harvested 28 days after application were below the LOQ of 0.05 mg/kg. No residues of AMPA above the LOQ were found in ground fallen olives harvested 7, 14, or 28 days after application. In raw and refined olive oil, no residues of glyphosate were found above the LOQ and no residues of AMPA were found above the LOD. Detailed residue levels are shown in the table below.

Table B.7.5.3.3-4: Residues of glyphosate and AMPA in olive processed fractions

Trial No. / Location / EU zone / Year	Crop/ Variety	Application rate kg a.s./ha	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues (mg/kg)	PFs	Residues (mg/kg)	PFs
AP/3065/ME/1 / [redacted], Malaga, Spain / SEU / 1995	Olive / Hoji-blanca	2.141	7	Fruit	0.14	-	<0.05	-
				Oil, raw	<0.05	<0.36	<0.05	-
				Oil, refined	<0.05	<0.36	<0.05	-
		2.188	14	Fruit	0.12	-	<0.05	-
				Oil, raw	<0.05	<0.42	<0.05	-
				Oil, refined	<0.05	<0.42	<0.05	-
		2.147	28	Fruit	<0.05	-	<0.05	-
				Oil, raw	<0.05	-	<0.05	-
				Oil, refined	<0.05	-	<0.05	-
AP/3065/ME/2 / [redacted], Cordoba, Spain / SEU /1995	Olive / Picual	2.341	7	Fruit	0.11	-	<0.05	-
				Oil, raw	<0.05	<0.45	<0.05	-
				Oil, refined	<0.05	<0.45	<0.05	-
		2.374	14	Fruit	0.11	-	<0.05	-
				Oil, raw	<0.05	<0.45	<0.05	-
				Oil, refined	<0.05	<0.45	<0.05	-
		2.160	28	Fruit	<0.05	-	<0.05	-
				Oil, raw	<0.05	-	<0.05	-
				Oil, refined	<0.05	-	<0.05	-

Table B.7.5.3.3-4: Residues of glyphosate and AMPA in olive processed fractions

Trial No. / Location / EU zone / Year	Crop/ Variety	Application rate kg a.s./ha	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues (mg/kg)	PFs	Residues (mg/kg)	PFs
AP/3065/ME/3 / [redacted] Cordoba, Spain / SEU /1995	Olive / Picual	2.281	7	Fruit	0.53	-	<0.05	-
				Oil, raw	<0.05	<0.09	<0.05	-
				Oil, refined	<0.05	<0.09	<0.05	-
		2.143	14	Fruit	0.13	-	<0.05	-
				Oil, raw	<0.05	<0.38	<0.05	-
				Oil, refined	<0.05	<0.38	<0.05	-
		2.056	28	Fruit	<0.05	-	<0.05	-
				Oil, raw	<0.05	-	<0.05	-
				Oil, refined	<0.05	-	<0.05	-
AP/3065/ME/4 / [redacted], Jaen, Spain / SEU /1995	Olive / Picual	2.279	7	Fruit	0.93	-	<0.05	-
				Oil, raw	<0.05	<0.05	<0.05	-
				Oil, refined	<0.05	<0.05	<0.05	-
		2.132	14	Fruit	0.93	-	<0.05	-
				Oil, raw	<0.05	<0.05	<0.05	-
				Oil, refined	<0.05	<0.05	<0.05	-
		2.151	28	Fruit	<0.05	-	<0.05	-
				Oil, raw	<0.05	-	<0.05	-
				Oil, refined	<0.05	-	<0.05	-

1 Days after last application
2 The processing factor is calculated by dividing the residue in the processed fraction by the residue in the RAC sample

III. Conclusion

The calculated processing factors of glyphosate for raw oil and refined oil were <1. Glyphosate does not concentrate in matrices destined for human consumption. For AMPA no residues above the LOQ were present in the raw agricultural commodity. Therefore, a calculation of processing factors was not possible.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study was previously evaluated at EU level. It was performed under GLP and is considered to be reliable and acceptable. Even though the sample sizes and the number of sampled trees were not specified, the sample sizes for processing were above 10 kg per sample. The oil samples were stored at temperatures <5 °C. Nevertheless processing factors could be derived. These can be regarded as reliable as it is not expected that residues of glyphosate will be found in high amounts in the oil due to the low log P_{ow} (-2.47). The study was not conducted at exaggerated application rates but residues of glyphosate were found in the RAC 7 and 14 days after the application so that processing factors could be derived for olive oil. Therefore, the study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 508. It adequately supports the representative processing processes for glyphosate and AMPA in olives.

The use of glyphosate in olives for oil production with harvesting of olives from treated soil is not supported in the dossier. Therefore, the study is not directly relevant to the representative uses and is considered as supportive.

Assessment and conclusion by RMS:

In the current study, the magnitude of residues during processing of olives was investigated in four trials following soil-directed application of Roundup, an SL formulation containing 360 g/L of glyphosate. Per trial, three plots were installed which differed in terms of PHI (7, 14, and 28 days), i.e. the three plots are not considered independent.

Specimens of olive fruits and oil were stored in accordance with the demonstrated period of storage stability for glyphosate (18 months in all plant commodities except dry matrices). In contrast, AMPA was shown to be stable in soybean seeds only and data are not sufficient to allow an extrapolation to all high oil content matrices or to all plant commodities. Therefore, the results for AMPA are pending an additional storage stability study. It is noted, however, that no processing factors are derived for AMPA since residue levels were below the LOQ in the RAC. Considering that storage stability was not adequately addressed for AMPA in olive/oil matrices, it is not possible to state whether residues were below the LOQ due to the fact that there were indeed no residues in/on the fruit or whether potential residues degraded during storage.

The applicant did not include any processing schemes in its summary, therefore, the RMS included screenshots from the study report (see below).

The analytical method was not fully validated for glyphosate and AMPA in olive fruits since recoveries were below 70% (see Vol. 3, B.5). In the frame of this processing study, however, mean concurrent/procedural recoveries were largely within the acceptable ranges. The only exception regarding procedural recoveries is the relative standard deviation determined for olive fruits spiked with glyphosate at 0.05 mg/kg (24%), i.e. the relative standard deviation is above the acceptable limit of 20%. Consequently, residue levels in olives might be less precise in case levels are around 0.05 mg/kg. In the current study, however, processing factors were only derived from trials where residue levels of glyphosate were clearly above the LOQ. Therefore, the deviation is accepted. Overall, the performance of the analytical method was sufficiently demonstrated and the method is therefore considered fit for purpose. It is noted, however, that additional information regarding the extraction efficiency is needed for confirmation.

No residues were detected in the control specimens. Residues of AMPA were below the LOQ in all samples; therefore no processing factors could be derived for AMPA. Residues of glyphosate were above the LOQ at PHIs of 7 and 14 days, but not at a PHI of 28 days. Therefore, processing factors can be calculated based on residue levels determined 7 and 14 days after application. It is debatable whether the highest processing factor or a mean processing factor should be selected per trial, but the RMS considers it adequate to average the processing factors per trial and subsequently calculate a median processing factor based on the different independent trials.

The average processing factors for raw olive oil from trial AP/3065/ME/1, AP/3065/ME/2, AP/3065/ME/3, and AP/3065/ME/4 are <0.39, <0.45, <0.24, and <0.05, respectively. Based on these processing factors, the median processing factor for glyphosate calculated for raw olive oil based on this study is <0.32.

The average processing factors for refined olive oil from trial AP/3065/ME/1, AP/3065/ME/2, AP/3065/ME/3, and AP/3065/ME/4 are <0.39, <0.45, <0.24, and <0.05, respectively. Based on these processing factors, the median processing factor for glyphosate calculated for refined olive oil based on this study is <0.32.

It is noted that the median processing factor is based on this study only. Additional studies investigating the processing of olive fruits were submitted and an overall median processing factor considering all available data will be calculated in Volume 1.

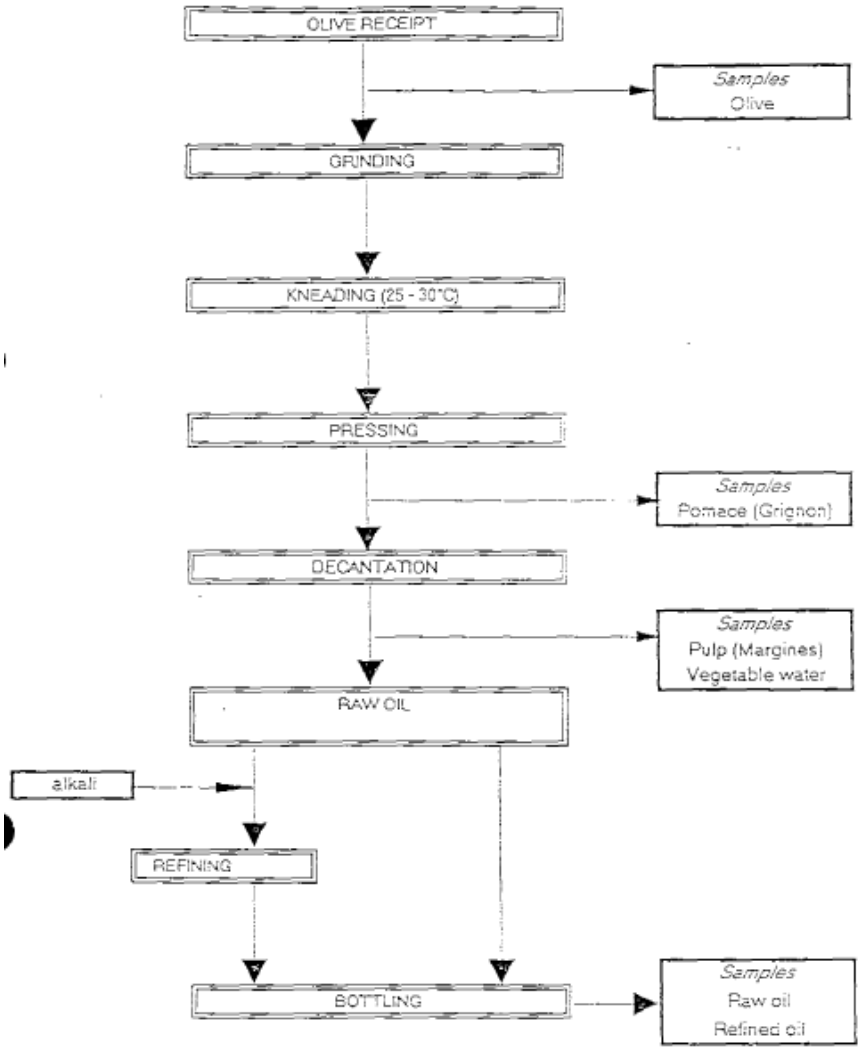


Figure B.7.5.3.3-1: Olive oil (raw and refined) processing protocol

B.7.5.3.4. Study 5

1. Information on the study

Data point:	CA 6.5.3/005
Report author	[redacted]
Report year	1993
Report title	Residues of glyphosate and AMPA in olives and olive oil, following a soil treatment with MON 65040 herbicide. Italian field trials, 1993
Report No	MLL 30319
Document No	93-GLY-01
Guidelines followed in study	No test guidelines cited in the report
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Testing of Chemicals, 508: • Storage conditions (temperature) of oil samples are unknown. • The study was not conducted at exaggerated application rates.
Previous evaluation	Yes, accepted in RAR (2015)

GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

The objective of the study was to determine the magnitude of the residues of glyphosate and AMPA in olive (fruit) and processed fraction olive oil after one application of MON 65040, an SL formulation containing 360 g/L of glyphosate and 20 g/L oxyfluorfen. No further analysis with regard to oxyfluorfen was conducted in the frame of the study.
The study included 2 field trials in the southern zone. There was one application to the soil under the olive trees at a target rate of 1.44 kg glyphosate per hectare. Olive samples were collected 7 and 14 days after the application from the soil (ground fallen). Residues of glyphosate in ground fallen olives harvested 7 or 14 days after application ranged from <0.05 mg/kg to 0.4 mg/kg. No residues of AMPA above the LOQ were found in ground fallen olives harvested 7 or 14 days after application.
The processing factors for glyphosate and AMPA in all trials were ≤ 1, indicating that there was no concentration of glyphosate or AMPA residue in raw or refined olive oil relative to the raw commodity, whole olive fruit collected from the ground.

I. Materials and Methods

A. Materials

1. Test material	
Description:	MON 65040
Active ingredient(s):	Glyphosate // oxyfluorfen
CAS number:	1071-83-6 // 42874-03-3
Content of a.s. nominal:	360 g/L // 20 g/L
Content of a.s. analysed:	Not provided
Formulation type:	SL

B. Methods

1. Field phase

Two residue trials were conducted on olives (outdoor) during the 1993 season in Italy ([redacted] Puglia; [redacted], Toscana). One application of MON 65040 (360 g/L glyphosate) was performed onto the soil under the olive trees (6-14 plants per plot) at 4.0 L product/ha (1.44 kg a.s./ha). The volume of water used to prepare the spray solution was in the range of 200-300 L/ha. The main application parameters are outlined in the table below.

Table B.7.5.3.4-1: Application information

Trial no.	Application code	Timing	Application rate kg a.s./ha	Water volume L/ha
[redacted], Puglia	T	6 days before harvest	1.44	200
[redacted], Toscana	T	7 days before harvest	1.44	300

Regions, varieties and cultivation were typical for the cultivation of olives. Care was taken that the spray solution was properly homogenised by mixing before application. Ground spray applications were made via knapsack sprayer with fan nozzles according to the label directions.

2. Sampling

Specimens of olive were taken by hand from treated and untreated plots on the day of application, at 6-7 and at 13-14 days after treatment. Specimens were collected from the ground underneath the tree. For the trial located in

[redacted] it is explicitly stated that stones were removed during sampling, whereas no statement with regard to the removal of the stone is given for the trial located in [redacted].
All samples were frozen within 24 hours from collection. Large samples for oil extraction were collected in double polyethylene bags and maintained at room temperature until analysis.

Table B.7.5.3.4-2: Crop sampling information

Trial	Crop	Commodity	DALA[1]	Quantity	Date of sampling
[redacted], Puglia	Olive	Fruit, from ground (for processing)	6 13	2.0 kg + 20 kg for processing	19.01.93 27.01.93
[redacted], Toscana	Olive	Fruit, from ground (for processing)	7 14	2.0 kg + 5.0 kg for processing	11.12.92 13.12.92

1 Days after last application

3. Processing

The olives were processed in raw olive oil. The technology used was a lab-scale process similar to the industrial process. After washing and shaking to remove excess of water, olives were thoroughly mashed with a laboratory size olive grindstone mill. In one trial ([redacted], Puglia; olives without stone), a quantity of NaCl equivalent to about the 10 % of the milled mass was added and accurately mixed to facilitate separation of liquid fraction from solids. The liquid fraction, an instable emulsion of oil and fruit water, was centrifuged to separate the oil.
As stated before, it is not known whether stoned or destoned olives were processed from the trial in [redacted].

4. Analytical phase

Residue analysis was conducted according to Monsanto method XA001. The residues of glyphosate and AMPA were extracted from the samples by water/dichloromethane partitioning/extraction followed by Chelex 100 resin isolation and anion exchange chromatographic clean-up. Quantification was based on a HPLC post column O-phthalaldehyde reaction system and comparison of peak area/height with known standards. Olives have been washed, the soil removed, and destoned before analysis. For glyphosate and AMPA in olives (fruit) and olive oil, the limit of quantitation (LOQ) was 0.05 mg/kg with a limit of detection (LOD) of 0.02 mg/kg each.
Treated and untreated olive specimens were maintained deep frozen and adequately separated during storage. Olives intended for processing were kept at room temperature, but were processed within 48 hours after harvest. Storage conditions of raw oil samples are unknown. The maximum sample storage interval from harvest to analysis was 171 days.
During analysis of olive (fruit) specimens, fortification experiments were performed with glyphosate and AMPA at fortification levels of 0.05, 0.1 and 0.5 mg/kg, with additional fortifications at 1.0 mg/kg for glyphosate alone. Concurrent recoveries for glyphosate and AMPA in olive oil were determined at fortification levels of 0.05 and 0.1 mg/kg. The results are summarised in the table below.

Table B.7.5.3.4-3: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Olives, fruit	Glyphosate	0.05	68-84	76	-	-	2
		0.1	77-93	85	-	-	2
		0.5	79	-	-	-	1
		1	77	-	-	-	1
		Overall	**68-93**	**80**	**8**	**10**	**6**
	AMPA	0.05	54-58	56	-	-	2
		0.1	71	71	-	-	1
		0.2	53	53	-	-	1
		Overall	**53-71**	**59**	**8**	**14**	**4**

Table B.7.5.3.4-3: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Olive, oil	Glyphosate	0.05	90	90	-	-	1
		0.1	95	95	-	-	1
		Overall	**90-95**	**93**	**-**	**-**	**2**
	AMPA	0.05	76	76	-	-	1
		0.1	80	80	-	-	1
		Overall	**76-80**	**78**	**-**	**-**	**2**

1 Residues of glyphosate and AMPA in blank matrix were below the limit of detection (< 0.02 mg/kg).
2 Mean and standard deviation values at each individual fortification level, as well as all relative standard deviation values, were calculated for this summary.

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA in processed commodities after usage of MON 65040 when applied as per the study.
Residues of glyphosate in ground fallen olives harvested 7 or 14 days after application ranged from <0.05 mg/kg to 0.43 mg/kg. No residues of AMPA were found above the LOQ in ground fallen olives harvested 7 or 14 days after application. Residues of glyphosate and AMPA in the unrefined oil were always below the LOQ of 0.05 mg/kg. Detailed residue levels are shown in the table below.

Table B.7.5.3.4-4: Residues of glyphosate and AMPA in olive processed fractions

Location / EU zone / Year	Crop/ Variety	Application rate kg a.s./ha	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found (mg/kg)	Processing factor[2]	Residues found (mg/kg)	Processing factor[2]
[redacted], Puglia, Italy / SEU /1993	Olive / Nardo	1.44	6	Fruit	0.54	-	<0.05	-
				Oil, unrefined	<0.05	<0.09	<0.05	-
		1.44	13	Fruit	0.35	-	<0.05	-
				Oil, unrefined	<0.05	<0.14	<0.05	-
[redacted], Toscana, Italy / SEU /1993	Olive / Leccino	1.44	7	Fruit	<0.05	-	<0.05	-
				Oil, unrefined	<0.05	-	<0.05	-
		1.44	14	Fruit	<0.05	-	<0.05	-
				Oil, unrefined	<0.05	-	<0.05	-

1 Days after last application
2 The processing factor is calculated by dividing the residue in the processed fraction by the residue in the RAC sample

III. Conclusion

The calculated processing factors of glyphosate for unrefined oil was <1 (based on one trial only). For AMPA no residues above the LOQ were present in the raw agricultural commodity. Therefore, a calculation of processing factors was not possible.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study was previously evaluated at EU level. It was performed under GLP and is considered to be reliable and acceptable. The oil samples were stored at room temperature. Nevertheless processing factors could be derived. These can be regarded as reliable as it is not expected that residues of glyphosate will be found in high amounts in the oil due to the low log P_{ow} (-2.47). The study was not conducted at exaggerated application rates but in one trial residues of glyphosate were found in the RAC 6 and 13 days after the application so that processing factors could be derived for olive oil. Therefore, the study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 508. It adequately supports the representative processing processes for glyphosate and AMPA in olives.

The use of glyphosate in olives for oil production with harvesting of olives from treated soil is not supported in the dossier. Therefore, the study is not directly relevant to the representative uses and is considered as supportive.

Assessment and conclusion by RMS:
In the current study, the magnitude of residues during processing of olives was investigated in two trials following soil-directed application of Roundup, an SL formulation containing 360 g/L of glyphosate and 20 g/L oxyfluorfen. No further analysis with regard to oxyfluorfen was conducted in the frame of the study. Ground-picked olives were sampled and processed to raw olive oil. A processing scheme was not included in the study report, but the processing steps are adequately described in the study report and in the summary above.
The analytical method was not fully validated for glyphosate and AMPA in olive fruits since recoveries were below 70% (see Vol. 3, B.5). In the frame of this processing study, however, mean concurrent/procedural recoveries were largely within the acceptable ranges. The only exception regarding procedural recoveries is the concurrent/procedural recovery of AMPA in olives which was below 70%. Since no processing factors are derived for AMPA, the impact on the study outcome is considered minor. Overall, the performance of the analytical method was sufficiently demonstrated and the method is therefore considered fit for purpose. It is noted, however, that additional information regarding the extraction efficiency is needed for confirmation.
Samples of olives were stored frozen until analysis. Storage conditions for olive oil in terms of temperatures, in contrast, were not reported. The impact on the study outcome is considered minor though. Specimens of olive fruits and oil were stored in accordance with the demonstrated period of storage stability for glyphosate (18 months in all plant commodities except dry matrices).
In contrast, AMPA was shown to be stable in soybean seeds only and data are not sufficient to allow an extrapolation to all high oil content matrices or to all plant commodities. Therefore, the results for AMPA are pending an additional storage stability study. It is noted that no processing factors are derived for AMPA since residue levels were below the LOQ in the RAC. Considering that storage stability was not adequately addressed for AMPA in olive/oil matrices, it is not possible to state whether residues were below the LOQ due to the fact that there were indeed no residues in/on the fruit or whether potential residues degraded during storage.
With regard to the results, the RMS notes the following: in the original summary of the study, residue levels corrected for recovery were shown. Also the study report only mentions corrected residue levels and no raw data are available. However, based on the corrected residue level and the corresponding procedural recovery, the RMS was able to calculate the uncorrected residue levels. Since all procedural recoveries were below 100%, it is also possible to conclude that corrected residue levels <0.05 mg/kg are also below the LOQ when residue levels would be uncorrected. In addition to this, the RMS notices that, based on the study report, it seems that three analytical replicates were measured whereas the applicant only included a single replicate in the original study summary. This was also accounted for by the RMS and values were amended accordingly.
No residues were detected in the control specimens. Residues of AMPA were below the LOQ in all samples; therefore no processing factors could be derived for AMPA. Residues of glyphosate were above the LOQ in the trial located in [redacted] but not in the trial located in [redacted] Therefore, processing factors are derived from one trial only. It is noted that two processing factors can be derived from the trial conducted in [redacted], however, the only difference would be that samples were taken at different PHIs. Since this is not considered an independent trial set-up, the RMS calculated a single processing factor only which is based on the mean processing factor of this trial.

The following processing factor for glyphosate was obtained for unrefined olive oil:
<0.12

It is noted that a median processing factor cannot be calculated based on this study only. Additional studies investigating the processing of olive fruits were submitted and an overall median processing factor considering all available data will be calculated in Volume 1.

B.7.5.3.5. Study 6

1. Information on the study

Data point:	CA 6.5.3/06
Report author	[redacted]
Report year	1992
Report title	Residues of glyphosate/AMPA in olives and olive oil following use of Sting SE - Spanish field trials 1990/1992
Report No	MLL 30297

Document No	90-GLY-02/92-GLY-01 SP
Guidelines followed in study	No test guidelines cited in the report
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Testing of Chemicals, 508: • Sample quantity and number of trees sampled were not provided. • No information provided regarding the storage conditions (temperature) of olive oil. • The study was not conducted at exaggerated application rates. • The mean concurrent recovery for AMPA in olive fruit was below 70 %.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

The objective of the study was to determine the magnitude of the residues of glyphosate and AMPA in olive (fruit) and processed fraction olive oil after one application of Sting SE, also referred to as MON 20072 or MON 14477, a formulation containing 120 g/L of glyphosate.
The study included 6 field trials in the southern zone. There was one application to the soil under the olive trees at a target rate of 0.36 kg glyphosate per hectare. Olive samples were collected 1 and 7 days after the application from the soil (ground fallen) in two trials and 0 and 24-41 days after the application in the four remaining trials. Residues of glyphosate in ground fallen olives harvested 0-1 days after application ranged from 0.08 mg/kg to 1.8 mg/kg and harvested 7-41 days after the application from 0.4 mg/kg to 2.0 mg/kg. No residues of AMPA above the LOQ were found in ground fallen olives harvested 0-41 days after application.
The processing factors for glyphosate and AMPA in all trials were ≤ 1, indicating that there was no concentration of glyphosate or AMPA residue in raw or refined olive oil relative to the raw commodity (whole olive fruit collected from the ground).

I. Materials and Methods

A. Materials

1. Test material	
Description:	Sting SE, also referred to as MON 20072 or MON 14477
Active ingredient(s):	Glyphosate
CAS number:	1071-83-6
Content of a.s. nominal:	120 g/L
Content of a.s. analysed:	Not provided
Formulation type:	SL

B. Methods

1. Field phase

Six residue trials were conducted on olives (*Olea europaea*) during 1990-1992 in Spain. One application of Sting SE (120 g/L glyphosate) was performed onto the soil under the olive trees at 3.0 L product/ha (0.36 kg a.s./ha). The volume of water used to prepare the spray solution was 200 L/ha. The main application parameters are outlined in the table below.

Table B.7.5.3.5-1: Application information

Trial no.	Application code	Timing	Application rate kg a.s./ha	Water volume L/ha
ES- [redacted], (Servilla)	T	01.02.90	0.36	200
ES- [redacted], (Servilla)	T	01.02.90	0.36	200
ES- [redacted], (Jaén)	T	13.12.91	0.36	200
ES- [redacted], (Málaga)	T	20.01.92	0.36	200
ES- [redacted], (Jaén)	T	15.01.92	0.36	200
ES- [redacted], (Córdoba)	T	15.01.92	0.36	200

Regions, varieties and cultivation were typical for the cultivation of olives. Care was taken that the spray solution was properly homogenised by mixing before application. Ground spray applications were made via knapsack sprayer with fan nozzles according to the label directions.

2. Sampling

Olive samples were collected 1 and 7 days after the application from the soil (ground fallen) in two trials and 0 and 24-41 days after the application in the four remaining trials. All samples were frozen within 24 hours from collection. Large samples for oil extraction were collected in double polyethylene bags and maintained at room temperature.

Table B.7.5.3.5-2: Crop sampling information

Trial	Crop	Commodity	DALA	Quantity	Date of sampling
ES - [redacted] (Servilla)	Olive	Fruit, from ground (for processing)	1 7	Not provided	02.02.90 08.02.90
ES - [redacted], (Servilla)	Olive	Fruit, from ground (for processing)	1 7	Not provided	02.02.90 08.02.90
ES - [redacted] (Jaén)	Olive	Fruit, from ground (for processing)	0 32	Not provided	13.12.91 14.01.92
ES - [redacted] (Málaga)	Olive	Fruit, from ground (for processing)	0 24	Not provided	20.01.92 13.02.92
ES- [redacted] (Jaén)	Olive	Fruit, from ground (for processing)	0 30	Not provided	15.01.92 14.02.92
ES - [redacted], (Córdoba)	Olive	Fruit, from ground (for processing)	0 41	Not provided	15.01.92 25.02.92

1 Days after last application

3. Processing

Processing was performed to obtain the processed fractions of raw olive oil. The technology used was a lab-scale process similar to the industrial process. Since the applicant did not include a processing scheme in this summary, the RMS copied a screenshot from the study report at the end of this summary. After washing, olives are mixed with a blender for 2 minutes. The so obtained paste was stirred for 20 min in a water bath at 50 °C, after adding water at 80 °C. After another addition of water at 80 °C , the stirring is continued for 10 minutes. The mixture is centrifuged for 10 min at 9000 rpm, to separate oil and water from solid residues.

4. Analytical phase

Residue analysis was conducted according to Monsanto method XA001. The residues of glyphosate and AMPA were extracted from the samples by water/dichloromethane partitioning/extraction followed by Chelex 100 resin isolation and anion exchange chromatographic clean-up. Quantification was based on a HPLC post column O-phthalaldehyde reaction system and comparison of peak area/height with known standards. For glyphosate and AMPA in olives (fruit) and olive oil, the limit of quantitation (LOQ) was 0.05 mg/kg with a limit of detection (LOD) of 0.02 mg/kg each. It is noted that residues of AMPA were not determined in the two trials conducted in 1990.

Treated and untreated specimens were maintained deep frozen and adequately separated during storage and shipment. The maximum sample storage interval from harvest to extraction was 171 days. No information regarding the storage of olive oil samples is available in the study report.
During analysis of olive (fruit) specimens, fortification experiments were performed with glyphosate and AMPA at fortification levels of 0.05, 0.1, 0.5, and 1.0 mg/kg, with additional fortifications at 5 mg/kg for glyphosate alone. Concurrent recoveries for glyphosate and AMPA in olive oil were determined at fortification levels of 0.05 and 0.1 mg/kg. The overall mean recovery value for each analyte and matrix was in the acceptable range of 70-110 % and RSDs were below 20 %. Only for AMPA in olive fruit the mean recovery was 61 %. The results are summarised in the table below.

Table B.7.5.3.5-3: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)	Standard deviation (%)[2]	Relative standard deviation (%)[2]	Number analyses (n)
Olives, fruit	Glyphosate	0.05	63-87	74	11	15	5
		0.1	62-104	76	15	20	6
		0.2	71	71	-	-	1
		0.5	60-70	65	4.8	7.3	4
		1	61-98	83	16	20	4
		5	70	70	-	-	1
		Overall	**60-104**	**74**	**13**	**17**	**21**
	AMPA	0.05	53-70	60	8.7	14	3
		0.1	57-76	67	9.6	14	3
		0.2	55-61	58	3.2	5.5	4
		0.5	68	68	-	-	1
		1	53	53	-	-	1
		Overall	**53-76**	**61**	**7.6**	**12**	**12**
Olive, oil	Glyphosate	0.05	66-96	81	10	12	7
		0.1	66-97	78	12	15	7
		Overall	**66-97**	**80**	**11**	**13**	**14**
	AMPA	0.05	57-95	72	12	16	7
		0.1	55-75	68	7.4	11	7
		Overall	**55-95**	**70**	**9.6**	**14**	**14**

1 Residues of glyphosate and AMPA in blank matrix were below the limit of detection (< 0.02 mg/kg).
2 Mean and standard deviation values at each individual fortification level, as well as all relative standard deviation values, were calculated for this summary.

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA in processed commodities after usage of MON 65040 when applied as per the study.

Table B.7.5.3.5-4: Residues of glyphosate and AMPA in olive processed fractions

Trial No. / Location / EU zone / Year	Crop/ Variety	Application rate kg a.s./ha	DALA[1] (days)	Processed commodity	Glyphosate		AMPA	
					Residues found (mg/kg)	Processing factor[2]	Residues found (mg/kg)	Processing factor[2]
ES - [redacted], (Sevilla) / SEU / 1990	Olive / Hojiblanca	0.36	1	Fruit	1.1	-	n.a.	-
				Oil, unrefined	<0.05	<0.05	n.a.	-
		0.36	7	Fruit	1.4	-	n.a.	-
				Oil, unrefined	<0.05	<0.04	n.a.	-
ES - [redacted] (Sevilla) / SEU / 1990	Olive / Lechin	0.36	1	Fruit	1.4	-	n.a.	-
				Oil, unrefined	<0.05	<0.04	n.a.	-
		0.36	7	Fruit	1.5	-	n.a.	-
				Oil, unrefined	<0.05	<0.03	n.a.	-
ES - [redacted], (Jaén) / SEU / 1991-1992	Olive / Picual	0.36	0	Fruit	2.6	-	<0.05	-
				Oil, unrefined	<0.05	<0.02	<0.05	-
		0.36	32	Fruit	1.2	-	<0.05	-
				Oil, unrefined	<0.05	<0.04	<0.05	-
ES - [redacted] (Málaga) / SEU / 1992	Olive / Hojiblanca	0.36	0	Fruit	0.4	-	<0.05	-
				Oil, unrefined	<0.05	<0.13	<0.05	-
		0.36	24	Fruit	0.6	-	<0.05	-
				Oil, unrefined	<0.05	<0.08	<0.05	-
ES - [redacted], (Jaén) / SEU / 1992	Olive / Picual	0.36	0	Fruit	0.3	-	<0.05	-
				Oil, unrefined	<0.05	<0.17	<0.05	-
		0.36	30	Fruit	2.9	-	<0.05	-
				Oil, unrefined	<0.05	<0.02	<0.05	-
ES - [redacted], (Córdoba) / SEU / 1993	Olive / Picual	0.36	0	Fruit	0.1	-	<0.05	-
				Oil, unrefined	<0.05	<0.50	<0.05	-
		0.36	41	Fruit	0.6	-	<0.05	-
				Oil, unrefined	<0.05	<0.08	<0.05	-

1 Days after last application
2 The processing factor is calculated by dividing the residue in the processed fraction by the residue in the RAC sample
n.a. Residue levels of AMPA were not determined.

III. Conclusion

The mean calculated processing factors of glyphosate for unrefined oil was ≤ 1. Glyphosate does not concentrate in matrices destined for human consumption. For AMPA no residues above the LOD were present in the raw agricultural commodity. Therefore, a calculation of processing factors was not possible.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study was previously evaluated at EU level. It was performed under GLP and is considered to be reliable and acceptable. The oil samples were stored at room temperature. Nevertheless processing factors could be derived. These can be regarded as reliable as it is not expected that residues of glyphosate will be found in high amounts in the oil due to the low log P_{ow} (-2.47). The study was not conducted at exaggerated application rates but in one trial residues of glyphosate were found in the RAC 6 and 13 days after the application so that processing factors could be derived for olive oil. Therefore, the study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 508. It adequately supports the representative processing processes for glyphosate and AMPA in olives.

The use of glyphosate in olives for oil production with harvesting of olives from treated soil is not supported in the dossier. Therefore, the study is not directly relevant to the representative uses and is considered as supportive.

Summary of olive processing factors

Source: DocID (trial reference)	**Olive oil, unrefined**	**Olive oil, refined**
MLL 30469 AP/3065/ME/1	<0.14 <0.17	<0.14 <0.17
MLL 30469 AP/3065/ME/2	<0.18 <0.18	<0.18 <0.18
MLL 30469 AP/3065/ME/3	<0.09 <0.15	<0.04 <0.38
MLL 30469 AP/3065/ME/4	<0.02 <0.02	<0.02 <0.05
MLL 30319 [redacted], Puglia	<0.17 <0.08	-
MLL 30297 ES-[redacted], (Servilla)	<0.06 <0.05	-
MLL 30297 ES-[redacted], (Servilla)	<0.05 <0.04	-
MLL 30297 ES-[redacted], (Jaén)	<0.03 <0.06	-
MLL 30297 ES-[redacted], (Málaga)	<0.17 <0.13	-
MLL 30297 ES-[redacted], (Jaén)	<0.25 <0.03	-
MLL 30297 ES-[redacted], (Córdoba)	<0.63 <0.13	-
Mean	**<0.13**	**<0.15**
Median	**<0.11**	**<0.15**

Assessment and conclusion by RMS:

In the current study, the magnitude of residues during processing of olives was investigated in six trials following soil-directed application of Sting SE, an SE formulation containing 120 g/L of glyphosate. After treatment at 360 g/ha, ground-picked olives were sampled and processed to unrefined olive oil. Olive samples were collected 1 and 7 days after the application from the soil (ground fallen) in two trials and 0 and 24-41 days after the application in the four remaining trials. Based on the study report, it is not possible to adequately retrieve whether olives were destoned at sampling, before processing, or whether they were destoned at all. A simplistic processing scheme was available in the study report which was not included by the applicant in the study summary, see figure below. Based on this scheme, however, it seems that olives were not destoned.

The analytical method was not fully validated for glyphosate and AMPA in olive fruits since recoveries were below 70% (see Vol. 3, B.5). In the frame of this processing study, however, mean concurrent/procedural recoveries were largely within the acceptable ranges. The only exception regarding procedural recoveries are the rather overall low recoveries for AMPA and the recovery for glyphosate in olive fruit at the fortification level of 0.5 mg/kg (65%, n = 4). Since no processing factors are derived for AMPA, the impact on the study outcome is considered minor. The procedural recovery for glyphosate which is below 70% is also considered to have minor impact on the study outcome considering that all other recoveries were acceptable. Overall, the performance of the analytical method was sufficiently demonstrated and the method is therefore considered fit for purpose. It is noted, however, that additional information regarding the extraction efficiency is needed for confirmation.

Samples of olives were stored frozen until analysis. Storage conditions for olive oil in terms of temperature, in contrast, were not reported. The impact on the study outcome is considered minor though. Specimens of olive fruits and oil were stored in accordance with the demonstrated period of storage stability for glyphosate (18 months in all plant commodities except dry matrices).

In contrast, AMPA was shown to be stable in soybean seeds only and data are not sufficient to allow an extrapolation to all high oil content matrices or to all plant commodities. Therefore, the results for AMPA are pending an additional storage stability study. It is noted that no processing factors are derived for AMPA since residue levels were below the LOQ in the RAC. Considering that storage stability was not adequately addressed for AMPA in olive/oil matrices, it is not possible to state whether residues were below the LOQ due to the fact that there were indeed no residues in/on the fruit or whether potential residues degraded during storage.

With regard to the results, the RMS notes the following: in the original summary of the study, residue levels corrected for recovery were shown. Also the study report only mentions corrected residue levels and no raw data are available. However, based on the corrected residue level and the corresponding procedural recovery, the RMS was able to calculate the uncorrected residue levels. Since all procedural recoveries were below 100%, it is also possible to conclude that corrected residue levels <0.05 mg/kg are also below the LOQ when residue levels would be uncorrected.

No residues were detected in the control specimens. Residues of AMPA were below the LOQ in all samples; therefore no processing factors can be derived for AMPA. Residues of glyphosate were above the LOQ in all trials so that processing factors can be calculated.

It is noted that two processing factors can be derived from each trial, however, the only difference would be that samples were taken at different PHIs. Since this is not considered an independent trial set-up, the RMS calculated one single processing factor per trial only which is based on the mean processing factor of the respective trial. The median processing factor is then calculated based on the different independent trials.

The following processing factors for glyphosate were obtained for unrefined olive oil:
<0.03, <0.04, <0.05, <0.10, <0.11, <0.29; median processing factor: <0.08

It is noted that the median processing factor is calculated based on this study only. Additional studies investigating the processing of olive fruits were submitted and an overall median processing factor considering all available data will be calculated in Volume 1.

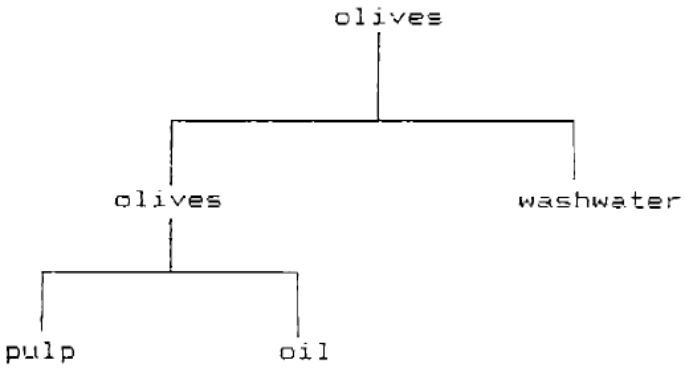
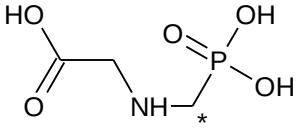
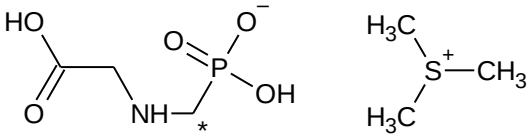


Figure B.7.5.3.5-1: Olive oil processing protocol

B.7.6. RESIDUES IN SUCCEEDING OR ROTATIONAL CROPS

B.7.6.1. Metabolism in rotational crops

No new rotational crop metabolism studies have been submitted within the current renewal of glyphosate.
Four confined rotational crop studies were conducted with various rotational crops using *N*-(phosphono-^{14}C-methyl)glycine; two rotational crop studies were conducted with various rotational crops using *N*-(phosphono-^{14}C-methyl)glycine trimesium salt:

Label	**Structural formula** (* indicates the label position)	**Code Number** **(Synonyms)** **That indicated in bold was used in the summary dossier**
Glyphosate CP 67573 *N*-(phosphono-methyl)glycine		
^{14}C-methane-label		• ***N*-(phosphono-^{14}C-methyl)glycine** • ^{14}C-methane-glyphosate
Available CRC studies	CA 6.6.1/001: [redacted]., 1998 CA 6.6.1/003: [redacted] 1990 CA 6.6.1/005: [redacted] 1978 CA 6.6.1/006: [redacted] 1976	
Glyphosate-trimesium ICIA0224 Trimesium salt of glyphosate *N*-(phosphono-methyl)glycine trimesium salt		
PMG-label		• ***N*-(phosphono-^{14}C-methyl)glycine trimesium salt** **(^{14}C-PMG-labelled glyphosate-trimesium)** • PMG • [^{14}C]-phosphonomethylene glyphosate trimesium • *N*-phosphono-methylglycine anion • [^{14}C-PMG]glyphosate-trimesium • ^{14}C-PMG-labeled glyphosate-trimesium
Available CRC studies	CA 6.6.1/002: [redacted] 1993 CA 6.6.1/004: [redacted] 1989	

B.7.6.1.1. Lettuce, radish, and wheat

1. Information on the study

Data point:	CA 6.6.1/001
Report author	[redacted]
Report year	1998
Report title	LX1146-02 (Glyphosate technical) Confined Rotational Crop Study on lettuce, radish, and wheat in California
Report No	1651-91-146-01-09B-17
Document No	459-GLY
Guidelines followed in study	Pesticide Assessment Guideline Subdivision N, Number 165-1
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Testing of Chemicals, 502: • TRR was only determined by combustion followed by LSC analysis but not determined after extraction of radioactive residues, but was for all samples ≥ 0.01 mg/kg and for all edible crops ≥ 0.05mg/kg; • The extractability of crop samples following extraction with chloroform and 0.1 M HCl was not determined and hence, majority of radioactivity (>99.9 %) is neither extracted, nor characterised, nor identified and no attempts to do so are reported. Peaks of metabolites other than AMPA and glyphosate were not integrated in the HPLC-chromatogram. Only glyphosate and AMPA are reported to be identified in very low amounts in all crop samples but the vast majority of remaining radioactivity is not further analysed. • Growth stage at sampling of immature crop samples is not given within the report • No flow chart depicting the overall extraction and fractionation strategies employed for each sample matrix analysed. • The limit of quantitation for HPLC analyses is due to the standards as high as 0.05 mg/kg • The storage duration of crop samples of the study was approx. 6 – 20 months, 2 – 16 months, and 7 – 10 months for the first, second and third rotation, respectively • The analytical residue method for analysis of glyphosate and AMPA was validated but mean validation recoveries of glyphosate and AMPA were between 72.3-101 % for all matrices except wheat chaff in which the mean recovery was 61.9 %
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	Yes 1) There was a field protocol and an analytical protocol until an amendment was issued on February 10, 1992, attaching or appending the analytical protocol to the field protocol. 2) Characterisation of the test and reference substances was not performed under GLP Standard §160.105. 3) Weather data was not collected from weather stations maintained under GLP procedures.
Acceptability/Reliability:	Conclusion applicant: supportive (Category 2a) Conclusion RMS: supportive only

2. Full summary of the study according to OECD format

Executive Summary

The metabolism of glyphosate was examined in rotational crops. The study was conducted to determine the amount of ^{14}C glyphosate and its major metabolite (AMPA) that are found in plants grown in pots of soil treated with ^{14}C

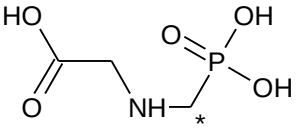
glyphosate. The treated soil was aged for 30 days, simulating a crop failure, 120 days, simulating a second crop planting in the same year, and 365 days, simulating a yearly rotational planting. This aging period prior to planting simulated a rotational planting scheme. The test crops for this study were radish, lettuce, and wheat. All crops were harvested at an immature stage of development, as well as at maturity, for the purpose of residue analysis in these crops. Soil samples were also taken at strategic points throughout the study to determine the amount and nature of the radiolabelled residue in the soil. Samples were frozen and shipped to PHARMACY LSR for analysis.

Total radioactive residues (TRR) were determined by combustion followed by LSC for all matrices of the crops of all rotations. The rotational crops from the 30 DALT pots contained 0.24 – 2.0 mg/kg TRR in edible matrices and 1.3 – 4.8 mg/kg TRR in inedible matrices. Crops from the 120 DALT planting contained TRR of 0.15 – 0.7 mg/kg and 0.17 – 1.4 mg/kg for edible and inedible matrices, respectively. Crops from the 365 DALT planting contained TRRs of 0.02 – 0.16 mg/kg and 0.01 – 0.19 mg/kg for edible and inedible matrices, respectively.

In mature, edible samples (lettuce leaves, wheat grain and radish root) of all three rotations, glyphosate was present only to amounts of < 0.05 mg/kg. In mature samples of wheat forage and chaff, glyphosate accounted for < 0.05 mg/kg, 0.3 – 0.4 mg/kg and < 0.05 – 0.06 mg/kg for the first, second and third rotation, respectively. AMPA residues were only seen at concentrations above the limit of quantification (0.05 ppm) in mature 30 and 120 day wheat forage, chaff, and seed, accounting for up to 0.4 mg/kg.

I. Materials and Methods

A. Materials

1. Test material	N-(phosphonomethyl)glycine; mixture of a) N-(phosphono-^{14}C-methyl)glycine (99 mg) b) N-(phosphono-^{12}C-methyl)glycine (2825 mg)
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	≥ 99 % (determined by HPLC)
Chemical purity	≥ 97 % (± 0.5 %)
Specific activity of the test substance applied:	11.03 MBq/mg (1.89 GBq/mmol or 51 mCi/mmol) 0.376 MBq/mg (22545 dpm/µg)

2. Test system	
Soil:	Sandy loam (pH: 7.9-8.0; cation exchange capacity: 5.1-5.6 meq./100 g; sand: 62 – 64 %; silt: 29 - 31 %; clay: 7 %; textural class (USDA): sandy loam)
Crop:	Radish (variety Cherry Belle), Lettuce (variety Waldmann's Green Leaf), Wheat (variety Yecora Rojo)
Botanical name:	*Latuca sativa* *Triticum aestivum* *Raphanus sativus*
Crop part(s):	Radish roots and leaves, lettuce leaves, wheat forage, chaff, grain (seeds)

B. Study design

1. In-life phase

The test substance contained 99 mg of N-(phosphono-^{14}C-methyl)glycine (^{14}C-glyphosate) with a specific activity of 11.03 MBq/mg (51 mCi/mmol) and 2825 mg of N (phosphonomethyl)glycine (^{12}C-glyphosate). Final specific activity of the test substance was 0.376 MBq/mg.

Labelled and unlabelled glyphosate was mixed in an aqueous solution to a concentration of total glyphosate of 1.19 mg/mL. ^{14}C-glyphosate was applied to the test plots at actual rates of 6.5 kg a.s./ha on bare soil. A pipette was used for the application of the aqueous solution to the soil surface in each of 61 treated pots. Additionally, untreated control pots were set up separate from the treated plots throughout the experiment.

All plants were grown outdoors in 30.5 cm diameter plastic pots which were moved into greenhouses when weather conditions became threatening or to maintain conditions conducive to plant growth. Pots were filled with sandy loam soil, levelled and watered. The seeds were then sown in four rows/pot. There were five treated and five non-treated pots for each planting interval for both the radish and lettuce. The wheat had nine treated and nine non-treated pots for each planting interval. There were a total of 38 pots for planting all three crops at each planting interval (soil aged 30, 120, and 365 days). Eight pots (four treated and four non-treated) were used solely for soil sampling at intervals prior to plant harvest. The pots were maintained in accordance with normal agricultural practice until a conventional harvest of each of these rotational crops was completed.

2. Sampling

The rotational crops were grown in [redacted] California, USA. The rotational crops were planted at 30 DALT, 120 DALT, and 365 DALT. Treated and non-treated mature and immature (50 % mature) radish, lettuce, and wheat were harvested. At half maturity, wheat forage was collected and at maturity above-ground growth was collected. Radish and lettuce crop samples were rinsed free of adhering soil and weighed. Radishes were separated into leaf and root, and above-growth of wheat plants at maturity was separated into forage, chaff and grain at the appropriate intervals. The same number of pots were harvested at the same intervals for the non-treated plants.

The soil in which the crops were growing was analysed at individual time points to follow the degradation of glyphosate and to identify the metabolites to which the rotational crops were exposed. Samples were taken prior to and immediately after treatment, at each rotational crop planting and harvest. Cores were collected to a depth of ca. 15.2 cm and separated into 0-7.6 cm and 7.6-15.2 cm sections. For collecting the soil cores, tubes (30.5 cm) were pushed by hand in the soil to the bottom of the pot (for soil samples till the first post-application interval) or entire pots were excavated.

All samples were bagged and frozen as soon as possible (usually within 1-2.5 hours) after collection. All treated post-application cores were sectioned approximately 14 hours later. All other cores were sectioned prior to freezing. Frozen samples were shipped to Pharmaco LSR. Samples were stored frozen below -20°C until preparation.

3. Analytical procedures

Total radioactive residues (TRR) in all plant samples were determined by liquid scintillation counting (LSC) following combustion.

Crop samples were prepared by grinding the whole frozen sample in a Waring type blender with dry ice to a homogenous mixtures. Crop samples containing significant levels of total radioactivity (≥ 0.01 mg/kg), were analysed further utilizing an analytical residue method. Subsamples of each crop sample were homogenised with chloroform and 0.1 M HCl. Samples were centrifuged and the aqueous layer was decanted, filtered if required and made up to volume with water to reach a pH of approx. 2.0.

The sample was purified using a Chelax resin column. The glyphosate and AMPA fractions were eluted with 6 M HCl and concentrated HCl was added to the collected fractions. For further purification, the sample was loaded on an anion exchange column (AG-1-X8) and eluted with 6 M HCl. The eluted sample was concentrated and prepared for HPLC.

The concentrated samples were analysed by HPLC and a post column reaction method specific for primary amines. Glyphosate was oxidised with sodium hypochlorite. The product from the oxidation reaction coil (glycine) and the AMPA were each coupled with o-phtalaldehyde in the presence of mercaptoethanol to give detectable fluorophors. HPLC was performed using two Aminex A-9 cation exchange columns.

Identification of glyphosate and AMPA in the sample extracts was done by retention time comparison with authentic standards.

Homogenised soil samples were analysed by combustion to determine the amount of ^{14}C residue. Moisture content was determined at the time combustion aliquots were taken. A pooled soil sample (of triplicates) was prepared for each sampling point, prior to combustion followed by LSC analysis.

II. Results and Discussion

A. Total radioactive residues (TRRs)

Total radioactive residue (measured as 14C glyphosate equivalents) was highest in the leafy crops or crop parts and generally declined from the earliest harvest dates to the latest harvest dates.

A summary of crop analyses through harvest of 30-, 120- and 365-day plantings is shown in Table B.7.6.1.1-1. After the first rotation, total radioactive residue (TRR) in edible commodities (lettuce leaves, radish roots and wheat grain) ranged from 0.24 to 2.0 mg/kg, and in non-edible commodities (radish leaves, wheat forage and wheat chaff) from 0.46 to 4.8 mg/kg. After the second rotation, TRR values in edible commodities (lettuce leaves, radish roots and wheat grain) ranged from 0.15 to 0.71 mg/kg, and in non-edible commodities (radish leaves, wheat forage and wheat chaff) from 0.17 to 1.4 mg/kg. After the third rotation, TRR values in edible commodities (lettuce leaves, radish roots and wheat grain) ranged from 0.02 to 0.16 mg/kg, and in non-edible commodities (radish leaves, wheat forage and wheat chaff) from 0.01 to 0.19 mg/kg.

Soil from untreated control and treated containers was assayed for total glyphosate equivalents. None of the soil or crop samples from control containers demonstrated radioactivity above background levels. A summary of soil analyses through harvest of 30-, 120- and 365-day plantings is shown in Table B.7.6.1.1-2. Residue levels for dry soil were calculated from assays of soil as received from the field and based on the moisture content determined from loss of weight from drying of corresponding samples. Levels of glyphosate equivalents found immediately after treatment of soil (pre-planting application) were 2.6 – 6.4 mg/kg in the upper 7 cm of the wet soil and 3.2 – 7.9 mg/kg in the upper 7 cm of the dry soil. Concentrations of glyphosate equivalents in the upper approx. 7 cm of the wet soil decreased from approximately 1.6 – 2.5 mg/kg at 55 – 120 DALT to approximately 0.6-1.1 mg/kg at 390 – 455 DALT. Concentrations of glyphosate equivalents in the upper approx. 7 cm of the dry soil decreased from approximately 1.8 – 3 mg/kg at 55 – 120 DALT to approximately 0.6-1.2 mg/kg at 390 – 455 DALT.

The soil characteristics indicated a sandy soil with a low organic matter content. After application to the surface, movement of glyphosate downward was minimal and residue levels in soil of ca. 7 – 15 cm depth accounted for most samples for ≤ 0.1 mg/kg and was for all samples in the range of 0.03 mg/kg to 1.0 mg/kg.

Table B.7.6.1.1-1: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
1st rotation	30	Lettuce	Leaves	55	0.46
				75	0.34
		Wheat	Forage	60	0.46
				120	1.3
			Chaff	120	1.6
			Grain	120	2.0
		Radish	Leaves	55	2.2
				75	4.8
			Root	55	0.38
				75	0.24
2nd rotation	120	Lettuce	Leaves	145	0.68
				165	0.25
		Wheat	Forage	150	0.45
				210	1.4
			Chaff	210	1.0
			Grain	210	0.7
		Radish	Leaves	145	0.33
				165	0.17
			Root	145	0.71
				165	0.15
3rd rotation	365	Lettuce	Leaves	390	0.02
				410	0.02

Table B.7.6.1.1-1: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
		Wheat	Forage	395	0.01
				455	0.08
			Chaff	455	0.19
			Grain	455	0.16
		Radish	Leaves	390	0.01
				410	0.02
			Root	390	0.06
				410	0.05

TRR – total radioactive residue, expressed as glyphosate equivalents
DALT – days after last treatment
PBI – plant back interval

Table B.7.6.1.1-2: Total radioactive residues in soil after application of ^{14}C-glyphosate to bare soil

Rotation. PBI	DALT	TRR (mg/kg) Soil wet depth 0 – 3 inches (ca. 0 – 7 cm)	Soil wet depth 0 – 6 inches (ca. 7 - 15 cm)	Soil dry depth 0 – 3 inches (ca. 0 – 7 cm)	Soil dry depth 0 – 6 inches (ca. 7 - 15 cm)
-	0 (post application, pre planting)	2.6 6.4	--- 0.1	3.2 7.9	--- 0.1
1st rotation PBI 30 days	55	2.2	0.1	2.5	0.1
	75	2.1	0.04	2.3	0.04
	55	2.5	0.04	3	0.05
	75	2.5	0.02	2.8	0.02
	60	1.6	0.03	1.8	0.03
	120	2.2	0.1	2.6	0.1
2nd rotation PBI 120 days	145	1.0	0.9	1.1	1.0
	165	2.0	0.09	2.2	0.1
	145	3.2	0.05	3.6	0.06
	165	1.5	0.1	1.8	0.1
	150	1.8	0.2	2.4	0.3
	210	1.9	0.1	2.1	0.1
3rd rotation PBI 365 days	390	0.9	0.03	1.0	0.03
	410	0.7	0.08	0.8	0.09
	390	1.1	0.04	1.2	0.04
	410	0.7	0.06	0.8	0.07
	395	0.7	0.04	0.8	0.04
	455	0.6	0.09	0.6	0.1

TRR – total radioactive residue, expressed as glyphosate equivalents; calculated within the report based on soil dry or wet weight
DALT – days after last treatment
PBI – plant back interval

B. Extraction and characterisation of residues

Plant samples were extracted and analysed for glyphosate and AMPA by HPLC (Table B.7.6.1.1-3). Validation results for recovery and determination of parent glyphosate and AMPA were obtained by analysing samples each previously fortified at the 0.1 mg/kg level. Results are shown in Table B.7.6.1.1-4 for analysis of mature crops. Mean validation recoveries of glyphosate were between 74.3 % and 101 % for all matrices except wheat chaff in which the mean recovery was 61.9 %. Mean validation recoveries for AMPA ranged from 72.3 % to 94.3 % for all matrices.

Glyphosate and AMPA were not detected in any radish root or lettuce leaf samples harvested at maturity at any of the three planting intervals. Mature wheat forage samples showed <0.05 mg/kg glyphosate and 0.2 mg/kg AMPA at the first rotation, 0.4 mg/kg glyphosate and 0.1 mg/kg AMPA at the second rotation and <0.05 mg/kg for both glyphosate and AMPA at the third rotation. Wheat chaff exhibited <0.05 mg/kg and 0.4 mg/kg glyphosate and AMPA, respectively, at the first rotation, 0.3 and 0.2 mg/kg at the second rotation and 0.06 mg/kg and < 0.05 mg/kg at the third rotation. Wheat seed did not exhibit glyphosate at concentrations greater than 0.05 mg/kg at the first, second or third rotations whereas AMPA was detected at 0.3 mg/kg in samples from the first rotation, 0.2 mg/kg in samples from the second rotation and at <0.05 mg/kg in samples from the third rotation in these samples.

For all untreated (control) crop samples, levels of glyphosate and AMPA were < 0.1 mg/kg.

Table B.7.6.1.1 -3: Radioactive residues of glyphosate and AMPA in lettuce leaves, wheat (forage, chaff and grain), and radish (leaves and roots) of rotational crop (first rotation, PBI 30 days; second rotation, PBI 120 days and third rotation, PBI 365 days) planted after application of glyphosate to bare soil

Rotation	PBI (days)	Crop	Sampled commodity	Sampling (DALT)	Glyphosate (mg/kg)	AMPA (mg/kg)
1st rotation	30	Lettuce	Leaves	75	< 0.05	< 0.05
		Wheat	Forage	120	< 0.05	0.2
			Chaff	120	< 0.05	0.4
			Grain	120	< 0.05	0.3
		Radish	Root	75	< 0.05	< 0.05
2nd rotation	120	Lettuce	Leaves	165	< 0.05	< 0.05
		Wheat	Forage	210	0.4	0.1
			Chaff	210	0.3	0.2
			Grain	210	< 0.05	0.2
		Radish	Root	165	< 0.05	< 0.05
3rd rotation	365	Lettuce	Leaves	410	< 0.05	< 0.05
		Wheat	Forage	455	< 0.05	< 0.05
			Chaff	455	0.06	< 0.05
			Grain	455	< 0.05	< 0.05
		Radish	Root	410	< 0.05	< 0.05

DALT – days after last treatment
PBI – plant back interval

Table B.7.6.1.1-4: Validation of Analytical Residue Method for Analysis of glyphosate and AMPA in various crop matrices

	Matrix[1]	Sample	% Recovery[2]	
			Glyphosate	AMPA
Radish	Root	control; PBI 30	82.9	80.45
	Leaf	control; PBI 30	73.5	76.1
Lettuce	Leaf	control; PBI 365	74.3	72.3
Wheat	Forage	control; PBI 30	87.1	86.0
	Chaff	control; PBI 30	61.9	94.3

Table B.7.6.1.1-4: Validation of Analytical Residue Method for Analysis of glyphosate and AMPA in various crop matrices

	Matrix[1]	Sample	% Recovery[2]	
			Glyphosate	AMPA
	Grain	control; PBI 30	101.0	77.4

PBI – Plant back interval
1 Duplicate samples analysed for each matrix except one analysis of wheat grain. Mean values were calculated upon dossier compilation.
2 Fortified with 0.1 mg/kg glyphosate and AMPA

C. Storage stability

All plant and soil samples were stored frozen below -20°C until preparation / LSC measurement. The storage period of the plant samples was approx. 6 – 20 months, 2 – 16 months, and 7 – 10 months for the first, second and third rotation, respectively. Radish samples were analysed 607, 162 and 259 days after sampling, for the first, second and third rotation, respectively. Lettuce samples were analysed 501, 50 and 286 days after sampling, for the first, second and third rotation, respectively. Wheat samples were analysed 186 -197, 485 and 202-203 days after sampling, for the first, second and third rotation, respectively.

D. Degradation pathway

Please refer to the overall pathway of glyphosate in rotational crops in Vol. 1, 2.7.7.

III. Conclusion

The rotational crops (lettuce, radish, and wheat) were planted into pots at 30, 120, and 365 days after herbicide treatment.

Total radioactive residues (TRR) were determined by combustion followed by LSC for all matrices of the crops of all rotations. The TRR detected in the various crops, in both mature and immature growth stages, was generally highest in the early planting and sampling intervals and lower in later intervals. The rotational crops from the 30 DALT planting contained 0.24 - 1.6 mg/kg TRR in edible matrices and up to 4.8 mg/kg TRR in inedible matrices. Crops from the 120 DALT planting contained TRRs of 0.15 – 0.7 mg/kg and 0.17 – 1.4 mg/kg for edible and inedible matrices, respectively. Crops from the 360 DALT planting contained TRRs of 0.02 – 0.16 mg/kg and 0.01 – 0.19 mg/kg for edible and inedible matrices, respectively. Hence, there was a more significant decrease in total radioactive residues between the 120 and 365 day planting interval than between the 30 day and 120 day planting interval as expected.

Rotational crop samples were extracted with chloroform and 0.1 M HCl and analysed by HPLC for the abundance of glyphosate and aminomethylphosphonic acid (AMPA). Even though TRRs were as high as several mg/kg, parent glyphosate was only detected at concentrations of < 0.05 mg/kg for mature, edible samples (lettuce leaves, wheat grain and radish root) of all three rotations. In mature samples of wheat forage and chaff, glyphosate accounted for < 0.05 mg/kg, 0.3 – 0.4 mg/kg and < 0.05 – 0.06 mg/kg for the first, second and third rotation, respectively. AMPA residues were only seen at concentrations above the limit of quantification (0.05 ppm) in mature 30 and 120 day wheat forage, chaff, and seed, accounting for 0.1 – 0.4 mg/kg.
This indicates that glyphosate and AMPA do not accumulate in the rotational crops tested and that the majority of carbon which was initially part of the glyphosate molecules applied to the soil that is taken up by these plants becomes incorporated into plant components or is converted into compounds other than glyphosate and AMPA.

Residues of glyphosate equivalents in soil showed little downward (i.e., through the soil profile) movement throughout the duration of the study. Total ^{14}C-glyphosate equivalents in soil declined steadily throughout the study period with little downward movement. Generally a correlation of levels in the crop compared to soil was not evident; however, rotational crops harvested at 390 – 455 days after soil treatment showed <0.1 mg/kg glyphosate equivalents corresponding to lower levels found in soil during the same time period.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study assessing the level of total residues of radioactive glyphosate equivalents in rotational crops (lettuce, wheat and radish) has been previously evaluated at EU level. It was performed under GLP. The study is deemed to largely comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 501 with deficits:

TRR was only determined by combustion followed by LSC analysis but not determined after extraction of radioactive residues, but was for all samples ≥ 0.01 mg/kg and for all edible crops ≥ 0.05mg/kg; The extractability of crop samples following extraction with chloroform and 0.1 M HCl was not determined and hence, majority of radioactivity (>99.9 %) is neither extracted, nor characterised, nor identified and no attempts to do so are reported. Peaks of metabolites other than AMPA and glyphosate were not integrated in the HPLC-chromatogram. However, the scope of the study was not to elucidate the metabolic pathway of glyphosate in CRC, but to estimate the amounts of glyphosate and AMPA in the different crop matrices, which was successfully completed for glyphosate and AMPA; Only glyphosate and AMPA are reported to be identified in very low amounts in all crop samples but the vast majority of remaining radioactivity is not further analysed. Although glyphosate and AMPA were not identified in two dissimilar HPLC systems, co-chromatographies (by comparison of retention times with reference compounds) strongly suggest the occurrence of glyphosate and its metabolite in the designated samples; The analytical residue method for analysis of glyphosate and AMPA was validated but mean validation recoveries of glyphosate and AMPA were between 72.3-101 % for all matrices except wheat chaff in which the mean recovery was 61.9 %.; The limit of quantitation for HPLC analyses is due to the standards as high as 0.05 mg/kg and therefore above the limit suggested by the OECD guidelines; The storage duration of crop samples of the study was approx. 6 – 20 months, 2 – 16 months, and 7 – 10 months for the first, second and third rotation, respectively; Growth stage at sampling of immature crop samples is not given within the report, but they can be roughly estimated based on planting and sampling dates; No flow chart provided depicting the overall extraction and fractionation strategies employed for each sample matrix analysed.

As the scope of the study was only to estimate the amounts of glyphosate and AMPA in the different crop matrices, the study is considered supportive for the assessment of the metabolic behaviour of glyphosate in rotational crops.

Assessment and conclusion by RMS:
The confined rotational crop study only provides information on the total radioactive residues in several crop parts after rotation. No investigation took place on extractability, and on the subsequent identification/characterization of other metabolites besides glyphosate and AMPA. Therefore, the majority of the residues has not been investigated, and as such no information on the metabolism can be abstracted from the study. Additional deficits are reported by the applicant. Altogether, the study is considered to only provide supportive information.

B.7.6.1.2. Lettuce, radish, and wheat

1. Information on the study

Data point:	CA 6.6.1/002
Report author	[redacted]
Report year	1993
Report title	[^{14}C-Anion] Glyphosate-Trimesium: Confined Accumulation Studies on Rotational Crops
Report No	RR92-096B
Document No	Not available
Guidelines followed in study	EPA Pesticide Registration Guideline Subdivision N, Number 165-1
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Metabolism in Rotational Crops, 502: • Developmental stages of the crops at application and harvesting are not reported, but could be roughly estimated based on planting and sampling dates. • Detailed information on sampling methods are not reported • Analysis of crop samples was not done within 6 months after sampling, but within 14 – 18 months. Storage stability data were generated to cover this period.

	• Extraction rates were low to moderate for crop samples (23.8 to 54.2 % of the TRR). Attempts (acid and/or basic hydrolysis) were made to characterise the non-extractable radioactivity, but final residues were between 13.1 to 59.4 % of the TRR, with absolute residues levels between 0.005 – 0.014 mg/kg.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	Conclusion applicant: valid (Category 2a) Conclusion RMS: acceptable

2. Full summary of the study according to OECD format

Executive Summary

The uptake and metabolism of glyphosate was examined in rotational crops. *N*-(phosphono-methyl)glycine labelled in the methylene position (^{14}C-PMG-label) was applied as its trimesium salt to two plots at different application rates; three additional plots received a comparable treatment of unlabelled active substance as control plots. The test item ^{14}C-PMG labelled glyphosate-trimesium was applied at a rate of 5.617 kg a.s./ha (3.87 kg a.s./ha expressed as glyphosate equivalents, plot 1.0) and at a total rate of 9.51 kg a.s./ha (three monthly applications, 6.56 kg a.s./ha expressed as glyphosate equivalents, plot 5.0).

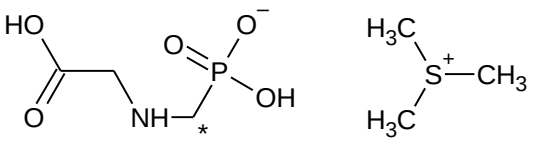
A primary crop of soybeans was planted prior to treatment in all plots containing sandy loam soil. After removal of the primary crop, the rotational crops lettuce, radish, and wheat were planted into the subplots at 35, 63, and 308 days after herbicide treatment (35, 63, and 308 plant-back intervals, PBI).

The soya cover crop was not analysed. For the characterisation and identification of residues in the rotated crops, samples were extracted using a mixture of 0.1N HCl and chloroform, followed by column fractioning using different solvents to separate residues and natural products. To characterise incorporation into natural products post-extraction solids were additionally hydrolysed under acid and basic conditions. The TRR levels in matrices obtained from rotational crops were relatively low, not exceeding 0.1 mg/kg, except for lettuce (0.127 mg/kg). The rotational crops from the 35 and 63 PBI plots contained TRR levels of 0.020 – 0.076 mg/kg and 0.021 – 0.127 mg/kg, respectively. Crops from the 308 PBI contained TRR levels of 0.010 – 0.038 mg/kg. All the residue levels were determined as N-phosphonomethylglycine (PMG) anion equivalents (mg ^{14}C-PMG anion equiv./kg, stated in the following only as mg/kg).

Analysis of rotational crop samples, after extraction with water and chloroform, revealed three residue components, PMG (glyphosate-anion), aminomethylphosphonic acid (AMPA) and a polar unknown metabolite (called metabolite 1 within the report). AMPA was found at levels of 8.7 - 34 % of the TRR. PMG was also detected in most samples, however its levels were <2.3 % of the TRR. Metabolite 1 was not identified as the amount was <0.01 mg/kg in all RAC's. Residues after extraction with water and chloroform were further investigated and were identified as being starch, lignins, amino acids and cellulose, as well as carbohydrates as glucose and fructose.

I. Materials and Methods

A. Materials

1. Test material	*N*-(phosphonomethyl)glycine trimesium salt; mixture of a) *N*-(phosphono-^{14}C-methyl)glycine as trimesium salt (82.3 mg, named ^{14}C-PMG labelled glyphosate as trimesium within this summary) b) *N*-(phosphono-^{12}C-methyl)glycine as trimesium salt (928 mg)
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	>97 %
Chemical purity	>99 %
Specific activity of the test substance applied:	0.68 MBq/mg (4.506 mCi/mmol)

2. Test system	

Soil:	Sandy loam (pH: 8.1; cation exchange capacity: 9.1 meq./100 g; sand: 54 %; silt: 36.1 %; clay: 9.9 %; textural class (USDA): sandy loam)
Crop:	*Primary crop:* Soybean (variety Pioneer 9271) *Rotational crops:* Lettuce (variety Fanfare), Radish (variety Icicle Short Top Radish), Wheat (variety Common Wheat, Germain's, W-444)
Botanical name:	*Glycine max* *Latuca sativa* *Raphanus sativus* *Triticum aestivum*
Crop part(s):	Lettuce leaves, wheat grain, wheat straw, wheat hay, wheat forage, radish root, radish top

B. Study design

1. In-life phase

The test substance contained 82.3 mg of ^{14}C-PMG labelled glyphosate-trimesium with a specific activity of 8.34 MBq/mg (55.3 mCi/mmol) and 928 mg of ^{12}C-glyphosate-trimesium. Final specific activity of the 1 % test substance was 0.68 MBq/mg (4.506 mCi/mmol).

Additionally, a test substance containing 1006.6 mg of unlabelled glyphosate-trimesium (^{12}C test substance) was prepared for application to the control plot.

The test area was divided into five plots which included two ^{14}C treated plots (plots 1.0 and 5.0), and three "control" plots treated with the non-radiolabelled material (plots 2.0, 3.0 and 4.0). Each of these plots was further subdivided into N.1, N.2, and N.3 subplots for planting the rotational crops. The sizes of the plots were 0.5 x 1 m for plots 1.0, 2.0 and 3.0, 0.7 x 1 m for plot 3.0 and 0.75 x 1 m for plot 5.0. Each plot area was clearly separated from other plot areas to avoid runoff of radioactive soil and mixing of the treated soils by aluminum or plastic lawn edgers and plexiglass. All plots were tilled and raked to depth of approximately 32 cm before each planting.

All applications were made to the soil. The test item ^{14}C-PMG labelled glyphosate-trimesium was applied to test plots 1.0 and 5.0. Plot 1.0 was treated once at a rate of 5.617 kg a.s./ha and plot 5.0 was treated three times at monthly intervals at rates of 6.361 kg a.s./ha, 2.08 kg a.s./ha and 1.07 kg a.s./ha, respectively (in total 9.51 kg a.s./ha). The control plots 2.0 and 4.0 were treated with nonradioactive glyphosate-trimesium, and served as controls in this study. Plot 2.0 was treated once at a rate of 5.79 kg a.s./ha and plot 4.0 was treated three times at monthly intervals at rates of 6.43 kg a.s./ha, 1.74 kg a.s./ha and 1.69 kg a.s./ha, respectively (in total 9.86 kg a.s./ha). Plot 3.0 was treated at a target rate of 33.88 kg a.s./ha of non-radioactive glyphosate-trimesium and served as an exaggerated rate treatment. An overview is given within the Table B.7.6.1.2-1.

All treatment solutions were applied using a repetitive stroke syringe fitted with a one-way valve and a manifold dispenser having 11 dispensing tips. The width of the manifold dispenser was 10 cm. The one-way valve was attached with tubing to a reservoir containing treatment solution, which was delivered using fairly rapid strokes on the syringe. Prior to application in the field, all manifold dispensers were calibrated. Treatment solutions were weighed before and after application to measure the exact amount of solution applied. At each date of application the first application was carried out on the non-radioactive plot, followed by application to the radioactive plot.

Table B.7.6.1.2-1: Overview of the different application scenarios

Plot No.	**Label**	**Application [1]**		**Primary crop for all plots**	**Rotational crop for all plots**
		Single rate (kg a.s./ha) [3]	**Total rate (kg a.s./ha)**		
1.0	^{14}C	5.617	5.617	Soybean	Lettuce Radish Wheat
2.0	^{12}C	5.79	5.79		
3.0	^{12}C	33.88	33.88		
4.0 [2]	^{12}C	6.43	9.86		
		1.74			
		1.69			
5.0 [2]	^{14}C	6.361	9.51		

Table B.7.6.1.2-1: Overview of the different application scenarios

Plot No.	Label	Application [1]		Primary crop for all plots	Rotational crop for all plots
		Single rate (kg a.s./ha) [3]	Total rate (kg a.s./ha)		
		2.08			
		1.07			

1 Application rates were calculated within the repot based on actual applied volumes.
2 The applications were conducted on a monthly interval.
3 The application rate is expressed as glyphosate-trimesium

As plots 2.0, 3.0 and 4.0 were treated with non-radioactive test item on the day before treatment of the radiolabelled plots 1.0 and 5.0 the calculated PBIs were one day longer than for the plots treated with radiolabelled test item. Plots 1.0, 2.0 and 3.0 were planted with rotational crops 35 or 36 days after treatment to simulate crop failure. Plots 4.0 and 5.0 were planted 64 or 63 and 309 or 308 days after the last treatment (125 and 371 days after the first treatment) to simulate rotation after harvest. The application dates were selected so that all rotational crops were planted at the same time to ensure the best climatic conditions for growing. Therefore, the multiple treatment plots (4.0 and 5.0) were treated first and the single treatment plots (1.0, 2.0, and 3.0) were treated last.
A cover crop of soybeans was planted in all subplots immediately prior to application of glyphosate-trimesium. Before planting the seeds were treated with "Garden Seed Inoculant Ni-Tro-Gen", a rhizobium inoculant of nitrogen fixing bacteria. The soybean cover crop on plots 4.0 and 5.0 germinated normally, but the soybeans did not germinate in plots 1.0, 2.0, and 3.0 because of cold outdoor temperature.
The rotational crops used in this study were lettuce, wheat, and radish, representing leafy, small grain, and root crops, respectively. Prior to planting, all five plots were divided into three equal subplots (N.1, N.2, and N.3). Radish was planted into subplots N.1, wheat into subplots N.2 and lettuce into subplots N.3. Before the second planting both plots were turned over with a hoe to the depth of about 15 cm and smoothed with a rake. Lettuce, wheat and radish crops were planted into subplots different from the first planting to simulate crop rotation. Lettuce was planted on N.2 subplots (planted on N.3 in the first rotation), radish was planted on N.3 subplots (planted on N.1 in the first rotation), and wheat was planted on N.1 subplots (planted on N.2 in the first rotation).

2. Sampling

Primary crop soybean (forage) was harvested from plots 4.0 and 5.0 at 35 DALT (days after last treatment), prior to the second application, by cutting at ground level at an immature stage. Soybean from plots 1.0, 2.0 and 3.0 failed to sprout, due to cool weather.

Rotational crops were harvested at maturity. In addition, wheat forage was harvested when the grain was in the milk stage. Detailed information on growth stages at harvest is missing. Samples of non-radiolabelled plots were taken first before harvesting samples of the ^{14}C-radioactive plots.

Harvest of radish was done at 132 DALT (plot 1.1, PBI 35), at 160 DALT (plot 5.1, PBI 63) and at 350 DALT (plot 5.3, PBI 308); raw agricultural commodity was separated into tops and roots directly. Harvest of lettuce was done at 147 DALT (plot 1.3, PBI 35), at 175 DALT (plot 5.3, PBI 63) and at 366 DALT (plot 5.2, PBI 308). Immature wheat forage was collected at 147 DALT (plot 1.2, PBI 35), at 175 DALT (plot 5.2, PBI 63) and at 373 DALT (plot 5.1, PBI 308); the remaining wheat plants were left on the plots until maturity. Harvest of mature wheat was carried out at 187 DALT (plot 1.2, PBI 35), at 215 DALT (plot 5.2, PBI 63) and at 454 DALT (plot 5.1, PBI 308) without separating grain from the straw. Detailed description of sampling methods is not reported.

Harvested and bagged crops were placed inside an ice-chest with dry-ice or water ice as they were harvested. Following weighing, crops were moved within one hour after harvest into a walk-in freezer located at the field station. Within a month, samples were shipped frozen to the analytical test facility. Upon receiving the samples, crops were transferred into lab freezers or walk-in freezer and remained frozen until analysis at temperatures usually at - 20 °C.

Soil samples were taken prior to and immediately after treatment, at crop planting and harvest time. Soil samples were collected with a soil core sampling device consisting of two different diameter hollow stainless steel tubes. Cores were collected to a depth of ca 16 inches (ca. 40 cm) and separated into two sections: a 0 - 4 inches and 2 inches in diameter section and a 4 - 16 inches and 1 inch in diameter section. Three different soil samples were taken: depth 0 - 4 inches (ca. 0 - 10 cm), depth 4 – 10 inches (ca. 10 - 25 cm) and depth 10 - 16 inches (ca. 25 - 40 cm). The deeper core was smaller in diameter to decrease the possibility of contamination of the deep core with surface soil. Soil samples were stored frozen until preparation.

3. Analytical procedures

Total radioactive residues (TRR) in all plant and soil samples were determined by LSC following combustion, except for soybean samples which were not analysed. Liquid samples were prepared for counting by combining aliquots (0.001 to 1 mL) with 15 - 20 mL Packard Scint-A XF cocktail. Samples were counted for a minimum of 5 minutes. After harvest, crops were divided into raw agricultural commodities (RAC's), processed and analysed by combustion. Samples of radish were separated into root and tops on the day of the harvest. The radish roots were ground to a powder with dry ice. Radish tops, lettuce and wheat forage were ground with to a powder dry-ice in a food chopper. Wheat grain was separated from straw and chaff by hand. Grain was ground in a coffee grinder or in a blender with dry-ice. Wheat straw was chopped with dry-ice using a meat cutter or a food processor.
Processed crops were left in open plastic bags in the freezers for a few days to allow the dry-ice to evaporate. When all of the CO_2 had evaporated, 5 subsamples of each crop matrix were weighed out for combustion.

The homogenised samples were extracted with an immiscible mixture of 0.1 M HCl and chloroform. The sample was blended for 15 minutes using a Tekmar tissue homogeniser and then centrifuged for 20 minutes. Aqueous and chloroform layers were pipetted off into separate plastic bottles. Extraction was repeated again and the nonextractable fraction (referred to as "pulp" within the report) was separated from the aqueous and chloroform layers by vacuum filtration. The aqueous and chloroform fractions were separately analysed by LSC to determine the amount of ^{14}C extractable residue. The non-extractable fraction was air-dried, weighed and combusted to determine the amount of ^{14}C non-extractable residue. Only pulp-fractions containing residues of more than 0.01 mg/kg were analysed further. The chloroform layer was not analysed further because the total ^{14}C residue in these fractions was insignificant (less than 0.01 mg/kg).
The aqueous (0.1 M HCl) extract was run through a Chelex® 100 column from which up to 7 fractions were collected. Fraction 1 containing carbohydrates was analysed by HPLC using an Aminex® carbohydrate analysis column and retention times of the radioactive peaks compared with sucrose, glucose, fructose and malic acid. The ^{14}C residues in fractions 2 (wash with deionised water) and 3 (elution with 0.2 M HCl) were negligible and not further analysed. Fraction 4 containing PMG and AMPA was eluted with 6 M HCl and cleaned up on an anion exchange column followed by HPLC analysis. Both compounds were characterised by comparing HPLC retention times with AMPA and PMG standards; TLC analysis was used for confirmation. Metabolite 1, a polar unknown metabolite, also appeared in the same fraction in all of the RAC's, but was not identified, as it was less than 0.01 mg/kg for all matrices. Fractions 5-7 were all eluted with 6 N HCl. As the ^{14}C residue was low in all three fractions no analysis was performed.
A subsample of the pulp fraction was air dried, weighed and combusted to determine the amount of ^{14}C non-extractable residue. This pulp fraction contained residues in excess of 0.01 mg/kg and was hydrolysed with 0.5 N HCl for 6-7 hours at reflux. After cooling to room temperature, the hydrolysis solution was filtered and aliquots analysed by LSC. The acid hydrolysate was passed through a Chelex® 100 column. Fraction 1 contained the majority of radioactivity, which was indicative of ^{14}C carbohydrates being released by acid hydrolysis. If the ^{14}C residue level was higher than 0.01 mg/kg, the fraction was analysed by HPLC for the presence of ^{14}C carbohydrates. To verify the presence of ^{14}C carbohydrate residue, glucosazone was also made from this fraction. The remaining hydrolysed pulp was combusted to determine again the amount of ^{14}C residue. The ^{14}C residue exceeded 0.01 mg/kg, and the pulp was hydrolysed with 20 % NaOH solution for 8 hours at reflux. The pulp and base hydrolysate were separated by filtering or centrifugation and aliquots from the base hydrolysate were taken for LSC analysis. The base hydrolysate was acidified and the resulting precipitate was removed by centrifugation. The precipitate contained the lignin fraction, and the soluble part contained the soluble amino acids fraction from base hydrolysis of proteins. The pulp remaining after acid and base hydrolysis consisted of cellulose, which was combusted to determine the amount of incorporated ^{14}C radioactivity.

As described above, in addition to the HPLC analysis, glucosazone was made from the carbohydrate fraction to verify the presence of ^{14}C glucose. For this purpose, milled wheat grain (35 and 63 PBI, respectively) was blended with DMSO and deionised water and then stirred overnight in a cold room. After centrifugation the supernatant was combined with ethanol to precipitate starch. The starch was washed with ethanol and dried. Isolated starch from 35 PBI wheat grain was hydrolysed with HC1 and the sample was refluxed with stirring for 7 hours. After cooling to room temperature the sample was divided in two. One half of the sample was neutralised with NaOH and sodium acetate and phenylhydrazine hydrochloride were added. The mixture was stirred and heated for 2 hours. The resulting product, a bright yellow solid, was filtered and washed with methanol. The glucosazone was recrystallised 8 times from methanol and hot water and out of each recrystallisation small subsample were weighed out for combustion. The glucosazone was analysed by MS and its structure was confirmed by comparison with glucosazone made directly from glucose. The other half of the hydrolysed starch was passed through a Chelex®100 column from which three fractions were collected. Fraction 1 contained 71.8 %, fraction 2 contained 1.7 %, and fraction 3 contained 26.5 % of the radioactivity applied to the column. Fraction 1 (not retained on Chelex®100) was reacted with phenylhydrazine and sodium acetate following neutralisation to pH 7. The overall yield of glucosazone was, which was recrystallised 4

times with methanol and hot water, and each subsample combusted. A sample was also submitted for MS confirmation of glucosazone. Isolated starch from 63 PBI was treated similarly yielding glucosazone. The final sample from recrystallisation was analysed and its structure confirmed by NMR and MS.
Soil samples from plots 1.0 and 5.0 were analysed by combustion to determine the ^{14}C residue. Similarly, the control soil cores from plot 2.0, 3.0, and 4.0 were combusted to determine if any ^{14}C contamination occurred on these plots during the study. Soil samples were extracted with different solvents: NH_4OH, HCl, H_2O, and a phosphate/ammonium hydroxide buffer (PAC). Best extractability was obtained with HCl, but these extracts were difficult to analyse by HPLC. Although extractability was not as good with water as it was with HCl, the water extracts were easily analysed. Consequently, the water extracts were further analysed. Soil extracts were quantified by HPLC by collecting fractions and counting by LSC; TLC analysis was used for confirmation.

II. Results and Discussion

A. Total radioactive residues (TRRs)

In crops, the TRR in the RAC's (raw agricultural commodities) was very low considering the application rates of 3.87 and 6.56 kg/ha radiolabelled glyphosate equivalents (as the trimesium salt) to the confined plots. These low TRR also show that the soil residues of PMG and AMPA were not concentrated in the crop RAC's. Total PMG and AMPA soil residues were 1.02 and 0.68 mg/kg at the time of planting in plots 1.0 (35 PBI) and 5.0 (63 PBI), respectively.

No analysis was undertaken on the cover crop of soybeans.

After the first rotation, total radioactive residues (TRR) in edible commodities (lettuce leaves, wheat grain, as well as radish roots) ranged from 0.020 to 0.076 mg/kg, and in non-edible commodities (wheat forage, wheat straw, wheat hay and radish tops) from 0.020 to 0.059 mg/kg. After the second rotation, TRR values in edible commodities ranged from 0.022 to 0.127mg/kg, and in non-edible commodities from 0.021 to 0.073 mg/kg. After the third rotation, TRR values in edible commodities ranged from 0.010 to 0.038 mg/kg, and in non-edible commodities from 0.016 to 0.034 mg/kg. Detailed results are presented in the following table. Control samples of plots 2.0 and 4.0 were also analysed for the TRR by combustion. TRR values were in the range of 0.00120 mg/kg (radish root, plot 2.1) and 0.0124 mg/kg (wheat straw, plot 4.2); plot 3.0 was not analysed.

Table B.7.6.1.2-2: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Plot ID	Subplot ID	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
1st rotation	35	1.0	1.1	Radish	Tops	*132*	0.020
					Root	*132*	0.020
			1.2	Wheat	Forage	*147*	0.024
					Hay	*187*	0.059
					Straw	*187*	0.050
					Grain	*187*	0.076
			1.3	Lettuce	Leaves	*147*	0.073
2nd rotation	63	5.0	5.1	Radish	Tops	*160*	0.021
					Root	*160*	0.022
			5.2	Wheat	Forage	*175*	0.033
					Hay	*215*	0.073
					Straw	*215*	0.063
					Grain	*215*	0.092
			5.3	Lettuce	Leaves	*175*	0.127
3rd rotation	308	5.0	5.1	Wheat	Forage	*373*	0.017
					Hay	*454*	0.034
					Straw	*454*	0.031
					Grain	*454*	0.038
			5.2	Lettuce	Leaves	*366*	0.017

Table B.7.6.1.2-2: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Plot ID	Subplot ID	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
			5.3	Radish	Tops	*350*	0.016
					Root	*350*	0.010

PBI Plant-back interval (meaning time between last treatment and planting of rotational crop)
DALT Days after last treatment
TRR Total radioactive residue, expressed as mg ^{14}C-PMG anion equiv./kg (by combustion)
1 The primary crop was not further analysed.
Values calculated upon dossier compilation are presented in italics.

In soil the combustion analysis showed a rapid decrease in radioactive residue, so that at the time of planting, 35 and 63 days after the last treatment, the total radioactive residue (TRR) had declined to 60 - 70 % of the applied radioactivity. Most of the radioactivity was in the top (0 - 10 cm) of the soil. The TRR in the 0 - 10 cm soil layer at the time of planting of the first rotation was 1.44 and 1.65 mg/kg in plot 1.0 and plot 5.0, respectively and at the second rotation 1.07 mg/kg in plot 5.0.

Table B.7.6.1.2-3: Total radioactive residues in soil after application of ^{14}C-glyphosate to bare soil

Plot ID	Days after treatment [1]	TRR (mg/kg)		
		Soil depth 0 – 4 inches (ca 0 – 10 cm)	Soil depth 4 – 10 inches (ca 10 - 25 cm)	Soil depth 10 – 16 inches (ca 25 - 40 cm)
1.0	-1	<0.0005	<0.0005	<0.0005
	0	3.55	0.003	0.001
	34 #	**1.44**	**0.001**	**<0.0005**
	132	0.806	0.010	0.001
	148	0.710	0.012	<0.0005
	188	0.590	0.007	<0.0005
5.0	*-1*	<0.0005	<0.0005	<0.0005
	0	3.78	0.013	0.004
	34 [2]	1.48	0.014	0.002
	34 [3]	2.97	0.005	0.001
	62 [4] (-1)	1.87	0.004	0.003
	63 [5] (0)	2.60	0.006	0.001
	***125* (62) #**	**1.65**	**0.016**	**<0.0005**
	223 (160)	1.41	0.009	0.002
	239 (176)	1.52	0.014	0.001
	279 (216)	0.557	0.008	0.001
	***370* (307) #**	**1.07**	**0.015**	**<0.0005**
	413 (350)	0.744	0.041	0.001
	436 (373)	0.271	0.019	0.001
	517 (454)	0.345	0.019	0.001

Table B.7.6.1.2-3: Total radioactive residues in soil after application of ^{14}C-glyphosate to bare soil

Plot ID	Days after treatment [1]	TRR (mg/kg)		
		Soil depth 0 – 4 inches (ca 0 – 10 cm)	Soil depth 4 – 10 inches (ca 10 - 25 cm)	Soil depth 10 – 16 inches (ca 25 - 40 cm)

TRR Total radioactive residue, expressed as mg ^{14}C-PMG anion equiv./kg (by combustion)
ND Not detected
1 For plot 1.0 values in this column refer to days after last treatment, for plot 5.0 values refer to days after first treatment (values in brackets refer to days after final treatment).
2 Before second treatment
3 After second treatment
4 Before third treatment
5 After third treatment
Indicating the day before planting of the rotational crops.
Values calculated upon dossier compilation are presented in italics.

B. Extraction and characterisation of residues

Plant matrices were extracted with a mixture of an aqueous and an organic solvent and the results are summarised in the tables below. In edible matrices portions of 44.7 – 54.2 % of the TRR were extractable with conventional extraction methods. For these samples, the major part of the residue was extracted with the aqueous solvent (40.6 – 51.4 % TRR). The remaining non-extractable residues (RRR) were further investigated with acidic and basic hydrolysis. (Calculation explanation: TRR within the following tables was calculated within the report as ERR + RRR. Total was calculated within the report as sum of single analyte results.)

Lettuce leaf

For the first plant-back intervals, 54.2 and 50.3 % of the TRR (0.039 and 0.064 mg/kg) in 35 and 63 PBI lettuce was found in the ERR, whereas 45.8 % and 49.7 % of the TRR (0.033 and 0.063 mg/kg) remained in the solids. The water phases of 35 and 63 PBI lettuce leaves contained 0.7 – 0.9 % of the TRR (0.001 mg/kg) PMG, 18.5 – 20.4 % of the TRR (0.015 – 0.024 mg/kg) AMPA, 5.1 – 6.5 % of the TRR (0.004 – 0.008 mg/kg) metabolite 1, 9.3 – 10.0 % of the TRR (0.007 – 0.012 mg/kg) glucose, 6.8 – 7.5 % of the TRR (0.005 – 0.009 mg/kg) fructose and 6.5 – 7.2 % of the TRR (0.005 – 0.008 mg/kg) malic acid. After acidic hydrolysis of the RRR 4.4 to 4.9 % of the TRR (0.003 and 0.006 mg/kg) PMG/AMPA were found in the acid hydrolysates. In 35 PBI lettuce 22.4 % (0.016 mg/kg) carbohydrates were solubilised and the acid hydrolysate of 63 PBI lettuce contained 16.7 % of the TRR (0.021 mg/kg) glucose and 7.0 % of the TRR (0.009 mg/kg) fructose. The precipitate remaining after HCl extraction was subjected to a basic hydrolysis solubilizing lignins (4.8 % of the TRR or 0.006 mg/kg), amino acids (10.2 % of the TRR or 0.013 mg/kg) and cellulose (6.0 % of the TRR or 0.008 mg/kg) in 63 PBI lettuce. For the 308 plant-back interval, 44.7 % of the TRR (0.008 mg/kg) in lettuce was found in the water phase. The TRR in the chloroform phase was below the detection limit. The remaining non-extractable residues (RRR) amounted to 55.3 % of the TRR (0.009 mg/kg). Results are presented in Table B.7.6.1.2-4.

Wheat grain

For all plant-back intervals, 40.6 to 50.0 % of the TRR (0.019 to 0.037 mg/kg) in wheat grain were found in the water phase and 0.8 to 7.1 % of the TRR (<0.001 to 0.007 mg/kg) in the chloroform phase. The RRR amounted to 49.2 to 52.3 % of the TRR (0.019 to 0.048 mg/kg). The water phase of wheat grain from 35 and 63 PBI contained 34.0 and 25.8 % of the TRR (0.026 and 0.024 mg/kg) of AMPA, 3.0 and 2.5 % of the TRR (0.002 mg/kg) of metabolite 1 and 9.8 % of the TRR (0.007 and 0.009) of carbohydrates, respectively. PMG was only detected in 63 PBI wheat grain amounting to 2.3 % of the TRR (0.002 mg/kg). In the water phase of wheat grain from the second plant-back interval (308 PBI) only carbohydrates were found with an amount of 8.4 % of the TRR (0.003 mg/kg). The remaining dissolved radioactive substances were detected in the Chelex fractions (41.7 % of the TRR and 0.016 mg/kg). Acidic hydrolysis of the RRR from the first plant-back interval led to solubilisation of 3.7 and 2.3 % of the TRR (0.003 mg/kg) PMG/AMPA and 32.3 and 39.8 % of the TRR (0.025 and 0.037 mg/kg) glucose. For wheat grain from the second plant-back interval (308 PBI) 36.1 % of the TRR (0.014 mg/kg) were found in the acid hydrolysate. Results are presented in Table B.7.6.1.2-5.

Wheat straw

In 35 and 63 PBI wheat straw 40.7 and 38.1 % of the TRR (0.021 and 0.025 mg/kg) and in 308 PBI 23.8 % of the TRR (0.0072 mg/kg) were found in the ERR, whereas only a minor part of the residues was extracted with chloroform (0.7 – 1.4 % TRR). The water phases of 35 and 63 PBI contained 0.3 – 0.4 % of the TRR (0.0002 mg/kg) PMG, 11.7 –

12.7 % of the TRR (0.006 – 0.008 mg/kg) AMPA, 4.1 – 4.2 % of the TRR (0.002 – 0.003 mg/kg) metabolite 1 and 0.1 % of the TRR (0.0001 mg/kg) other anions. Carbohydrates were also found in the water phases of all wheat straw extracts. Wheat straw of the 35 and 308 PBI contained carbohydrates with amounts of 22.9 and 15.0 % of the TRR (0.012 and 0.004 mg/kg) and the carbohydrates in the 65 PBI sample were identified as glucose (3.5 % TRR and 0.002 mg/kg), fructose (3.7 % TRR and 0.002 mg/kg), malic acid (4.6 % TRR and 0.003 mg/kg) and metabolite 2 (8.3 % TRR and 0.005 mg/kg). The remaining dissolved radioactive substances of 308 PBI wheat straw were detected in the Chelex fractions (8.5 % of the TRR and 0.003 mg/kg) and were not further investigated. After acidic hydrolysis of the RRR 16.0 and 19.1 % of the TRR (0.008 and 0.012 mg/kg) were found in the acid hydrolysates. The precipitate remaining after HCl extraction was subjected to a basic hydrolysis to solubilise amino acids (20.7 – 15.6 % or 0.010 mg/kg), cellulose (22.6 – 13.9 % of the TRR or 0.011 – 0.009 mg/kg) and in case of 63 PBI wheat straw also lignins (12.8 % of the TRR or 0.008 mg/kg). Results are presented in Table B.7.6.1.2-6.

Wheat forage

For wheat forage of the 35 and 63 PBI, 46.2 and 47.3 % of the TRR (0.011 and 0.016 mg/kg) were found in the water phase and 3.1 and 2.0 % of the TRR (0.001 mg/kg) in the chloroform phase. The RRR amounted to 50.7 % of the TRR (0.012 and 0.016 mg/kg). The water phase contained 20.5 % of the TRR (0.005 and 0.007 mg/kg) of AMPA, 1.9 and 2.0 % of the TRR (0.0003 and 0.001 mg/kg) of metabolite 1, 2.2 and 3.3 % of the TRR (0.001 mg/kg) of other anions and 17.9 and 21.6 % of the TRR (0.004 and 0.007 mg/kg) of carbohydrates. Only in the water phase of wheat forage at the 35 PBI PMG was found with an amount of 0.5 % of the TRR (0.0001 mg/kg). Acidic hydrolysis of the RRR from 63 PBI wheat forage led to solubilisation of 23.1 % of the TRR (0.008 mg/kg) carbohydrates. The precipitate remaining after HCl extraction was subjected to a basic hydrolysis solubilizing lignins (4.4 % of the TRR or 0.001 mg/kg), amino acids (10.5 % of the TRR or 0.003 mg/kg) and cellulose (12.7 % of the TRR or 0.004 mg/kg) in 63 PBI wheat forage. Due to low TRR values (0.017 mg/kg) wheat forage from the plant-back interval 308 PBI was not further extracted and analysed. Results are presented in Table B.7.6.1.2-7.

Radish root

In radish root of all plant-back intervals, 46.7 to 51.4 % of the TRR (0.010 to 0.005 mg/kg) were found in the ERR, whereas only a minor part of the residues was extracted with chloroform (0.4 – 1.4 % of the TRR). The water phases of radish root of 35 and 63 PBI contained 1.7 – 1.8 % of the TRR (0.0004 mg/kg) PMG, 8.7 – 11.0 % of the TRR (0.002 mg/kg) AMPA, 1.0 – 1.2 % of the TRR (0.0002 – 0.0003 mg/kg) metabolite 1, 1.6 -2.1 % of the TRR (0.0004 mg/kg) other anions and 31.8 – 32.7 % of the TRR (0.007 mg/kg) carbohydrates. In the water phase of radish root samples of the 308 PBI 43.2 % of the TRR (0.004 mg/kg) were characterised as carbohydrates. Due to low TRR values the remaining non-extractable residues (RRR) were not further investigated with acidic and basic hydrolysis. Results are presented in Table B.7.6.1.2-8.

Radish top

In radish top of all plant-back intervals, 40.4 to 45.0 % of the TRR (0.007 to 0.009 mg/kg) were found in the ERR, whereas only a minor part of the residues was extracted with chloroform (3.1 – 5.5 % of the TRR). The water phases of radish top samples of the 35 and 63 PBI contained 0.9 – 1.1 % of the TRR (0.0002 mg/kg) PMG, 9.5 – 12.3 % of the TRR (0.002 mg/kg) AMPA, 1.0 – 1.4 % of the TRR (0.0002 – 0.0003 mg/kg) metabolite 1, 1.8 % of the TRR (0.0004 mg/kg) other anions and 21.0 – 25.7 % of the TRR (0.004 - 0.005 mg/kg) not further characterised carbohydrates. In the water phase of radish top samples of the 308 PBI 27.4 % of the TRR (0.004 mg/kg) were characterised as carbohydrates. Due to low TRR values the remaining non-extractable residues (RRR) were not further investigated with acidic and basic hydrolysis. Results are presented in Table B.7.6.1.2-9.

Table B.7.6.1.2-4: Extraction of the radioactive residues in lettuce leaves of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in lettuce leaves					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	147		175		366	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.072	100	0.127	100	0.017	100
ERR	***0.039***	***54.2***	***0.064***	***50.3***	***0.009***	***44.7***
Chloroform phase	0.002	2.8	0.002	1.6	<0.001	ND
Water phase	0.037	51.4	0.062	48.7	0.008	44.7

Table B.7.6.1.2-4: Extraction of the radioactive residues in lettuce leaves of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

Chelex fractions [1]	*0.020*	*26.8*	*0.033*	*26.1*	0.003	19.6
PMG	0.001	0.7	0.001	0.9	NP	NP
AMPA	0.015	20.4	0.024	18.5		
Metabolite 1	0.004	5.1	0.008	6.5		
Other anion	0.0004	0.6	0.0003	0.2		
Carbohydrates [2]	*0.017*	*24.7*	*0.029*	*22.6*	0.004	25.1
Glucose	0.007	10.0	0.012	9.3	NP	NP
Fructose	0.005	7.5	0.009	6.8		
Malic acid	0.005	7.2	0.008	6.5		
RRR (extracted pulp)	**0.033**	**45.8**	**0.063**	**49.6**	**0.009**	**55.3**
Acid hydrolysate [3]	*0.019*	*26.8*	*0.036*	*28.6*	NP	NP
PMG/AMPA	0.003	4.4	0.006	4.9	-	-
Carbohydrates [2]	0.016	22.4	*0.030*	*23.7*	-	-
Glucose	NP	NP	0.021	16.7	-	-
Fructose			0.009	7.0	-	-
Pulp 2 (acid hydrolysed pulp)	0.014	19.0	-	-	-	-
Base hydrolysate [4]	NP	NP	*0.027*	*21.0*	NP	NP
Lignins	-	-	0.006	4.8	-	-
Amino acids	-	-	0.013	10.2	-	-
Cellulose	-	-	0.008	6.0	-	-
Identified [5]	*0.036*	*50.2*	*0.090*	*70.6*	-	-
Characterised [6]	*0.022*	*30.9*	*0.037*	*29.3*	*0.008*	*44.7*
Final residue	**0.014**	**19.0**	**-**	**-**	**0.009**	**55.3**
Total	**0.071**	**100.1**	**0.127**	**99.9**	**0.016**	**100.0**
Recovered radioactivity	**83.7**		**90.5**		**91.2**	

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Carbohydrates were calculated as sum of results of glucose, fructose and malic acid.
3 Acid hydrolysate was calculated as PMG/AMPA, carbohydrates (glucose, fructose).
4 Base hydrolysate was calculated as sum of lignins, amino acids and cellulose.
5 Identified was calculated as sum of PMG, AMPA, malic acid, PMG/AMPA, glucose and fructose.
6 Characterised was calculated as sum of chloroform phase, metabolite 1, other anion, carbohydrates, lignins, amino acids and cellulose.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.2-5: Extraction of the radioactive residues in wheat grain of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in wheat grain					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	187		215		454	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.076	100	0.092	100	0.038	100
ERR	***0.038***	***50.0***	***0.044***	***47.7***	***0.020***	***50.8***

Table B.7.6.1.2-5: Extraction of the radioactive residues in wheat grain of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

Chloroform phase	0.002	3.1	0.007	7.1	<0.001	0.8
Water phase	0.036	46.9	0.037	40.6	0.019	50.0
Chelex fractions [1]	*0.030*	*37.0*	*0.029*	*30.6*	0.016	41.7
PMG	<0.001	ND	0.002	2.3	NP	NP
AMPA	0.026	34.0	0.024	25.8		
Metabolite 1	0.002	3.0	0.002	2.5		
Other anion	<0.001	ND	<0.001	ND		
Carbohydrates	0.007	9.8	0.009	9.8	0.003	8.4
RRR (extracted pulp)	**0.038**	**50.1**	**0.048**	***52.3*** [6]	**0.019**	**49.2**
Acid hydrolysate [2]	*0.029*	*36.0*	*0.041*	*42.1*	0.014	36.1
PMG/AMPA	0.003	3.7	0.003	2.3	NP	NP
Carbohydrates [3]	*0.026*	*32.3*	*0.038*	*39.8*		
Glucose	0.025	32.3	0.037	39.8		
Fructose	<0.001	ND	<0.001	ND		
Pulp 2 (acid hydrolysed pulp)	0.011	14.1	0.009	10.2	0.005	13.1
Identified [4]	*0.056*	*70.0*	*0.067*	*70.2*	-	-
Characterised [5]	*0.012*	*15.9*	*0.019*	*19.4*	*0.034*	*87.0*
Final residue	**0.011**	**14.1**	**0.009**	**10.2**	**0.005**	**13.1**
Total	**0.076**	**100.0**	**0.093**	**99.8**	**0.038**	**99.3**
Recovered radioactivity	**99.7**		**97.5**		**92.2**	

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Acid hydrolysate was calculated as PMG/AMPA, carbohydrates (glucose, fructose).
3 Carbohydrates were calculated as sum of results of glucose, and fructose.
4 Identified was calculated as sum of PMG, AMPA, PMG/AMPA, glucose and fructose.
5 Characterised was calculated as sum of chloroform phase, metabolite 1, other anion, and carbohydrates.
6 This value was recalculated as the value given in the report (51.9 %) does not fit to the given single values.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.2-6: Extraction of the radioactive residues in wheat straw of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in wheat straw					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	187		175		366	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.050	100	0.063	100	0.031	100
ERR	***0.021***	***40.7***	***0.025***	***38.1***	***0.0072***	***23.8***
Chloroform phase	0.001	1.4	0.001	0.9	0.0002	0.7
Water phase	0.020	39.3	0.024	37.2	0.007	23.1
Chelex fractions [1]	*0.008*	*16.3*	*0.011*	*17.3*	0.003	8.5
PMG	0.0002	0.4	0.0002	0.3	NP	NP
AMPA	0.006	11.7	0.008	12.7		

Table B.7.6.1.2-6: Extraction of the radioactive residues in wheat straw of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in wheat straw					
PBI	35		63		308	
Metabolite 1	0.002	4.1	0.003	4.2		
Other anion	0.0001	0.1	0.0001	0.1		
Carbohydrates [2]	0.012	22.9	*0.012*	*20.1*	0.004	15.0
Glucose	NP	NP	0.002	3.5	NP	NP
Fructose			0.002	3.7		
Malic acid			0.003	4.6		
Metabolite 2			0.005	8.3		
RRR (extracted pulp)	**0.030**	**59.3**	**0.039**	***61.9*** [6]	**0.024**	**76.1**
Acid hydrolysate	0.008	16.0	0.012	19.1	0.011	34.5
Pulp 2 (acid hydrolysed pulp)	-	-	-	-	0.013	41.7
Base hydrolysate [3]	*0.021*	*43.3*	*0.027*	*42.3*	NP	NP
Lignins	included with Cellulose		0.008	12.8	-	-
Amino acids	0.010	20.7	0.010	15.6		
Cellulose	0.011	22.6	0.009	13.9		
Identified [4]	*0.006*	*12.1*	*0.015*	*24.8*	-	-
Characterised [5]	*0.044*	*87.8*	*0.048*	*74.9*	*0.018*	*58.7*
Final residue	**-**	**-**	**-**	**-**	**0.013**	**41.7**
Total	**0.050**	**99.9**	**0.063**	**99.6**	**0.031**	**99.9**
Recovered radioactivity	**106.8**		**88.8**		**100.3**	

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Carbohydrates were calculated as sum of results of glucose, and fructose.
3 Base hydrolysate was calculated as sum of lignins, amino acids and cellulose.
4 Identified was calculated as sum of PMG, AMPA, PMG/AMPA, glucose, fructose and malic acid.
5 Characterised was calculated as sum of chloroform phase, metabolite 1+2, other anion, acid hydrolysate, lignins, amino acids and cellulose.
6 This value was recalculated as the value given in the report (61.5 %) does not fit to the given single values.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.2-7: Extraction of the radioactive residues in wheat forage of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in wheat forage					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	147		175		373	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.024	100.0	0.033	100.0	0.017	100.0
ERR	***0.012***	***49.3***	***0.017***	***49.3***	NP	NP
Chloroform phase	0.001	3.1	0.001	2.0		
Water phase	0.011	46.2	0.016	47.3		
Chelex fractions [1]	*0.0064*	*25.1*	*0.010*	*25.8*		
PMG	0.0001	0.5	<0.001	ND		

Table B.7.6.1.2-7: Extraction of the radioactive residues in wheat forage of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in wheat forage					
PBI	35		63		308	
AMPA	0.005	20.5	0.007	20.5		
Metabolite 1	0.0003	1.9	0.001	2.0		
Other anion	0.001	2.2	0.001	3.3		
Carbohydrates [2]	0.004	17.9	0.007	21.6		
RRR (extracted pulp)	***0.012*** [6]	***50.7*** [6]	**0.016**	**50.7**		
Acid hydrolysate [3]	NP	NP	*0.008*	*23.1*		
PMG/AMPA			ND	ND		
Carbohydrates			0.008	23.1		
Pulp 2 (acid hydrolysed pulp)			-	-		
Base hydrolysate [3]			*0.008*	*27.6*		
Lignins			0.001	4.4		
Amino acids			0.003	10.5		
Cellulose			0.004	12.7		
Identified [4]	*0.005*	*21.0*	*0.007*	*20.5*		
Characterised [5]	*0.006*	*25.1*	*0.026*	*79.6*		
Final residue	**0.012**	**50.7**	**-**	**-**		
Total	**0.024**	**99.9**	**0.033**	**100.1**		
Recovered radioactivity	**89.8**		**91.7**			

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Acid hydrolysate was calculated as PMG/AMPA, carbohydrates.
3 Base hydrolysate was calculated as sum of lignins, amino acids and cellulose.
4 Identified was calculated as sum of PMG, AMPA, PMG/AMPA.
5 Characterised was calculated as sum of chloroform phase, metabolite 1, other anion, carbohydrates, lignins, amino acids and cellulose.
6 These values were recalculated based on the assumption that ERR+RRR=TRR (values given in the report: 0.013 mg/kg, 53.8 %).
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.2-8: Extraction of the radioactive residues in radish root of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in radish root					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	132		160		350	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.020	100.0	0.022	100.0	0.010	100.0
ERR	***0.010***	***46.7***	***0.010***	***48.5***	***0.005***	***51.4***
Chloroform phase	0.0001	0.4	0.0003	1.4	0.0001	0.8
Water phase	0.010	46.3	0.010	47.1	0.005	50.6
Chelex fractions [1]	*0.0030*	*13.6*	*0.0031*	*15.5*	0.001	7.4
PMG	0.0004	1.8	0.0004	1.7	NP	NP
AMPA	0.002	8.7	0.002	11.0		

Table B.7.6.1.2-8: Extraction of the radioactive residues in radish root of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

Metabolite 1	0.0002	1.0	0.0003	1.2		
Other anion	0.0004	2.1	0.0004	1.6		
Carbohydrates	0.007	32.7	0.007	31.8	0.004	43.2
RRR (extracted pulp)	**0.011**	**53.3**	**0.011**	**51.5**	**0.005**	**48.6**
Identified [2]	*0.002*	*10.5*	*0.002*	*12.7*	-	-
Characterised [3]	*0.008*	*36.2*	*0.008*	*36.0*	*0.005*	*51.4*
Final residue	**0.011**	**53.3**	**0.011**	**51.5**	**0.005**	**48.6**
Total	**0.021**	**100.0**	**0.021**	**100.0**	**0.010**	**100.0**
Recovered radioactivity	**109.1**		**112.5**		**76.7**	

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Carbohydrates were calculated as sum of results of glucose, fructose and malic acid.
3 Acid hydrolysate was calculated as PMG/AMPA, carbohydrates (glucose, fructose).
4 Base hydrolysate was calculated as sum of lignins, amino acids and cellulose.
5 Identified was calculated as sum of PMG, AMPA, malic acid, PMG/AMPA, glucose and fructose.
6 Characterised was calculated as sum of chloroform phase, metabolite 1, other anion, carbohydrates, lignins, amino acids and cellulose.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.2-9: Extraction of the radioactive residues in radish top of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in radish top					
PBI	35		63		308	
Plot-ID	1.3		5.3		5.2	
DALT (sampling)	132		160		350	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.020	100.0	0.021	100.0	0.016	100.0
ERR	***0.008***	***41.7***	***0.009***	***45.0***	***0.007***	***40.4***
Chloroform phase	0.001	4.8	0.001	5.5	0.001	3.1
Water phase	0.007	36.9	0.008	39.5	0.006	37.3
Chelex fractions [1]	*0.0028*	*16.0*	*0.0029*	*13.8*	0.002	10.0
PMG	0.0002	0.9	0.0002	1.1	NP	NP
AMPA	0.002	12.3	0.002	9.5		
Metabolite 1	0.0002	1.0	0.0003	1.4		
Other anion	0.0004	1.8	0.0004	1.8		
Carbohydrates	0.004	21.0	0.005	25.7	0.004	27.4
RRR (extracted pulp)	**0.012**	**58.2**	**0.012**	**54.9**	**0.010**	**59.4**
Identified [2]	*0.002*	*13.2*	*0.002*	*10.6*	-	-
Characterised [3]	*0.005*	*23.8*	*0.006*	*28.9*	*0.007*	*40.5*
Final residue	**0.012**	**58.2**	**0.012**	**54.9**	**0.010**	**59.4**
Total	**0.020**	**99.9**	**0.022**	**99.9**	**0.017**	**99.9**
Recovered radioactivity	**102.4**		**88.8**		**102.9**	

Table B.7.6.1.2-9: Extraction of the radioactive residues in radish top of rotational crop planted after application of ^{14}C-PMG labelled glyphosate to bare soil

	Residues in radish top		
PBI	35	63	308

DALT – days after last treatmentPBI – plant-back interval TRR – Total radioactive residue
RRR – Residual radioactive residue ND – not detected NP – not performed
ERR – Extractable radioactive residue (calculated as sum of water phase and chloroform phase)
1 Chelex fractions were calculated as sum of PMG, AMPA, metabolite 1 and other anion.
2 Identified was calculated as sum of PMG, and AMPA.
3 Characterised was calculated as sum of chloroform phase, metabolite 1, other anion, and carbohydrates (for PBI 308 chloroform phase, Chelex fraction, and carbohydrates).
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values given as <0.001 mg/kg were set as 0.001 mg/kg.

Soil

The extractability of soil samples analysed for plot 1.0 was between 58.4 to 70.6 % of the TRR and for plot 5.0 between 30.4 to 41.2 % of the TRR. The only components found in soil extracts were PMG and AMPA. The amount of PMG in soil was at the highest level directly after application and decreased over time in all the samples. In plot 1.0 the amount of PMG decreased from 59.5 % of the TRR (2.11 mg/kg) to 3.54 % of the TRR (0.021 mg/kg) and in plot 5.0 the amount decreased from 31.9 % of the TRR (1.21 mg/kg) to 2.98 % of the TRR (0.045 mg/kg) in the period from 0 to 176 DALT and then slightly increased to 8.85 % of the TRR (0.049 mg/kg) for 216 DALT. In plot 1.0 AMPA was not detected directly after application. Afterwards, the amount of AMPA increased over time to 58.6 % of the TRR (0.844 mg/kg) for sample 34 DALT and then slightly decreased to 54.9 % of the TRR (0.324 mg/kg) for sample 183 DALT. In plot 5.0 AMPA residues increased from 6.31 % of the TRR (0.239 mg/kg) to 27.5 % of the TRR (0.564 mg/kg) and then slightly decreased to 24.1 % of the TRR (0.134 mg/kg) for sample 216 DALT. Results are presented in the following tables (Table B.7.6.1.2-10 and Table B.7.6.1.2-11).

Table B.7.6.1.2-10: PMG and AMPA residues determined by HPLC analysis in 0 – 10 cm soil cores collected after treatment of plot 1.0 with 3.87 kg/ha ^{14}C-PMG (5.617 kg/ha glyphosate-trimesium)

Plot 1.0	**Residues in soil cores (0 - 10 cm)**					
DALT	0		34		188	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
TRR	**3.55**	**100**	**1.44**	**100**	**0.590**	**100**
ERR	2.11	59.5	1.02	70.6	0.345	58.4
PMG	2.11	59.5	0.172	12.0	0.021	3.54
AMPA	0.000	0.0	0.844	58.6	0.324	54.9
RRR	1.44 [1]	40.5 [1]	0.423 [2]	29.4 [2]	0.245 [2]	41.6 [2]
TRR	**3.55**	**100**	**1.44**	**100**	**0.590**	**100**

DALT days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue
1 These values were determined by difference within the report.
2 These values were determined by combustion analysis.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg

Table B.7.6.1.2-11: PMG and AMPA residues determined by HPLC analysis in 0 – 10 cm soil cores collected after treatment of plot 5.0 with 6.56 kg/ha ^{14}C-PMG (9.51 kg/ha glyphosate-trimesium)

Plot 5.0	**Residues in soil cores (0 - 10 cm)**									
DALT	0		62 (125)		160 (223)		176 (239)		216 (279)	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
TRR	**3.78**	**100**	**1.65**	**100**	**1.41**	**100**	**1.52**	**100**	**0.557**	**100**
ERR	**1.44**	**38.2**	**0.681**	**41.2**	**0.446**	**31.6**	**0.461**	**30.4**	**0.183**	**32.9**
PMG	1.21	31.9	0.117	7.10	0.058	4.09	0.045	2.98	0.049	8.85

Table B.7.6.1.2-11: PMG and AMPA residues determined by HPLC analysis in 0 – 10 cm soil cores collected after treatment of plot 5.0 with 6.56 kg/ha ^{14}C-PMG (9.51 kg/ha glyphosate-trimesium)

Plot 5.0	**Residues in soil cores (0 - 10 cm)**									
DALT	0		62 (125)		160 (223)		176 (239)		216 (279)	
AMPA	0.239	6.31	0.564	34.1	0.388	27.5	0.416	27.4	0.134	24.1
RRR [1]	2.34	61.8	0.973	58.8	0.693	68.4	1.06	69.6	0.374	67.1
Total (TRR)	**3.78**	**100**	**1.65**	**100**	**1.41**	**100**	**1.52**	**100**	**0.557**	**100**

DALT days after last treatment (meaning after the third treatment; values in parenthesis correspond to the days after first treatment)
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue
1 These values were determined by difference; selected cores were combusted for verification of this approach.
All residue data are expressed as mg ^{14}C-PMG anion equiv./kg

C. Storage stability

All of the crop samples were analysed by combustion within 3 to 83 days after sampling, except 308 PBI radish, which was combusted 12 months after sampling. All of the RAC's were extracted and analysed 14 - 18 months (406 – 552 days) after sampling. All of the soil cores were analysed by combustion within 3 to 135 days after sampling. All of the soil cores that were extracted and analysed by chromatography were done 4 - 14 months (114 – 426 days) after sampling. Throughout the study all samples, crops and soils and extracts, were stored in freezers usually at -20 °C.

Analysis of crop extracts showed that glyphosate and AMPA were the major residues beside natural products (see storage stability investigations depicted below). For the stability of glyphosate and AMPA, a high number of storage stability investigations are available in storage stability studies summarised under 7.1.
For the storage stability of natural products, two crop samples, 63 PBI wheat straw and 63 PBI lettuce leaf were used. These samples were removed from the freezer and extracted at 184 and 654 days (wheat straw) and 230 and 692 days (lettuce leaf) after harvest. These crops were extracted using a modified method, which fractionated the crop into its main natural products components. At first, the crop was extracted with cold water and acetonitrile and filtrated, so that a soluble and an insoluble fraction were generated. Then ethanol was added in excess to the soluble fraction to precipitate pectic substances, starch, gums, and fructans. The precipitate and the supernatant, containing monosaccharides and soluble acids, were separated by centrifugation. The insoluble fraction was extracted with NaOH solution and centrifuged or filtered. The precipitate remaining after base extraction contained alpha-cellulose. The supernatant after base extraction was acidified to precipitate high molecular weight hemicellulose, and centrifuged. The supernatant contained low molecular weight hemicellulose. All terminal fractions were analysed for ^{14}C residue by combustion (solid fractions) or by LSC (liquid fractions). This extraction scheme was repeated at a much later date and compared with the results from the first extraction for the two selected crops, lettuce leaf and wheat straw. It is evident that the distribution of residue in these natural product fractions were similar at the two extraction dates for the same crop. This shows that the components of the residue in straw and lettuce were stable under frozen storage for approximately two years (22 to 23 months). The results of these extractions are shown in Table B.7.6.1.2-12.

Table B.7.6.1.2-12: Extraction of the radioactive residues of glyphosate in wheat straw and lettuce leaf – storage stability assessment

	Wheat straw (PBI 63)		**Lettuce leaf (PBI 63)**	
	184 (6 months)	**654 (22 months)**	**230 (8 months)**	**692 (23 months)**
	% TRR	**% TRR**	**% TRR**	**% TRR**
TRR	100	100	100	100
ERR	***44***	***38***	***47***	***55***
Acetone Layer	2	3	3	-
Juice layer	42	35	*44* [2]	55
Pectins	5	13	23	22
Soluble monosaccharides	36	21	21	33
RRR	**56**	**63**	**53**	**45**
Alpha cellulose	36	34	20	27

Table B.7.6.1.2-12: Extraction of the radioactive residues of glyphosate in wheat straw and lettuce leaf – storage stability assessment

	Wheat straw (PBI 63)		**Lettuce leaf (PBI 63)**	
	184 (6 months)	**654 (22 months)**	**230 (8 months)**	**692 (23 months)**
	% TRR	**% TRR**	**% TRR**	**% TRR**
Hemicellulose (low molecular weight)	19	28	26	12
Hemicellulose (high molecular weight)	1	1	4	6
NaOH wash	-	-	3	-
Total	**100**	**100**	**100**	**100**

TRR Total radioactive residue (expressed as mg ^{14}C-PMG anion equiv./kg)
ERR Extractable radioactive residue (calculated as sum of acetone layer and juice layer)
RRR Residual radioactive residue
1 Storage intervals were calculated using date of harvest and date of HPLC analysis (the latest event, reflecting the longest storage duration as the most critical scenario)
2 Too dark to LSC. Recoveries determined from subsequent fractions.
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding.

D. Degradation pathway

Please refer to the overall pathway of glyphosate in rotational crops in Vol. 1, 2.7.7.

III. Conclusion

The uptake and metabolism of glyphosate was examined in rotational crops. *N*-(phosphono-methyl)glycine labelled in the methylene position (^{14}C-PMG-label) was applied as its trimesium salt to two plots at different application rates; three additional plots received a comparable treatment of unlabelled active substance as control plots. The test item ^{14}C-PMG labelled glyphosate-trimesium was applied at a rate of 5.617 kg a.s./ha (3.87 kg a.s./ha expressed as glyphosate equivalents, plot 1.0) and at a total rate of 9.51 kg a.s./ha (three monthly applications, 6.56 kg a.s./ha expressed as glyphosate equivalents, plot 5.0) to bare soil.

A primary crop, soybean, was planted prior to treatment in all plots containing sandy loam soil. After removal of the primary crop, the rotational crops lettuce, radish and wheat were planted into the subplots at 35, 63, and 308 days after herbicide treatment (35, 63, and 308 plant-back intervals, PBI).

The soya cover crop was not analysed. Residues samples from the rotated crops were extracted using a mixture of 0.1N HCl and chloroform, followed by column fractioning using different solvents to separate residues and natural products. To characterise incorporation into natural products, postextraction solids were additionally hydrolysed under acid and basic conditions. The TRR levels in matrices obtained from rotational crops were relatively low, not exceeding 0.1 mg/kg, except for lettuce (0.127 mg/kg). The rotational crops from the 35 and 63 PBI plots contained TRR levels of 0.020 – 0.076 mg/kg and 0.021 – 0.127 mg/kg, respectively. Crops from the 308 PBI contained TRR levels of 0.010 – 0.038 mg/kg. All the residues were determined as *N*-phosphonomethylglycine (PMG) anion equivalents (mg ^{14}C-PMG anion equiv./kg, stated in the following only as mg/kg).

Analysis of rotational crop samples, after extraction with water and chloroform, revealed three residue components: PMG (glyphosate-anion), aminomethylphosphonic acid (AMPA) and a polar unknown metabolite (called metabolite 1 within the report). AMPA was found at levels of 8.7 - 34 % of the TRR. PMG was also detected in most samples, but its levels were <2.3 % of the TRR. Metabolite 1 was not identified as the amount was <0.01 mg/kg in all RAC's. Residues after extraction with water and chloroform were further investigated and were identified as being starch, lignins, amino acids and cellulose, as well as carbohydrates as glucose and fructose.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study assessing the metabolic behavior of ^{14}C-labelled glyphosate in rotational crops of lettuce, wheat and radish has been previously evaluated at EU level. It was performed under GLP. The study is deemed to largely comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Metabolism in Rotational Crops, 502 with some deficits (minor deviations from the current guideline are listed above in section 1, Information on the study): No attempts were made to further solubilise the non-extractable radioactivity, so final residues were only analysed by combustion in some cases and were between 29.4 to 69.6 % of the TRR (0.245 – 2.34 mg/kg). Analysis of crop samples was not done within 6 months after sampling, but within 14 – 18 months. A storage stability investigation was conducted for natural products. Beside natural products only the test item PMG and AMPA were found in crop samples. For PMG and AMPA storage stability is covered by separate storage stability studies for up to 18 months ([redacted], 2010, CA 6.1/03, [redacted] 1991, CA 6.1/13 and [redacted], J.C., 1989, CA 6.1/14), so the storage periods in this study are adequately covered. Extraction rates were low to moderate for crop samples (23.8 to 54.2 % of the TRR), several attempts (acid and/or basic hydrolysis) were made to characterise the non-extractable radioactivity. However, final residues were between 13.1 to 59.4 % of the TRR, with absolute residues levels between 0.005 – 0.014 mg/kg.

The study is considered to be reliable for the assessment of the metabolic behaviour of glyphosate in rotational crops

Assessment and conclusion by RMS:
Extractability with conventional methods was up to 54.2% TRR, which is considered not very high. Further investigation of the non-extractable residues resulted in additional characterization of the residues. Since the final residue was only up to 0.014 mg glyphosate-trimesium equiv./kg, and sufficient attempts to identify/characterize have been undertaken, this is considered acceptable. The remark from the applicant that final residues were up to 2.34 mg/kg concerns soil samples, which are not required to be analysed according to the guidelines. The assessment of the applicant on storage stability should be considered in the light of the evaluation of the RMS in Vol. 1, 2.7.1. Glyphosate is shown to be stable in watery matrix (including lettuce leaves, wheat forage and radish tops) for approximately 24 months, which covers the maximum storage time in the study. Similarly, storage stability of AMPA in watery crops is demonstrated for 18 months, thereby also covering the maximum storage period. Glyphosate is demonstrated to be stable in dry commodities (including hay and straw) for 24 months, which covers the storage duration in the current study. In contrast, storage stability of AMPA in straw and hay is variable, making it difficult to draw a general conclusion. Regarding the storage period of wheat grain and radish roots, glyphosate is considered stable for 24 months in starch containing crops, thus covering the max. possible storage time in this study. Storage of AMPA in crops with a high starch content is demonstrated for max. 10-12 months, which is not covering the time period of the current study. Although there is only 4-8 months difference between the demonstrated storage stability period and the max. period of sample storage, results of AMPA in roots and grains are considered less reliable. Altogether, storage stability is considered to not impact this metabolism study to a large extent, except for the results of AMPA in roots, grain, straw and hay. Therefore, these results should be considered with caution, since the levels might be underestimated. In addition, in several other metabolism studies (see also references in assessment of applicant), it was shown that degradation of radioactive residues was not an issue.
Therefore, altogether, the study is considered acceptable.

B.7.6.1.3. Carrot, lettuce and barley

1. Information on the study

Data point:	CA 6.6.1/003
Report author	[redacted]
Report year	1990
Report title	Confined Rotational Crop Study of Glyphosate. Part I: In-Field Portion. (Part II: MSL-9811)
Report No	MSL-9810
Document No	-
Guidelines followed in study	Pesticide Assessment Guideline Subdivision N, Number 165-1
Deviations from current test guideline	See the table below

Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	Conclusion applicant: valid (Category 2a) Conclusion RMS: acceptable

Data point:	CA 6.6.1/003
Report author	[redacted]
Report year	1990
Report title	Confined Rotational Crop Study of Glyphosate. Part II: Quantitation, Characterisation, and Identification of Glyphosate and Its Metabolites in Rotational Crops, (Part I: MSL-9810)
Report No	MSL-9811
Document No	-
Guidelines followed in study	Pesticide Assessment Guideline Subdivision N, Number 165-1
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Testing of Chemicals, 501: • Growth stage at sampling of immature crop samples is not given within the report; however could be roughly estimated based on planting and sampling dates. • The frozen samples were stored at -5°C or below and not at -18°C, however, storage stability at -5°C was demonstrated for the study duration. • For barley straw and carrot tops the initial extraction for storage analysis was performed 7 months after sampling and not within the 6 months, for which the frozen samples are assumed to be stable. • No date of analysis is given within the report. Less than 90 % of TRR was identified or characterised. Several attempts were made to characterise the bound radioactivity for selected matrices, but at least 20.7 – 28.7 % TRR (0.0278 – 0.0581 mg/kg) remained non-extracted.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	Conclusion applicant: valid (Category 2a) Conclusion RMS: acceptable

2. Full summary of the study according to OECD format

Executive Summary

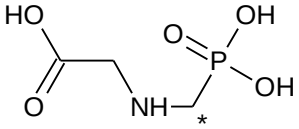
The metabolism of glyphosate was examined in rotational crops. ^{14}C-glyphosate formulated as Roundup® was applied to plots of bare sandy loam soil at a rate of 4.16 kg a.s./ha. A primary crop of soybeans was planted 7 days after application. The primary crop was harvested and the plots rototilled before planting rotational crops of lettuce, carrots, and barley into the subplots at 30, 119, and 364 days after herbicide treatment.

The primary and rotational crops were sampled for analysis. The rotational crops from the 30 DALT plots contained 0.037 – 0.188 mg/kg of glyphosate equivalent residues. Crops from the 119 DALT planting contained residues of 0.017 – 0.078 mg/kg. Carrots, barley, and lettuce from the 364 day planting contained residues of 0.0096 to 0.061 mg/kg. Analysis of rotational crop samples revealed two residue components, aminomethylphosphonic acid (AMPA) and a polar metabolite (called Metabolite 1 within the report) characterised as being a mixture of sugars, primarily glucose and fructose. Glyphosate was present only in lettuce, barley straw and grain of the first rotation 1.0 – 9.8 % TRR (0.0018 – 0.0184 mg/kg) and in lettuce DALT 167 of the second rotation 1.6 % TRR (0.0009 mg/kg). AMPA ranged from 3.7 – 17.9, 1.1 – 14.2 and 7.7 – 20.0 % TRR (0.0007 – 0.0336, 0.0003 – 0.0111 and 0.0045 – 0.0093 mg/kg) in the matrices of the crops of the first, second and third rotation, respectively. Metabolite 1 amounted

to 7.7 – 40.8, 6.3 – 24.9, 6.6 – 31.9 % TRR (0.0136 – 0.0327, 0.0039 – 0.0147 and 0.0031 – 0.0182 mg/kg) in the matrices of the crops of the first, second and third rotation, respectively.
Residues after extraction with water and chloroform were further investigated and were identified as being starch, lignin and cellulose, as well as biopolymers of glucose.

I. Materials and Methods

A. Materials

1. Test material	N-(phosphonomethyl)glycine; mixture of a) N-(phosphono-^{14}C-methyl)glycine (227.3 mg) b) N-(phosphono-^{12}C-methyl)glycine (3681.7 mg)
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	96.5 % (contained 2.4 % AMPA, 1 % nonionic component)
Chemical purity	>99 %
Specific activity of the test substance applied:	0.29 MBq/mg (1.31 mCi/mmol)

2. Test system	
Soil:	Sandy loam (pH: 6.2-7.3; cation exchange capacity: 3.6-4.4 meq./100 g; sand: 62 – 70 %; silt: 23 - 31 %; clay: 9 %; textural class (USDA): sandy loam)
Crop:	Primary crop: Soybean (var. Williams) Rotational crops: Carrot (var. Goldmine), Lettuce (var. Waldmann's Green Leaf), Barley (variety Barley Blend BB88-2, 425:X1275)
Botanical name:	*Glycine max (L.) Merr.* *Daucus carota subsp. sativus.* Hoffm. *Latuca sativa* L. *Hordeum vulgare* L.
Crop part(s):	Carrot roots and tops, lettuce leaves, barley forage, straw, seeds

B. Study design

1. In-life phase

The in-life phase of the study was conducted outdoors in [redacted], California.
The test substance contained 227.3 mg of N-(phosphono-^{14}C-methyl)glycine (^{14}C-glyphosate) with a specific activity of 4.93 MBq/mg (22.53 mCi/mmol) and 3681.7 mg of N-(phosphonomethyl)glycine (^{12}C-glyphosate). Final specific activity of the test substance was 0.29 MBq/mg (1.31 mCi/mmol).
Additionally, a test substance containing 3.909 g of unlabelled glyphosate (^{12}C test substance) was prepared for application to the control plot.
Glyphosate in both test substances was formulated as the isopropylamine salt, and a tallowamine surfactant was added. The relative proportions of glyphosate, isopropylamine, and surfactant in the test substance were prepared to be the same as in the formulation of Roundup®.
At study initiation, the plots were planted with annual rye grass. The rye grass was allowed to grow to a height of 4-6 inches prior to the application of glyphosate.
^{14}C-glyphosate was applied to the test plots at a target rate of 4.16 kg a.s./ha on bare soil. The control plots were treated with ^{12}C-glyphosate at the same rate. A CO_2 backpack sprayer was used for the application of the aqueous spray solutions.
Primary crop soybean was planted in all subplots 7 days after the application. For each subplot a single row of soybean seeds was hand planted to a depth of 2.5 cm. The plots were rototilled to a depth of 10 cm prior to planting.

To assess the results of crop failure, 30 days after treatment, the soybean crop foliage in one-third of the plot was collected and the plot then rototilled in both the control and ^{14}C treated plots. Each subplot was further divided into three equal mini-plots (0.61 x 1.69 m) which were planted with one of the rotational crops (carrots, lettuce, or barley). A second subplot from each plot was prepared for planting 119 days after treatment. All soybean foliage within each of the 119 day subplots was collected. Three mini-plots (0.61 x 1.69 m) were each rototilled and planted with one of the rotational crops (carrots, lettuce, or barley).
The mature soybean crop was harvested from the final subplot and the plot prepared for planting 364 days after treatment. Three mini-plots (0.61 x 1.69 m) were each planted with one of the rotational crops (carrots, lettuce, or barley).
Each of the mini-plots was maintained in accordance with normal agricultural practice until a conventional harvest of each of these rotational crops was completed.

2. Sampling

The soybean foliage sample was harvested from the first subplot at 30 DALT. Soybean samples from the second subplot were harvested at 119 DALT, and mature soybeans from the third subplot were harvested at 182 DALT. The samples were divided in leaves, stems, pods and seeds. A soybean foliage sample from the second and third subplots was also harvested at 70 DALT. The rotational crops were planted at 30 DALT in the first subplot, at 119 DALT in the second subplot, and at 364 DALT in the third subplot. Lettuce was harvested at two intermediate times and at maturity in each subplot, while carrots were harvested only at maturity. Barley was harvested only at maturity in the 30 and 119 DALT subplots due to shortage of sample. In addition to the harvest at maturity, a barley forage sample was collected from the 365 DALT subplot. Carrots were separated into tops and root, and barley was separated into heads and straw. All samples were double-bagged with plastic and cloth bags and frozen immediately after collection. Frozen samples were placed in insulated containers with dry ice and shipped to the analytical facility via overnight delivery.
Samples were either immediately prepared for analysis or stored frozen below -5°C until preparation.
The soil in which the crops were growing was analysed at individual time points to follow the degradation of glyphosate and to identify the metabolites to which the rotational crops were exposed. Samples were taken prior to and immediately after treatment, at primary crop planting, and at each rotational crop planting and harvest. Soil samples were collected with a zero-contamination corer measuring one inch (ca 3 cm) in diameter. Cores were collected to a depth of ca 30 cm and separated into 0-15 cm and 15-30 cm sections. Soil samples were shipped frozen to the analytical facility.

3. Analytical procedures

Total radioactive residues (TRR) in all plant samples were determined by liquid scintillation counting (LSC) following combustion. Moisture content was determined at the time combustion aliquots were taken.
Samples were prepared by grinding the whole frozen sample in a vertical cutter mixer with dry ice until the sample was a fine powder. Subsamples of each crop sample were homogenised first with water, than with chloroform. Samples were centrifuged and the aqueous layer was decanted and filtered. The aqueous extract was concentrated and an aliquot removed for LSC analysis. The concentrated samples were analysed by HPLC using an Aminex A-5 cation exchange column.
Unextractable residues were further analysed from a representative 105 DALT lettuce sample. This sample was chosen because of the quantity of sample available and the residue levels present. The solid plant tissue remaining after aqueous extraction was homogenised with 5.0 M NH_4OH. After centrifugation, the supernatant was decanted and concentrated. The concentrated extract was analysed by HPLC on an Aminex A-5 column. The solid tissue remaining from the ammonium hydroxide extraction was extracted with DMSO at 100°C for 16 hours to remove starch and lignin. After centrifugation, decantation and filtration, the supernatant was concentrated and an aliquot was taken for LSC analysis. The concentrated extract was lyophilised, and aliquots of the resultant solid were combusted. The solid residue from the lyophilisation of the DMSO extract was incubated with amyloglucosidase (from *Aspergillus niger*) in a sodium acetate buffer pH 4.5 at 55°C for 6 hours. This enzyme liberates glucose from starch. After centrifugation, the sample supernatant was concentrated and the concentrate applied to a Bio-Gel P-2 column. The column was eluted with water and 1.0 min fractions were collected and analysed by LSC. Fractions were also analysed for glucose using Chemstrips.
The 125 DALT barley grain and straw samples were analysed using the same procedure described for the 105 DALT lettuce sample. In addition, the straw tissue remaining after the DMSO extraction was washed with water to remove any residual DMSO. The sample was incubated with cellulase (from *Aspergillus niger*) in a 0.05 M sodium acetate buffer pH 5.0 at 37°C for 16 hours. This enzyme liberates glucose from cellulose. The mixture was centrifuged, the supernatant decanted, and concentrated. An aliquot of the concentrate was applied to a Bio-Gel P-2 column. The column was eluted with water and 1.0 min fractions were collected. The fractions were analysed for glucose using Chemstrips and by LSC.

The identity of the AMPA component was verified by co-elution with standards on two chromatography systems: Aminex A-5 cation exchange column and an anion exchange column packed with Dowex AG-1 in the chloride form. Metabolite 1 eluting near the void volume was isolated from the 105 DAT lettuce sample by making repeated injections on the HPLC, using the Aminex A-5 column, and collecting the effluent from 1 – 15 min. The effluent was concentrated and aliquots were analysed by anion exchange chromatography on a Spherisorb S-5 SAX column and by reverse phase chromatography on a Cl8 reverse phase column. The effluent was further purified by HPLC using the Cl8 reverse phase column. Aliquots of the combined and concentrated effluent were analysed by LSC, Chemstrips and HPLC (Bio-Rad HFX-87H column). Glucose, fructose and sucrose standards were also analysed by HPLC to compare retention times. Another aliquot, after additional purification (HPX-87H columns) was analysed by NMR. A mixture of glucose and fructose was also analysed by NMR for comparison. Another portion of the isolated metabolite/matrix was analysed on a Bio-Gel P-2 sizing column (molecular weight range 100-1800). The sample was applied to the C18 column and eluted with water. Fractions were collected and analysed by LSC. Again, glucose, fructose, and sucrose standards were also analysed to compare retention times.
Homogenised soil samples were analysed by combustion to determine the amount of ^{14}C residue. Moisture content was determined at the time combustion aliquots were taken. A pooled soil sample was prepared for each sampling point. An aliquot was taken from each soil core of a given sampling time, and these aliquots were combined to generate the pooled sample. Each pooled sample was extracted twice with 0.5 M NH_4OH. Samples were centrifuged and the supernatant decanted. The supernatants were combined and an aliquot taken for LSC analysis. Concentrated extracts were analysed by LSC and HPLC using an Aminex A-9 cation exchange column.
Identification of glyphosate and AMPA was confirmed by co-elution with authentic standards under two separate chromatographic conditions (HPLC using an Aminex A-9 cation exchange column and column chromatography on Dowex AG-1 chloride form).
The metabolite eluting near the void volume (Metabolite A) was isolated from the 147 DAT soil sample by making repeated injections on the HPLC, using an Aminex A-9 column, and collecting the effluent from 1 – 8 min. The effluent was concentrated and an aliquot was reanalysed on an Aminex A-9 column. An aliquot of Metabolite A, isolated from the cation exchange column, was analysed by reverse phase chromatography on a C18 reverse phase column. Other aliquots of the metabolite were analysed by HPLC on a Bio-Rad HPX-87H organic acids column used for carbohydrate analysis and chromatographed on a Bio-Gel P-2 sizing column.

II. Results and Discussion

A. Total radioactive residues (TRRs)

After the first rotation, total radioactive residue (TRR) in edible commodities (lettuce leaves, carrot roots as well as barley grain) ranged from 0.048 to 0.188 mg/kg, and in non-edible commodities (carrot tops and barley straw) from 0.051 to 0.175 mg/kg. After the second rotation, TRR values in edible commodities (lettuce leaves, carrot roots as well as barley grain) ranged from 0.017 to 0.078 mg/kg, and in non-edible commodities (carrot tops and barley straw) from 0.028 to 0.056 mg/kg.
After the third rotation, TRR values in edible commodities (lettuce leaves, carrot roots as well as barley grain) ranged from 0.0096 to 0.057 mg/kg. In non-edible commodities (carrot tops, barley forage and straw) from 0.018 to 0.061 mg/kg.
Residue levels in soil of 0 – 15 cm depth decrease from approximately 0.74 mg/kg to approximately 0.18 mg/kg over the course of this study. The soil characteristics of the [redacted] soil indicated a sandy loam soil with a low organic matter content. The radioactivity found in the 15-30 cm cores may be due to contamination from the 0-15 cm level during sampling since the 0 DALT post application sample has the highest residue level of any 15-30 cm core.

Table B.7.6.1.3-1: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
Primary crop	-	Soybean	Foliage	30	0.4329
				70	0.3309
			Soybean	119	0.2327
			Leaves	177	0.0918
			Stems	182	0.0822
			Pods	182	0.2276
			Seeds	182	0.3185

Table B.7.6.1.3-1: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)
1st rotation	30	Lettuce	Leaves	70	0.108
				90	0.048
				105	0.097
		Barley	Straw	125	0.175
			Grain	125	0.188
		Carrot	Tops	154	0.051
			Root	154	0.037
2nd rotation	119	Lettuce	Leaves	147	0.059
				167	0.055
				181	0.037
		Barley	Straw	314	0.056
			Grain	314	0.078
		Carrot	Tops	210	0.028
			Root	210	0.017
3rd rotation	364	Lettuce	Leaves	399	0.057
				425	0.043
				455	0.028
		Barley	Forage	412	0.056
			Straw	482	0.061
			Grain	482	0.047
		Carrot	Tops	482	0.018
			Root	482	0.0096

TRR – total radioactive residue, expressed as glyphosate, calculated within the report based on sample fresh weight
DALT – days after last treatment
PBI – plant back interval

Table B.7.6.1.3-2: Total radioactive residues in soil after application of ^{14}C-glyphosate to bare soil

Rotation. PBI	DALT	TRR (mg/kg)	
		Soil depth 0 – 6 inches (ca 0 – 15 cm)	Soil depth 6 – 12 inches (ca 15 - 30 cm)
-	0 (post application)	0.711	0.0453
	7	0.738	0.0088
1st rotation, PBI 30 days	30	0.518	0.0017
	76	0.518	0.0036
	90	0.354	0.0016
	105	0.526	0.0021
	125	0.625	0.0009
	154	0.250	0.0010
2nd rotation, PBI 119 days	119	0.142	0.0011
	147	0.589	0.0001
	167	0.372	0.0006
	181	0.203	0.0009
	210	0.277	0.0006

Table B.7.6.1.3-2: Total radioactive residues in soil after application of ^{14}C-glyphosate to bare soil

Rotation. PBI	DALT	TRR (mg/kg)	
		Soil depth 0 – 6 inches (ca 0 – 15 cm)	Soil depth 6 – 12 inches (ca 15 - 30 cm)
	314	0.250	ND
3rd rotation, PBI 365 days	364	0.184	0.0009
	399	0.297	ND
	412	0.211	0.0032
	425	0.248	0.0011
	455	0.172	0.0021
	482	0.179	ND

TRR – total radioactive residue, expressed as glyphosate; calculated within the report based on soil dry weight
DALT – days after last treatment
ND – not detected

B. Extraction and characterisation of residues

Lettuce leaves

From the first rotation, 44.6 to 49.1 % of the TRR (0.0219 to 0.0530 mg/kg) in lettuce collected 70, 90 and 105 DALT was found in the water phase, whereas 48.9 % – 59.9 % of the TRR (0.0288 – 0.0551 mg/kg) remained in the solids. The water phase contained 8.1 – 14.6 % TRR (0.0039 – 0.0158 mg/kg) AMPA and 28.6 – 33.8 % TRR (0.0162 – 0.0327 mg/kg) Metabolite 1. Glyphosate was only found in lettuce leaves sampled 70 and 105 DALT and amounted to 2.9 to 3.8 % TRR (0.0028 – 0.0041 mg/kg) (Table B.7.6.1.3-3).

The metabolite eluting near the void volume, Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Since the aqueous extraction removed less than 50 % of the total radioactive residue, the extraction was repeated and the residue was further examined for lettuce 105 DALT. 11.3 % TRR (0.0110 mg/kg) was additionally released by NH_4OH. The profiles of the NH_4OH extracts analysed by cation exchange chromatography were very similar to the profiles of the water extracts. The same components were extracted with water and ammonium hydroxide, although the ratios of the components were different. The residue remaining after the NH_4OH treatment was stirred with DMSO to solubilise starch and lignin. The solvent was removed from the solubilised material and the remaining solid was subjected to enzymatic degradation with amyloglucosidase. This enzyme liberates glucose from starch. Chromatography, on a Bio-Gel P-2 column, of the radioactivity in solution after degradation shows a broad peak which elutes in the region of glucose. Testing of the collected fractions with Chemstrips showed the co-elution of glucose with a significant fraction of the radioactivity. This result suggests that DMSO extracted starch, into which the label had been reincorporated. The material that was not digested by the enzyme could be lignin. 14.8 % TRR (0.0144 mg/kg) was released after DMSO treatment. The final residue amounted to 28.7 % of the TRR (0.0278 mg/kg) (Table B.7.6.1.3-10).

From the second rotation, 34.3 to 40.1 % of the TRR (0.0148 to 0.0208 mg/kg) in lettuce collected 147, 167 and 181 DALT was found in the water phase. Non-extractable residues amounted to 57.0 to 70.3 % (0.0231 to 0.0415 mg/kg) of the TRR. The water phase contained 4.6 – 12.4 % TRR (0.0027 – 0.0050 mg/kg) AMPA and 15.1 – 24.9 % TRR (0.0083 – 0.0147 mg/kg) Metabolite 1. Glyphosate was found only in lettuce leaves sampled 167 DALT and amounted to 1.6 % TRR (0.0009 mg/kg) (Table B.7.6.1.3-5).

From the third rotation, 37.2 to 56.4 % of the TRR (0.0158 to 0.0279 mg/kg) was found in the water phase. Non-extractable residues amounted to 45.3 to 62.2 % of the TRR (0.0127 to 0.0311 mg/kg). The water phase contained 10.5 – 20.0 % TRR (0.0045 – 0.0076 mg/kg) AMPA and 18.6 – 31.9 % TRR (0.0079 – 0.0182 mg/kg) Metabolite 1. Glyphosate was not detected (Table B.7.6.1.3-7).

Barley grain

From the first, second and third rotations, 15.3, 25.2 and 24.8 % of the TRR (0.0288, 0.0197 and 0.0117 mg/kg) in barley grain was found, respectively, in the water phase. Non-extractable residues amounted to 82.8, 61.7 and 69.6 % of the TRR (0.1557, 0.1160 and 0.0327 mg/kg), respectively. The water phase of barley grain from the first, second and the third rotations contained 17.9, 14.2 and 15.7 % TRR (0.0336, 0.0111 and 0.0074 mg/kg) AMPA and 7.7, 6.3 and 6.6 % TRR (0.0144, 0.0049 and 0.0031) Metabolite 1, respectively. Glyphosate was only identified in grain of the first rotation at 9.8 % TRR (0.0184 mg/kg) (Table B.7.6.1.3-4, Table B.7.6.1.3-6 and Table B.7.6.1.3-8).

For barley grain collected 125 DALT (first rotation) further treatment procedures were employed 22.5 % TRR (0.0423 mg/kg) was additionally released by NH_4OH. Similar to lettuce leaves the profiles of the NH_4OH extracts of barley grain analysed by cation exchange chromatography were very similar to the profiles of the water extracts. The same components were extracted with water and ammonium hydroxide, although the ratios of the components were different. The residue remaining after the NH4OH treatment was stirred with DMSO to solubilise starch and lignin. The solvent was removed from the solubilised material and the remaining solid was subjected to enzymatic degradation with amyloglucosidase. Chromatography, on a Bio-Gel P-2 column, of the radioactivity in solution after degradation showed a broad peak which elutes in the region of glucose. Testing of the collected fractions with Chemstrips showed the co-elution of glucose with a significant fraction of the radioactivity. This result suggests that DMSO extracted starch, into which the label had been reincorporated. The material that was not digested by the enzyme could be lignin. 20.4 % TRR (0.0384 mg/kg) was released after treatment with DMSO. The final residue after solvent extraction and further treatments for solubilisation amounted to 30.9 % of the TRR (0.0581 mg/kg) (Table B.7.6.1.3-10).

Carrot roots

From the first, second and third rotations, 54.4, 32.1 and 39.9 % of the TRR (0.0201, 0.0055 and 0.0038 mg/kg) in carrot roots was found in the water phase. Non-extractable residues amounted to 49.7, 61.9 and 64.3 % of the TRR (0.0184, 0.0105 and 0.0062 mg/kg), respectively. The water phase of carrot roots from the first and the second rotations contained 11.1 and 8.2 % TRR (0.0041 and 0.0014 mg/kg) AMPA and 40.8 and 22.9 % TRR (0.0151 and 0.0039) Metabolite 1, respectively. Glyphosate was not detected. (Table B.7.6.1.3-4, Table B.7.6.1.3-6 and Table B.7.6.1.3-9).

For the carrot roots after the third rotation no metabolite elucidation was performed due to low TRRs found.

Barley forage

Barley forage was sampled only after the third rotation. A total of 31.1 % of the TRR (0.0174 mg/kg) in barley forage was found in the water phase. Non-extractable residues amounted to 67.3 % of the TRR (0.0377 mg/kg). The water phase of barley forage contained 16.6 % TRR (0.0093 mg/kg) AMPA and 14.6 % TRR (0.0082 mg/kg) Metabolite 1. Glyphosate was not detected (Table B.7.6.1.3-8).

Barley straw

From the first, second and third rotation, 25.9, 19.2 and 22.7 % of the TRR (0.0453, 0.0108 and 0.0138 mg/kg) in barley straw was found, respectively, in the water phase. Non-extractable residues amounted to 74.3, 78.3 and 80.9 % of the TRR (0.1300, 0.0438 and 0.0493 mg/kg), respectively.

The water phase of barley straw from the first, second and the third rotations contained 3.7, 9.6 and 7.7 % TRR (0.0065, 0.0054 and 0.0047 mg/kg) AMPA and 16.8, 8.2 and 11.1 % TRR (0.0294, 0.0046 and 0.0068 mg/kg) Metabolite 1, respectively. Glyphosate was detected only in the straw of the first rotation at 1.0 % TRR (0.0018 mg/kg) (Table B.7.6.1.3-4, Table B.7.6.1.3-6 and Table B.7.6.1.3-8).

For barley straw collected 125 DALT (first rotation) further treatment procedures were employed. 8.9 % TRR (0.0156 mg/kg) was additionally released by NH_4OH. The residue remaining after the NH_4OH treatment was stirred with DMSO to solubilize starch and lignin. Another 17.0 % TRR (0.0298 mg/kg) was released. NH_4OH and DMSO fractions from barley straw (125 DALT) were further analysed in the same way as the corresponding fractions from lettuce (105 DALT) and barley grain (125 DALT). The same findings were observed. Additionally, the unextracted barley straw tissue remaining after DMSO extraction was subjected to enzymatic digestion using a cellulase, which liberates glucose from cellulose. 36.0 % TRR (0.0630 mg/kg) was released. Chromatography on a BioGel P-2 sizing column again showed a broad peak which eluted in the region of glucose. Testing of the fractions with Chemstrips showed again that glucose co-eluted with a major portion of the sample radioactivity. The final residue amounted to 20.7 % of the TRR (0.0362 mg/kg) (Table B.7.6.1.3-10).

Carrot tops

From the first, second and third rotation, 27.3, 24.5 and 22.7 % of the TRR (0.0139, 0.0069 and 0.0041 mg/kg) in carrot tops was found, respectively, in the water phase. Non-extractable residues amounted to 70.7, 83.2 and 67.0 % of the TRR (0.0361, 0.0233 and 0.0121 mg/kg), respectively.

The water phase of carrot tops from the first and the second rotations contained 1.4 and 1.1 % TRR (0.0007 and 0.0003 mg/kg) AMPA and 26.7 and 17.5 % TRR (0.0136 and 0.0049 mg/kg) Metabolite 1, respectively. Glyphosate was not detected.

For the carrot roots after the third rotation no metabolite elucidation was performed due to low TRRs found (Table B.7.6.1.3-4, Table B.7.6.1.3-6 and Table B.7.6.1.3-9).

Table B.7.6.1.3-3: Identification and characterisation of the radioactive residues of glyphosate in lettuce leaves of rotational crop (first rotation, PBI 30 days) planted after application of glyphosate to bare soil

	First rotation, PBI 30 days					
	Residues in lettuce leaves					
DALT	**70**		**90**		**105[3]**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.108	100	0.048	100	0.097	100
Extraction with water and chloroform[1]						
Water phase	*0.0530*	49.1	*0.0219*	45.7	*0.0433*	44.6
Metabolite 1[2]	0.0309	*28.6*	0.0162	*33.8*	0.0327	*33.7*
Glyphosate	0.0041	*3.8*	ND	*ND*	0.0028	*2.9*
AMPA	0.0158	*14.6*	0.0039	*8.1*	0.0137	*14.1*
Identified	*0.0199*	*18.4*	*0.0039*	*8.1*	*0.0165*	*17.0*
Characterised	*0.0309*	*28.6*	*0.0162*	*33.8*	*0.0327*	*33.7*
ERR	*0.0530*	49.1	*0.0219*	45.7	*0.0433*	44.6
RRR	*0.0528*	48.9	*0.0288*	59.9	*0.0551*	*56.2[4]*
Total	0.1058	98.0	0.0507	105.6	0.0978	100.8

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] only water phase was analysed. Chloroform phase was not analysed.
[2] Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.
[3] Amounts of glyphosate and its metabolites presented for lettuce leaves 105 DALT reflect the sum of the component levels of all extractions (see also Table B.7.6.1.3-10)
[4] within the report erroneously referred as 5.6 % TRR

Table B.7.6.1.3-4: Identification and characterisation of the radioactive residues of glyphosate in barley and carrot commodities of rotational crop planted after application of glyphosate to bare soil

	First rotation, PBI 30 days							
	Barley straw		**Barley grain**		**Carrot tops**		**Carrot roots**	
DALT	**125**				**154**			
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.175	100	0.188	100	0.051	100	0.037	100
Extraction with water and chloroform[1]								
Water phase	*0.0453*	25.9	*0.0288*	15.3	*0.0139*	27.3	*0.0201*	54.4
Metabolite 1[2]	0.0294	*16.8*	0.0144	*7.7*	0.0136	*26.7*	0.0151	*40.8*
Glyphosate	0.0018	*1.0*	0.0184	*9.8*	ND	*ND*	ND	*ND*
AMPA	0.0065	*3.7*	0.0336	*17.9*	0.0007	*1.4*	0.0041	*11.1*
Identified	*0.0083*	*4.7*	*0.0520*	*27.7*	*0.0007*	*1.4*	*0.0041*	*11.1*
Characterised	0.0294	*16.8*	0.0144	*7.7*	0.0136	*26.7*	0.0151	*40.8*
ERR	*0.0453*	25.9	*0.0288*	15.3	*0.0139*	27.3	*0.0201*	54.4
RRR	*0.1300*	74.3	*0.1557*	82.8	*0.0361*	70.7	*0.0184*	49.7

Table B.7.6.1.3-4: Identification and characterisation of the radioactive residues of glyphosate in barley and carrot commodities of rotational crop planted after application of glyphosate to bare soil

	First rotation, PBI 30 days							
Total	*0.1754*	100.2	*0.1844*	98.1	*0.0481*	94.3	*0.0385*	104.1

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] only water phase was analysed. Chloroform phase was not analysed.
[2] Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Table B.7.6.1.3-5: Identification and characterisation of the radioactive residues of glyphosate in lettuce leaves of rotational crop (second rotation, PBI 119 days) planted after application of glyphosate to bare soil

	Second rotation, PBI 119 days					
	Lettuce leaves					
DALT	**147**		**167**		**181**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.059	100	0.055	100	0.037	100
Extraction with water and chloroform[1]						
Water phase	*0.0208*	35.3	*0.0189*	34.3	*0.0148*	40.1
Metabolite 1[2]	0.0147	*24.9*	0.0083	*15.1*	0.0087	*23.5*
Glyphosate	ND	*ND*	0.0009	*1.6*	ND	*ND*
AMPA	0.0027	*4.6*	0.0050	*9.1*	0.0046	*12.4*
Identified	0.0027	*4.6*	*0.0059*	*10.7*	0.0046	*12.4*
Characterised	0.0147	*24.9*	0.0083	*15.1*	0.0087	*23.5*
ERR	*0.0208*	35.3	*0.0189*	34.3	*0.0148*	40.1
RRR	*0.0415*	70.3	*0.0314*	57.0	*0.0231*	62.4
Total	*0.0623*	105.6	*0.0502*	91.3	*0.0379*	102.5

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] only water phase was analysed. Chloroform phase was not analysed.
[2] Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Table B.7.6.1.3-6: Identification and characterisation of the radioactive residues of glyphosate in barley and carrot commodities of rotational crop (second rotation, PBI 119 days) planted after application of glyphosate to bare soil

	Second rotation, PBI 119 days							
	Barley straw		**Barley grain**		**Carrot tops**		**Carrot roots**	
DALT	**314**				**210**			
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**

Table B.7.6.1.3-6: Identification and characterisation of the radioactive residues of glyphosate in barley and carrot commodities of rotational crop (second rotation, PBI 119 days) planted after application of glyphosate to bare soil

	Second rotation, PBI 119 days							
TRR	0.056	100	0.078	100	0.028	100	0.017	100
Extraction with water and chloroform[1]								
Water phase	*0.0108*	19.2	*0.0197*	25.2	*0.0069*	24.5	*0.0055*	32.1
Metabolite 1[2]	0.0046	*8.2*	0.0049	*6.3*	0.0049	*17.5*	0.0039	*22.9*
Glyphosate	ND	*ND*	ND	*ND*	ND	*ND*	ND	*ND*
AMPA	0.0054	*9.6*	0.0111	*14.2*	0.0003	*1.1*	0.0014	*8.2*
Identified	0.0054	*9.6*	0.0111	*14.2*	0.0003	*1.1*	0.0014	*8.2*
Characterised	0.0046	*8.2*	0.0049	*6.3*	0.0049	*17.5*	0.0039	*22.9*
ERR	*0.0108*	19.2	*0.0197*	25.2	*0.0069*	24.5	*0.0055*	32.1
RRR	*0.0438*	78.3	*0.1160*	61.7	*0.0233*	83.2	*0.0105*	61.9
Total	*0.0546*	97.5	*0.0679*	86.9	*0.0302*	107.7	*0.0160*	94.0

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in italics
1 only water phase was analysed. Chloroform phase was not analysed.
2 Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Table B.7.6.1.3-7: Identification and characterisation of the radioactive residues of glyphosate in lettuce leaves of rotational crop (third rotation, PBI 364 days) planted after application of glyphosate to bare soil

	Third rotation, PBI 364 days					
	Lettuce leaves					
DALT	**399**		**425**		**455**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.057	100	0.043	100	0.028	100
Extraction with water and chloroform[1]						
Water phase	*0.0279*	49.0	*0.0160*	37.2	*0.0158*	56.4
Metabolite 1[2]	0.0182	*31.9*	0.0080	*18.6*	0.0079	*28.2*
Glyphosate	ND	*ND*	ND	*ND*	ND	*ND*
AMPA	0.0076	*13.3*	0.0045	*10.5*	0.0056	*20.0*
Identified	0.0076	*13.3*	0.0045	*10.5*	0.0056	*20.0*
Characterised	0.0182	*31.9*	0.0080	*18.6*	0.0079	*28.2*
ERR	*0.0279*	49.0	*0.0160*	37.2	*0.0158*	56.4
RRR	*0.0311*	54.5	*0.0267*	62.2	*0.0127*	45.3
Total	*0.0590*	103.5	*0.0427*	99.4	*0.0285*	101.7

Table B.7.6.1.3-7: Identification and characterisation of the radioactive residues of glyphosate in lettuce leaves of rotational crop (third rotation, PBI 364 days) planted after application of glyphosate to bare soil

	Third rotation, PBI 364 days		
	Lettuce leaves		
DALT	**399**	**425**	**455**

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] only water phase was analysed. Chloroform phase was not analysed.
[2] Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Table B.7.6.1.3-8: Identification and characterisation of the radioactive residues of glyphosate in barley commodities of rotational crop (third rotation, PBI 364 days) planted after application of glyphosate to bare soil

	Third rotation, PBI 364 days					
	Barley forage		**Barley straw**		**Barley grain**	
DALT	**412**		**482**		**482**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.056	100	0.061	100	0.047	100
Extraction with water and chloroform[1]						
Water phase	*0.0174*	31.1	*0.0138*	22.7	*0.0117*	24.8
Metabolite 1[2]	0.0082	*14.6*	0.0068	*11.1*	0.0031	*6.6*
Glyphosate	ND	ND	ND	ND	ND	ND
AMPA	0.0093	*16.6*	0.0047	*7.7*	0.0074	*15.7*
Identified	0.0093	*16.6*	0.0047	*7.7*	0.0074	*15.7*
Characterised	0.0082	*14.6*	0.0068	*11.1*	0.0031	*6.6*
ERR	*0.0174*	31.1	*0.0138*	22.7	*0.0117*	24.8
RRR	*0.0377*	67.3	*0.0493*	80.9	*0.0327*	69.6
Total	*0.0551*	98.4	*0.0632*	103.6	*0.0444*	94.4

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
ND – not detected
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in italics
1 only water phase was analysed. Chloroform phase was not analysed.
2 Metabolite 1 was isolated from a 105 DALT lettuce sample for characterisation. An extensive analysis showed that the major component of Metabolite 1 is glucose. Metabolite 1 also contains a closely related material of similar molecular weight which could be fructose.

Table B.7.6.1.3-9: Identification and characterisation of the radioactive residues of glyphosate in carrot commodities of rotational crop (third rotation, PBI 364 days) planted after application of glyphosate to bare soil

	Third rotation, PBI 364 days	
	Carrot tops	**Carrot roots**
DALT	**482**	

Table B.7.6.1.3-9: Identification and characterisation of the radioactive residues of glyphosate in carrot commodities of rotational crop (third rotation, PBI 364 days) planted after application of glyphosate to bare soil

	Third rotation, PBI 364 days			
	mg/kg	**% TRR**	**mg/kg**	**% TRR**
TRR	0.018	100	0.0096	100
Extraction with water and chloroform[1]				
Water phase	*0.0041*	22.7	*0.0038*	39.9
ERR	*0.0041*	22.7	*0.0038*	39.9
RRR	*0.0121*	67.0	*0.0062*	64.3
Total	*0.0161*	89.7	*0.0100*	104.2

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue
PBI – plant back interval
Total – sum of ERR and RRR
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] only water phase was analysed. Chloroform phase was not analysed.

Table B.7.6.1.3-10: Additional treatments of the radioactive residues of glyphosate in lettuce leaves, barley straw and grain planted after application of glyphosate to bare soil

	First rotation, PBI 30 days					
	Lettuce leaves		**Barley straw**		**Barley grain**	
DALT	**105**		**125**		**125**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.097	100	0.175	100	0.188	100
Extraction with water and chloroform[1]						
Water phase	*0.0438*	45.2	*0.0305*	17.4	*0.0493*	26.2
NH_4OH extract[2]	*0.0110*	11.3	*0.0156*	8.9	*0.0423*	22.5
DMSO[3]	*0.0144*	14.8	*0.0298*	17.0	*0.0384*	20.4
Extract after cellulase treatment	-	-	*0.0630*	36.0	-	-
Characterised	***0.0692***	**71.3**	***0.1388***	**79.3**	***0.1299***	**69.1**
Final residue	***0.0278***	***28.7***	***0.0362***	***20.7***	***0.0581***	***30.9***
Total	***0.097***	***100***	***0.175***	***100***	***0.188***	***100***

DALT – days after last treatment
TRR – total radioactive residue
ERR – extractable radioactive residue
RRR – residual radioactive residue, calculated assuming that there were no losses during extraction and purification
ND – not detected
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
1 only water phase was analysed. Chloroform phase was not analysed.
2 The profiles of the NH_4OH extracts analysed by cation exchange chromatography were very similar to the profiles of the water extracts. The same components were extracted with water and ammonium hydroxide, although the ratios of the components were different (see also Table B.7.6.1.3-3 and Table B.7.6.1.3-4).
3 The solvent was removed from the solubilised material and the remaining solid was subjected to enzymatic degradation with amyloglucosidase. This enzyme liberates glucose from starch. Chromatography, on a Bio-Gel P-2 column, of the radioactivity in solution after degradation shows a broad peak which elutes in the region of glucose. Testing of the collected fractions with Chemstrips showed the coelution of glucose with a significant fraction of the radioactivity. This result suggests that DMSO extracted starch, into which the label had been reincorporated.

Soil

The extractability of soil samples varied with time: initially, at early sampling times, a very high percentage of the radioactivity from the soil was extracted (98 %). Over time the extractability decreased and then levelled off to the range of 45 - 76 % TRR. The only components found in soil extracts were non-metabolised glyphosate, AMPA, and an early eluting component referred to as Metabolite A. The amount of glyphosate in soil was the highest directly after application (90.5 % TRR, 0.6431 mg/kg) and decreased over time in the samples from each subplot. Thus, in the first, second and third rotation the glyphosate decreased from 28.1 (0.1453 mg/kg) to 3.2 % TRR (0.0081 mg/kg), 31.6 (0.0449 mg/kg) to 1.0 % TRR (0.026 mg/kg) and from 17.8 % TRR (0.0327 mg/kg) to not detected, respectively.
As for AMPA, only 4.7 % TRR (0.0334 mg/kg) could be detected directly after the application. Afterwards, the amount of AMPA slightly increased over time in the samples of each subplot. Thus, in the first, second and third rotation the AMPA residue increased from 39.0 % (0.2020 mg/kg) to 46.8 % TRR (0.1170 mg/kg), 27.4 % (0.0389 mg/kg) to 47.8 % TRR (0.1194 mg/kg) and from 45.6 % (0.0839 mg/kg) to 56.5 % TRR (0.1012 mg/kg), respectively. The maximum amount of AMPA detected in 30, 119 and 365 PBI subplots was 51.7, 56.5 and 58.4 % TRR (0.3014, 0.2287 and 0.1449 mg/kg). Thus, the absolute amount of AMPA (expressed as mg/kg TRR) slightly decreased over the time of study conduction.
The metabolite eluting near the void volume, Metabolite A, was isolated from a 147 DALT soil sample for characterisation. After isolation by cation exchange chromatography, the metabolite was analysed by reverse phase chromatography on a C18 column. The radioactivity eluted near the void volume, suggesting that this material was very polar. It was also observed that the metabolite fraction contained two components. Another portion of the isolated metabolite was analysed by HPLC chromatography on a HPX-87H carbohydrate column. Again two major components were observed one of which elutes in the same region as glucose. Another portion of the isolated metabolite was analysed by chromatography on a Bio-Gel P-2 (weight range 100-1800). Unlabelled glucose was added to the sample applied to the column to determine where glucose elutes from the column. A single major peak was observed with approximately half of the activity eluting in the same region as glucose. The results of these analyses show that Metabolite A is made up of two components, one of which seems to be glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative. It ranged from 2.5 to 9.0 % TRR (0.0087 – 0.0465 mg/kg), from 2.2 to 8.2 % TRR (0.0080 – 0.0483 mg/kg) and from 3.3 to 7.1 % TRR (0.0057 – 0.0141 mg/kg) in the first, second and third rotation, respectively. Thus, the absolute amount of Metabolite A (expressed as mg/kg TRR) decreased over the time of study conduction.

Table B.7.6.1.3-11: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	-		First rotation, PBI 30 days			
DALT	**0**		**30**		**76**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.711	100	0.518	100	0.518	100
Extraction with 0.5 M NH_4OH						
Aqueous extract	*0.6968*	98	*0.3937*	76	*0.3937*	76
Metabolite A[1]	0.2020	*28.4*	0.0315	*6.1*	0.0465	*9.0*
Glyphosate	0.6431	*90.5*	0.1453	*28.1*	0.1189	*23.0*
AMPA	0.0334	*4.7*	0.2020	*39.0*	0.2224	*42.9*
Identified	*0.6765*	*95.1*	*0.3473*	*67.0*	*0.3413*	*65.9*
Characterised	0.2020	*28.4*	0.0315	*6.1*	0.0465	*9.0*
ERR	*0.6968*	98	*0.3937*	76	*0.3937*	76
RRR	*0.0142*	*2*	*0.1243*	*24*	*0.1243*	*24*
Total	0.711	100	0.518	100	0.518	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

Table B.7.6.1.3-12: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	First rotation, PBI 30 days							
DALT	**90**		**105**		**125**		**154**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.354	100	0.526	100	0.625	100	0.250	100
Extraction with 0.5 M NH_4OH								
Aqueous extract	*0.2230*	63	*0.3103*	59	*0.3500*	56	*0.1375*	55
Metabolite A[1]	0.0087	*2.5*	0.0196	*3.7*	0.0210	*3.4*	0.0111	*4.4*
Glyphosate	0.0268	*7.6*	0.0456	*8.7*	0.0266	*4.3*	0.0081	*3.2*
AMPA	0.1831	*51.7*	0.2442	*46.4*	0.3014	*48.2*	0.1170	*46.8*
Identified	*0.2099*	*59.3*	0.2898	*55.1*	*0.3280*	*52.5*	*0.1251*	*50.0*
Characterised	0.0087	*2.5*	0.0196	*3.7*	0.0210	*3.4*	0.0111	*4.4*
ERR	*0.2230*	63	*0.3103*	59	*0.3500*	56	*0.1375*	55
RRR	*0.1310*	*37*	*0.2157*	*41*	*0.2750*	*44*	*0.1125*	*45*
Total	0.354	100	0.526	100	0.625	100	0.250	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in italics
1 Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

Table B.7.6.1.3-13: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	Second rotation, PBI 119 days					
DALT	**119**		**147**		**167**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.142	100	0.589	100	0.372	100
Extraction with 0.5 M NH_4OH						
Aqueous extract	*0.0951*	67	*0.3181*	54	*0.1674*	45
Metabolite A[1]	0.0066	*4.6*	0.0483	*8.2*	0.0080	*2.2*
Glyphosate	0.0449	*31.6*	0.0083	*1.4*	ND	*ND*
AMPA	0.0389	*27.4*	0.2287	*38.8*	0.1590	*42.7*
Identified	*0.0838*	*59.0*	*0.237*	*40.2*	*0.1590*	*42.7*
Characterised	0.0066	*4.6*	0.0483	*8.2*	0.0080	*2.2*
ERR	*0.0951*	67	*0.3181*	54	*0.1674*	45
RRR	*0.0469*	*33*	*0.2709*	*46*	*0.2046*	*55*
Total	0.142	100	0.589	100	0.372	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
ND – not detected
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

Table B.7.6.1.3-14: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	Second rotation, PBI 119 days					
DALT	**181**		**210**		**314**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.203	100	0.277	100	0.250	100
Extraction with 0.5 M NH_4OH						
Aqueous extract	*0.1137*	56	*0.1911*	69	*0.1425*	57
Metabolite A[1]	0.0107	*5.3*	0.0176	*6.4*	0.0171	*6.8*
Glyphosate	0.0017	*0.8*	0.0029	*1.0*	0.0026	*1.0*
AMPA	0.0983	*48.4*	0.1565	*56.5*	0.1194	*47.8*
Identified	*0.1000*	*49.3*	*0.1594*	*57.5*	*0.1220*	*48.8*
Characterised	0.0107	*5.3*	0.0176	*6.4*	0.0171	*6.8*
ERR	*0.1137*	56	*0.1911*	69	*0.1425*	57
RRR	*0.0893*	*44*	*0.0859*	*31*	*0.1075*	*43*
Total	0.203	100	0.277	100	0.250	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
ND – not detected
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

Table B.7.6.1.3-15: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	Third rotation, PBI 365 days					
DALT	**364**		**399**		**412**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.184	100	0.297	100	0.211	100
Extraction with 0.5 M NH_4OH						
Aqueous extract	*0.1325*	72	*0.1455*	49	*0.1329*	63
Metabolite A[1]	0.0111	*6.0*	0.0141	*4.7*	0.0122	*5.8*
Glyphosate	0.0327	*17.8*	0.0413	*13.9*	0.0072	*3.4*
AMPA	0.0839	*45.6*	0.0853	*28.7*	0.0957	*45.4*
Identified	*0.1166*	*63.4*	*0.1266*	*42.6*	*0.1029*	*48.8*
Characterised	0.0111	*6.0*	0.0141	*4.7*	0.0122	*5.8*
ERR	*0.1325*	72	*0.1455*	49	*0.1329*	63
RRR	*0.0515*	*28*	*0.151*	*51*	*0.0781*	*37*
Total	0.184	100	0.297	100	0.211	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
ND – not detected
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

Table B.7.6.1.3-16: Identification and characterisation of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil

	Third rotation, PBI 365 days					
DALT	**425**		**455**		**482**	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.248	100	0.172	100	0.179	100
Extraction with 0.5 M NH_4OH						
Aqueous extract	*0.1587*	64	*0.1015*	59	*0.1128*	63
Metabolite A[1]	0.0124	*5.0*	0.0057	*3.3*	0.0127	*7.1*
Glyphosate	ND	*ND*	0.0010	*0.6*	ND	*ND*
AMPA	0.1449	*58.4*	0.0757	*44.0*	0.1012	*56.5*
Identified	*0.1449*	*58.4*	*0.0767*	*44.6*	*0.1012*	*56.5*
Characterised	0.0124	*5.0*	0.0057	*3.3*	0.0127	*7.1*
ERR	*0.1587*	64	*0.1015*	59	*0.1128*	63
RRR	*0.0893*	*36*	*0.0705*	*41*	*0.0662*	*37*
Total	0.248	100	0.172	100	0.179	100

DALT – days after last treatment
TRR – Total radioactive residue
ERR – Extractable radioactive residue
RRR – Residual radioactive residue, calculated assuming that there were no losses during extraction and purification
PBI – plant-back interval
ND – not detected
All residue data are expressed as mg/kg glyphosate equivalents
Values calculated upon dossier compilation are presented in *italics*
[1] Metabolite A is made up of two components, one of is glucose and the other a closely related material of similar molecular weight, possibly a glucose derivative

C. Storage stability

Three crop samples, 105 DALT lettuce, 125 DALT barley straw, and 154 DALT carrot tops were used for storage stability testing in crops. These samples were removed from the freezer (<-5°C) and extracted at 5 to 7 months and again at 12 to 15 months after receipt. The extracts of each sample were analysed by HPLC and no significant difference between the respective profiles was detected. The combustion analysis and extractabilities of the respective samples were nearly identical as well. These results indicate that the crop metabolites are stable in the freezer over an extended period of time.

The storage stability test for soil was done in a similar manner. 76 DALT soil was used as the test sample. This sample was extracted with 0.5 M NH4OH and analysed by HPLC within 7 months of receipt. After storage below -5°C for 17 months, a fresh aliquot was extracted and analysed as before. Residue levels and the amount of radioactivity extracted again were compared as were the HPLC profiles of the original extraction. No significant difference between the respective profiles was detected. The combustion analysis and extractabilities of the respective samples were similar.

The dates of analysis are not indicated within the report, therefore the storage period was calculated from the date of sampling to the date of report finalisation, as the worst case. In this case, the storage duration for crops from the third rotation was 11 – 14 months and is covered by storage stability analysis conducted within the study. For the second rotation and first rotation the storage duration is 17 – 22 and 22 – 25 months, respectively. Although in some cases the maximum storage periods are longer than are covered by the available storage stability data, because there was no visible change in profile after 12 months (lettuce leaves) and 15 months (barley straw and carrot tops) storage, it is reasonable to suppose that the residues remained stable throughout the duration of the study.

Table B.7.6.1.3-17: Extraction of the radioactive residues of glyphosate in lettuce leaves, barley straw and carrot tops – storage stability assessment

	Lettuce leaves 105 DALT		**Barley straw 125 DALT**		**Carrot tops 154 DALT**	
Storage interval (months)	**5**	**12**	**7**	**15**	**7**	**15**
	% TRR	**% TRR**	**% TRR**	**% TRR**	**% TRR**	**% TRR**
TRR	100	100	100	100	100	100
ERR	45.2	45.2	25.9	27.6	32.4	34.6
RRR	*54.8*	*54.8*	*74.1*	*72.4*	*67.6*	*65.4*
Total	**100**	**100**	**100**	**100**	**100**	**100**

TRR Total radioactive residue (expressed as glyphosate equivalents)
ERR Extractable radioactive residue
RRR Residual radioactive residue, calculated based on the assumption that there were no losses during extraction.

Table B.7.6.1.3-18: Extraction of the radioactive residues of glyphosate in soil – storage stability assessment

	Soil 76 DALT	
Storage interval (months)	**7**	**17**
	% TRR	**% TRR**
TRR	100	100
ERR	75.9	76.7
RRR	*24.1*	*23.3*
Total	**100**	**100**

TRR Total radioactive residue (expressed as glyphosate equivalents)
ERR Extractable radioactive residue
RRR Residual radioactive residue

D. Degradation pathway

Please refer to the overall pathway of glyphosate in rotational crops in Vol. 1, 2.7.7.

III. Conclusion

The metabolism of glyphosate was examined in rotational crops. ^{14}C-glyphosate formulated as Roundup® was applied to plots of bare sandy loam soil at a rate of 4.16 kg a.s./ha. A primary crop of soybeans was planted 7 days after application. The primary crop was harvested and the plots rototilled before planting rotational crops of lettuce, carrots, and barley into the subplots at 30, 119, and 364 days after herbicide treatment.

The primary and rotational crops were sampled for analysis. The rotational crops from the 30 DALT plots contained 0.037 – 0.188 mg/kg of glyphosate equivalent residues. Crops from the 119 DALT planting contained residues of 0.017 – 0.078 mg/kg. Carrots, barley, and lettuce from the 364 day planting contained residues of 0.0096 to 0.061 mg/kg. Analysis of rotational crop samples revealed two residue components, aminomethylphosphonic acid (AMPA) and a polar metabolite (called Metabolite 1 within the report) characterised as being a mixture of sugars, primarily glucose and fructose. Glyphosate was present only in lettuce, barley straw and grain of the first rotation 1.0 – 9.8 % (0.0018 – 0.0184 mg/kg) and in lettuce DALT 167 of the second rotation 1.6 % TRR (0.0009 mg/kg). AMPA ranged from 3.7 – 17.9, 1.1 – 14.2 and 7.7 – 20.0 % TRR (0.0007 – 0.0336, 0.0003 – 0.0111 and 0.0045 – 0.0093 mg/kg) in the matrices of the crops of the first, second and third rotation, respectively. Metabolite 1 amounted to 7.7 – 40.8, 6.3 – 24.9, 6.6 – 31.9 % TRR (0.0136 – 0.0327, 0.0039 – 0.0147 and 0.0031 – 0.0182 mg/kg) in the matrices of the crops of the first, second and third rotation, respectively.

Residues after extraction with water and chloroform were further investigated and were identified as being starch lignin and cellulose, as well as biopolymers of glucose.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study assessing the metabolic behaviour of glyphosate in rotational crops lettuce, barley and carrot has been previously evaluated at EU level. It was performed under GLP. The study is deemed to largely comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Testing of Chemicals, 501 with some deficits: the frozen samples were stored at -5°C and not at -20°C, however, storage stability at -5°C or below was demonstrated within the study for at least 12 to 15 months, for barley straw and carrot tops (as well as for soil) the initial extraction for storage analysis was performed 7 months after storage and not within the 6 months, for which the frozen samples are assumed to be stable and no dates of analysis are given within the report.
Less than 90 % of TRR was identified or characterised and relatively large amounts of radioactivity remained unextracted. Several attempts were made to characterise the residual radioactive residue after extraction with water for lettuce leaves, barley straw and barley grain. After additional solubilisation procedures, 20.7– 30.9 % TRR (0.0278 – 0.0581 mg/kg) remained unextracted. Significant attempts to characterize non-extracted residues were performed with sequential treatments with NH_4OH, DMSO, amyloglucosidase and cellulase. The DMSO and enzyme treatments released up to 14.8, 20.4 % and 36.0 % of TRR (0.0144, 0.0384 and 0.0630 mg/kg) in lettuce leaves, barley grain and straw, respectively. It seems likely that a significant part of the non-extracted radioactivity could be attributed to natural plant constituents.

Storage stability
No date of analyses is stated within the report. However, since the storage stability was tested within the study, it is reasonable to assume according to the study design that at the time the storage stability was tested the final analyses have already been performed. For purposes of storage stability testing the initial extraction of barley straw and carrot tops was conducted after a storage period of 7 months. Assuming the worst case, the storage period can be calculated from the date of sampling to the date of report finalisation. In this case, the storage duration for crops from the third rotation is 11 – 14 months and is covered by storage stability analysis conducted within the study. For the second rotation and first rotation the storage duration is 17 – 22 and 22 – 25 months, respectively which is longer than the tested period.
In all of the plant matrices from all rotations, the same analytes were found: glyphosate, AMPA and Metabolite 1. Glyphosate and its primary metabolite AMPA are likely to be stable, as shown in storage stability studies. Metabolite 1, which was characterised as being a mixture of sugars, primarily glucose and fructose, is formed through plant anabolism and is unlikely to be a degradation product. Therefore, in total for all plant matrices from all rotations it is likely that the stored samples were stable from sampling to analysis.
The study is considered reliable for the assessment of the metabolic behaviour of glyphosate in rotational crops.

Assessment and conclusion by RMS:
The RMS largely agrees with the assessment provided by the applicant. Sufficient attempts have been undertaken to identify/characterize the residual radioactive residues. Storage stability has been sufficiently addressed. The confined rotational crop study is considered acceptable.

B.7.6.1.4. Wheat and turnips

1. Information on the study

Data point:	CA 6.6.1/004
Report author	[redacted]
Report year	1989
Report title	[^{14}C-Anion]ICIA0224 - Confined Accumulation Studies on Rotational Crops
Report No	WRC 89-25
Document No	VV-320956
Guidelines followed in study	Pesticide Assessment Guideline Subdivision N, Number 165-1
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Metabolism in Rotational Crops, 502: • A crop representing leafy vegetables was not included in the study. • Growth stage of crops at planting is not given. • The study was intended only to study uptake of radio-labelled residues and no attempt was made to characterise the residues.

	• The frozen samples were stored at -10°C and not at -18°C. Storage stability was not investigated, although the samples were kept frozen longer than 6 months. • No dates of analyses are given within the report. • Residue levels of the control samples are not included in the report. • The radiochemical purity of the application solution was <95 %; no specifications of the impurities were given.
Previous evaluation	No
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	Conclusion applicant: supportive (Category 2a) Conclusion RMS: supportive only

2. Full summary of the study according to OECD format

Executive Summary

A rotational crop uptake study was conducted to measure the uptake of glyphosate from soil by rotational crops, namely wheat and turnips, and to determine the identity of these residues. In this study only two crops were planted - wheat and turnips, representing a root crop and a small grain cereal.

For the investigations, a loamy sand soil was treated with *N*-(phosphono-^{14}C-methyl)glycine as its trimesium salt (^{14}C-glyphosate-trimesium). Treatment was performed at a nominal rate of 6 mg/kg ^{14}C-glyphosate-trimesium (corresponding to 4 mg/kg of ^{14}C-glyphosate).

Wheat and turnips were planted 35, 95 and 370 days after treatment. At each interval, soil core samples (0 - 15 cm) were taken from the pots. The average radioactive residue in soil declined from 1.58 mg/kg (day 0) to 0.803 mg/kg (day 370).

The plants were harvested at maturity and the radioactive residues were determined in commodities of wheat (seed, chaff and stalks/leaves) and of turnips (leaves and bulbs).

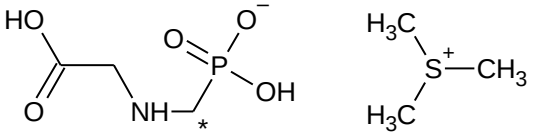
Total glyphosate equivalent residues in wheat seeds, chaff and stalks/leaves were 0.25, 0.29 and 0.46 mg/kg grown on soil aged for 35 days, 0.28, 0.25 and 0.51 mg/kg (on soil aged for 95 days) and 0.06, 0.1 and 0.11 mg/kg (on soil aged for 370 days).

In turnip leaves and bulbs the radioactive residues amounted to 0.02 mg/kg for both commodities of turnips grown on soil aged for 35 days, to 0.09 and 0.03 mg/kg (on soil aged for 95 days) and were detected at 0.03 and 0.02 mg/kg (on soil aged for 370 days).

The radioactive residues in the plant matrices were not extracted and investigated for their identity, since the residue levels were considered to be too low for reasonable analyses.

I. Materials and Methods

A. Materials

1. Test material	**Glyphosate-trimesium** • N-(phosphono-^{14}C-methyl)glycine trimesium salt (^{14}C-PMG-labelled glyphosate-trimesium) Mixture of a) N-(phosphono-^{14}C-methyl)glycine trimesium salt (5.7 mg) b) N-(phosphono-^{12}C-methyl)glycine trimesium salt (138.5 mg)
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	a) 94 % (by TLC) radiochemical purity of applied solution is not given

Chemical purity	a) not given b) 95.7 %
Specific activity:	Applied solution: 0.53 MBq/mg (32000 dpm/µg)

2. Test material	
Soil:	Loamy sand (pH: 6.9; cation exchange capacity: 12.2 meq/100 g; OM: 0.6 %, sand: 79.90 %; silt: 13.90 %; clay: 6.20 %; textural class (USDA): not given)
Crop:	*Primary crop*: No primary crops were planted *Rotational crops*: Wheat (variety Anza) Turnips (variety Purple-top White Globe)
Botanical name:	*Brassica rapa* *Triticum aestivum*
Crop part(s):	Wheat (chaff, seeds, stalks/leaves), turnips (leaves, bulbs)

B. Study design

1. In-life phase

Application solutions

Two application solutions were prepared, one containing a mixture of radiolabelled and unlabelled test item and another containing only unlabelled test item (used for control soil).

For preparation of the application solution containing ^{14}C-PMG-labelled glyphosate-trimesium, 5.7 mg of the radiolabelled test item with a specific activity of 4.53 MBq/mg (30 mCi/mmol) and 138.5 mg of unlabelled glyphosate-trimesium (equivalent to about 79.5 mg pure test item) were mixed in 100 mL distilled water. After assaying, the specific activity was calculated to be 0.37 MBq/mg (21911 dpm/µg) for glyphosate-trimesium or 0.53 MBq/mg (32000 dpm/µg) for ^{14}C-glyphosate.

For preparation of the application solution for the control pots, unlabelled glyphosate-trimesium (130.5 mg, equivalent to about 74.9 mg pure test item) were dissolved in 100 mL distilled water.

Soil preparation, treatment, aging and planting

Fifteen pots, each with a hole in the bottom for drainage were used. A total of 59 kg soil was mixed with 185 g of "17-17-17" fertilizer in a mixing drum, sifted through a 2 mm screen, and divided into the fifteen pots. Glass tubes were positioned down the centre of each of the pots and were secured in the bottom hole with packed cotton while the soil was added. These tubes served to drain any water in excess of 1 cm deep from the top of the soil, thereby avoiding unrealistic flooded conditions. Plastic buckets into which the pots were placed caught this drainage water as well as a leachate.

The top 7.5 cm of soil was removed from twelve of the pots and weighed. This was done in order to calculate the amount of glyphosate-trimesium needed to add to the soil to achieve a 6 mg/kg concentration to approximate the intended rate of 6 kg/ha glyphosate-trimesium, equivalent to 4.12 kg glyphosate/ha.

Radiolabelled glyphosate-trimesium was incorporated into half the removed soil (enough for 6 pots) using a twin shell blender. Unlabelled glyphosate-trimesium was incorporated into the other half. After mixing, the treated soil samples were subsampled for combustion analysis and placed back into the original twelve pots to give 6 radiolabelled and 6 "cold" treated pots of soil.

The actual treatment rate was calculated to be 5.8 mg/kg ^{14}C-glyphosate-trimesium or equivalent to 4.0 mg/kg glyphosate. The actual treatment rate of unlabelled glyphosate-trimesium was calculated to be 5.8 mg/kg glyphosate-trimesium or equivalent to 4.0 mg/kg glyphosate.

The pots of soil were aged outdoors for three intervals: 35, 95 or 370 days. Four pots of soil, two treated with ^{14}C-PMG-labelled glyphosate-trimesium and two with non-radioactive test item, were sampled and planted with crops at each interval.

After each aging period, a soil core sample was taken for analysis. All of the remaining soil was then taken out of each pot, mixed separately in a blender, sampled and returned to the pot. Two pots of soil were planted with wheat, one treated with ^{14}C- glyphosate-trimesium and the other with non-radioactive glyphosate-trimesium. Similarly, two pots of soil were planted with turnips. Plants were grown outside and were watered and thinned as needed until they reached maturity and were harvested. The plants were also treated with allethrin for insect control. Plant thinnings and leachates (from rain or irrigation) were collected and analysed during the growing period.

2. Sampling

Samples were collected from mature wheat plants by clipping the heads off the stalks and then clipping the stalks off at soil level. The heads were threshed, and the chaff, seeds, and stalks (including leaves) were weighed separately. These three crop parts were then ground separately in a lab mill and subsamples were taken for combustion.
The turnips were harvested when the roots were full and mature. Turnip leaves were clipped off the roots, weighed, frozen with liquid nitrogen, homogenised with a mortar and pestle, then subsampled for combustion analysis. The roots were rinsed free of dirt with distilled water, patted dry, weighed and ground using a food processor.
The processed plant materials were put in separate plastic bags, one bag for each plant-back interval: 35-day, 95-day, 370-day and controls. All samples were stored at <-10°C.

The soil in which the crops were growing was analysed to follow the degradation of glyphosate-trimesium and to identify the metabolites to which the rotational crops were exposed.
After each aging period, a soil core sample (1 cm diameter x 15 cm deep) was taken from each of the four pots (two pots containing soil treated with ^{14}C- glyphosate-trimesium and two with non-radioactive test item). The 0 - 7.5 cm and the 7.5 – 15 cm segments were separated, placed into separate bags, subsampled for future combustion, and frozen at <-10°C until analysed. After harvesting the plants, the soil was removed from the pots, weighed, sub-sampled for combustion analysis and stored at <-10°C.

3. Analytical procedures

Two or more aliquots of all soil and plant samples were combined with an approximately equal volume of cellulose in combustion thimbles and combusted. The $^{14}CO_2$ generated was trapped with Carbosorb, then mixed with Permafluor scintillation cocktail. Liquid samples, including those resulting from combustion, were assayed by liquid scintillation counting (LSC).

The extractability of the ^{14}C-glyphosate from soil was tested by separately extracting 2 g subsamples from the 0-time soil with each of the following solvents: H_2O, 1 M HCl, 1 M NH_4Cl, 0.5 M NaOH, 0.5 M NaOH in methanol and 0.5 M HCl in methanol.

Each soil sample was weighed into a centrifuge tube, combined with an adequate volume of solvent, and extracted by shaking. The tubes were centrifuged and the solvent separated from the soil by decantation. Three successive extractions were done in this way using fresh solvent each time. Soils were combusted and analysed as previously described while soil extracts were analysed by LSC.

Large scale extractions (20 - 200 g soil) were done in a similar manner using a volume of solvent (in mL) equal to 5 times the weight of the soil (in g). 1 M HCl was determined to be the most suitable solvent for these extractions.
Zero-day, 30-day, 95-day, and 365-day soils were sampled just prior to planting with wheat and turnips. Extracts and soils were analysed by LSC. The extracts were concentrated by rotary vacuum evaporation (RVE) and reassayed by LSC.

The extracts were passed through individual, plastic disposable columns containing C-18 bonded silica, which had been pre-rinsed with methanol and 1 M HCl. Radioactivity was eluted with 1 M HCl and concentrated by RVE. The concentrate was diluted with distilled water and mixed with Dowex AG-50-WX8 (hydrogen form) and swirled at 25°C for 2 hours. The resin was filtered, dried, and combusted to determine residual radioactivity. The filtrate was concentrated by RVE for TLC analysis.

Thin-layer chromatography (TLC) was performed on 250 µ Merck silica gel plates utilizing the following solvent systems developed both one- and two-dimensionally:
N-propanol/diethylamine/water (5/2/3) and N-propanol/triethylamine/water (5/2/3).

II. Results and Discussion

A. Total radioactive residues (TRRs)

The radioactive residues detected in commodities of wheat and turnips after three rotations are shown in table B.7.6.1.4-1.
For the commodities sampled from wheat (chaff, seeds and stalks/leaves), the total radioactive residues were comparable for samples taken from the first and the second rotation. The residues in seeds, chaff and stalks/leaves amounted to 0.25, 0.29 and 0.46 mg/kg (harvest 133 DALT) and to 0.28, 0.25 and 0.51 mg/kg (harvest 195 DALT). In samples from the one-year plant-back, harvested at 469 DALT lower residue levels were detected, amounting to 0.06, 0.1 and 0.11 mg/kg for seeds, chaff and stalks/leaves, respectively.

Turnips were harvested 195, 257 and 469 days after treatment. The radioactive residues in turnip leaves and bulbs were comparable (0.02 - 0.03 mg/kg for leaves and 0.02 - 0.03 mg/kg for bulbs) for samples of all three rotations, except for leaves of the second rotation, where radioactive residues amounted to 0.09 mg/kg.

Table B.7.6.1.4-1: Total radioactive residues in rotational crops planted after application of 14C-PMG labelled glyphosate-trimesium to bare soil

PBI (days)	Crop	Sampled commodity	Sampling (DALT)	TRR (mg/kg)	% AR
35	Wheat	Seeds	133	0.25	0.020
		Chaff		0.29	0.008
		Stalks/leaves		0.46	0.064
	Turnip	Leaves	257	0.02	0.011
		Bulbs		0.02	0.016
95	Wheat	Seeds	195	0.28	0.013
		Chaff		0.25	0.004
		Stalks/leaves		0.51	0.025
	Turnip	Leaves	195	0.09	0.023
		Bulbs		0.03	0.015
370	Wheat	Seeds	469	0.06	0.005
		Chaff		0.1	0.003
		Stalks/leaves		0.11	0.017
	Turnip	Leaves	469	0.03	0.064
		Bulbs		0.02	0.026

PBI Plant back interval in days (time interval between treatment and planting)
DALT Days after treatment
AR Applied radioactivity
TRR Total radioactive residue

The radioactive residues detected in soil at the time points of planting and harvest are shown in table B.7.6.1.4-2.
At zero-time, all of the radioactive residue was confined to the top 7.5 cm of soil. The amount of material present in this top 7.5 cm was equal to 4.59 mg/kg glyphosate-trimesium or 3.15 mg/kg glyphosate. Averaged through the whole pot of soil (0-15 cm depth) the 0-day residue was 1.58 mg/kg glyphosate equivalents.
The soil residue declined from 1.58 to 0.68 mg/kg, representing a decline of 57 % over the course of the study (496 days). This is probably due to soil microbial degradation of glyphosate-trimesium.
Dissipation by leaching was not a significant mode for loss of the radiolabel from the soil. Analysis of the soil cores (0 - 7.5 cm and 7.5 – 15 cm depth) at each aging interval revealed, that most of the residues remained in the treated layer (0 - 7.5 cm) at all sampling intervals. However, significant residues were found in the lower layer (7.5 – 15 cm). These were probably not due to leaching, but rather to contamination from the treated layer because it was impossible to totally separate the two layers.
The amount of radioactivity found in the leachate was insignificant, indicating that movement of the chemical through the soil did not occur.

Table B.7.6.1.4-2: Total radioactive residues in soil after application of ^{14}C-PMG labelled glyphosate-trimesium to bare soil

Rotation, PBI	DALT	Pot [1]	TRR, mg/kg		
			Soil core (0.0 - 7.5 cm)	Soil core (7.5 - 15 cm)	Mixed soil
Before application	**0**		**3.15**	n.a.	**1.58**
1st rotation, PBI 35 days	**35**	1	2.04	0.45	1.43
		2	2.76	0.566	1.23
		Mean	**2.4**	**0.508**	**1.33**

Table B.7.6.1.4-2: Total radioactive residues in soil after application of ^{14}C-PMG labelled glyphosate-trimesium to bare soil

Rotation, PBI	DALT	Pot [1]	TRR, mg/kg		
			Soil core (0.0 - 7.5 cm)	Soil core (7.5 - 15 cm)	Mixed soil
2nd rotation, PBI 95 days	**95**	1	1.91	1.73	1.34
		2	1.84	0.534	1.25
		Mean	**1.89**	**1.13**	**1.30**
harvest of wheat (**1st rotation)**	133	1	n.a.	n.a.	0.96
		2	n.a.	n.a.	n.a.
		Mean	n.a.	n.a.	--
harvest of wheat and turnips **(2nd rotation)**	195	1	n.a.	n.a.	1.1
		2	n.a.	n.a.	0.64
		Mean	n.a.	n.a.	**0.87**
harvest of turnips **(1st rotation)**	257	1	n.a.	n.a.	n.a.
		2	n.a.	n.a.	0.52
		Mean	n.a.	n.a.	--
3rd rotation, PBI 370 days	**370**	1	1.43	0.23	0.878
		2	1.46	0.091	0.727
		Mean	**1.45**	**0.161**	**0.803**
harvest of wheat and turnips **(3rd rotation)**	469	1	n.a.	n.a.	0.64
		2	n.a.	n.a.	0.71
		Mean	n.a.	n.a.	**0.68**

PBI Plant back interval in days (time interval between treatment and planting)
DALT Days after treatment
TRR Total radioactive residue, expressed in glyphosate equivalents
n.a. Not analysed The pots of soil were aged outdoors for three intervals of 35, 95 or 370 days
[1] Pot 1: wheat, pot 2: turnips

B. Extraction and characterisation of residues

Determination of the extractability of radioactive residues from soil with different solvents resulted in an extractability of >95 % with 1 M HCl, <5 % with distilled water, <5 % with 1 M NH_4Cl and >90 % with 0.5 M NaOH. Therefore, radioactive residues were extracted with 1 M HCl.

Soil samples, taken prior to planting of the crops from all six pots treated with radiolabelled test item, were separately extracted. The extracts contained large quantities of substances, presumably dissolved soil organic matter, which interfered with TLC analysis. Attempts were made to purify the extracts using first a C-18 column and then a cation-exchange batch method. The efforts to purify the material resulted in losses which left about 25% of the original extracted radioactivity. TLC analysis of one of the resulting "purified" extracts was not successful in characterizing soil metabolites because almost all of the radioactivity remained at the origin of the plate. In many other cases that are not shown in the report, streaking over the length of the plate occurred and it was assumed, that co-extractants interfered with this chromatography.

AMPA and the ^{14}C-glyphosate chromatographed as expected when they were run alone in the TLC systems. However, when AMPA or glyphosate standards were used to spike the purified soil extracts, the standards did not move from the origin. Therefore the authors concluded, that the soil extracts contained some components which complexed with glyphosate-trimesium and its metabolites and prevented analysis by TLC. No further attempts for analyses of components in soil were performed.

Because of the comparatively low level of radioactivity in plant samples of wheat and turnips and previous experience with the difficulty of purifying the ^{14}C-glyphosate and its metabolites from soil the authors stated that no characterisation of the residue in these samples was going to be successful. No attempts at analysis of the crop samples was made.

C. Storage stability

Throughout the study all samples of crops and soils, were stored in freezers usually at -10°C. No storage stability investigations were performed. The storage duration is not indicated in the report. Harvest of crop samples was between 24.03.1983 and 23.02.1984. The study was completed on 08.10.1985 and finalised on 27.07.1989. Thus the maximum storage time was 929 days.

III. Conclusion

N-(phosphono-^{14}C-methyl)glycine as its trimesium salt (^{14}C-glyphosate-trimesium) was incorporated into a loamy sand soil at a concentration of 4.6 mg/kg glyphosate-trimesium or 3.15 mg/kg of glyphosate. The ^{14}C-glyphosate concentration declined gradually to leave a ^{14}C residue of 1.45 mg/kg over a 370 day interval determined in the soil layer of 0 - 7.5 cm.

Wheat and turnips were planted in the treated soil at intervals of 35, 95, and 370 days after treatment (plant back intervals (PBI): 35, 95 and 370 days) and harvested at maturity. Although considerable residues of ^{14}C-glyphosate remained in the soil at these planting times, uptake of radioactivity by these crops was comparatively low. For the commodities sampled from wheat (chaff, seeds and stalks/leaves), the total radioactive residues were comparable for samples taken from the first and the second rotation. The residues in seeds, chaff and stalks/leaves amounted to 0.25, 0.29 and 0.46 mg/kg (PBI 35 days) and to 0.28, 0.25 and 0.51 mg/kg (PBI 95 days). In samples from the third rotation (PBI 370 days), lower residue levels were detected, amounting to 0.06, 0.1 and 0.11 mg/kg for seeds, chaff and stalks/leaves, respectively. The radioactive residues in turnip leaves and bulbs were comparable (0.02 - 0.03 mg/kg for leaves and 0.02 - 0.03 mg/kg for bulbs) for samples of all three rotations, except for leaves of the second rotation (PBI 95 days), where radioactive residues amounted to 0.09 mg/kg.
The radioactive residues in the plant matrices were not extracted, so no characterisation or identification of radioactive residues was performed.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study assessed the uptake of glyphosate in rotational crops of turnips and wheat. It was performed under GLP.
The study has significant deviations when compared with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Metabolism in Rotational Crops, 502:
No representative leafy vegetable crop was included in the study.
The radiochemical purity of the application solution was <95 %; no specifications of the impurities were given.
Developmental stages of the crops at planting and harvesting are not reported, though they could be roughly estimated based on sampling dates. No information on storage duration of plant samples and dates of analyses is given. The storage stability is not covered.
No attempts were made to identify or characterise the radioactive residues in the plant matrices. Especially in wheat matrices (seed, chaff and stalks/leaves), comparatively high residue levels were detected amounting to up to 0.28 mg/ kg in wheat seeds, up to 0.29 mg/kg in wheat chaff and up to 0.51 mg/kg in wheat stalks/leaves. In turnip leaves residues were between 0.02 and 0.06 mg/kg.
The analysis of the control experiments was not reported.

The study is considered to provide only supporting uptake data for the assessment of the metabolic behavior of glyphosate in rotational crops.

Assessment and conclusion by RMS:
The study is considered to only give some qualitative information on glyphosate uptake by rotational crops. The main deficit is the fact that no characterization or identification of the residues was conducted on the plant samples, while in particular in wheat commodities the TRR was sufficiently high. The study is, therefore, only considered as supportive.

B.7.6.1.5. Beet, cabbage and wheat

1. Information on the study

Data point:	CA 6.6.1/005
Report author	[redacted]
Report year	1978
Report title	Uptake and metabolism of Glyphosate in root, leaf and cereal type rotation crops

Report No	MSL-0882
Document No	-
Guidelines followed in study	None
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Metabolism in Rotational Crops, 502: • The radiochemical purity of the test item(s) is not clearly specified. • Details on application (formulation on test item) are missing within the report. • Information about timing of the second treatment (emergency crop) is missing. • Developmental stages of the crop at harvesting are not reported. • Harvest samples of wheat were not separated into grain and straw, no intermediate samples (green material) were collected; only results for the whole plant at harvest are available. • Details about sampling of cabbage is missing, it is assumed that the whole plant was sampled. • No information on the storage stability for all major components of the total radioactive residues. Storage conditions and duration of plant samples is not given. • Date of analysis is missing within the report. • Extraction rates are 32 – 76 %. The water extracts were only analysed by ion-exchange chromatography. No attempts were made to characterise the bound radioactivity. • The water extracts of the primary crop soybean were not further analysed by ion-exchange chromatography. • Identification of glyphosate and AMPA was done by comparison with elution volumes of respective standards; no additional analytic method was established. • Unextracted radioactive residues for each sample not precisely quantified (not analysed by combustion/further extraction, only calculated from TRR - ERR). • No quantification of the residues as concentration (mg/kg, as active ingredient equivalents) in the original sample matrix analysed (re-calculation possible) • No flow chart depicting the overall extraction and fractionation strategies employed for each sample matrix analysed.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory at the time the study was performed
Acceptability/Reliability:	Conclusion applicant: invalid (Category 2a) Conclusion RMS: not acceptable

2. Full summary of the study according to OECD format

Executive Summary

The uptake and metabolism of glyphosate was examined in rotational crops. Glyphosate radio-labelled with ^{14}C in the methyl position [*N*-(phosphono-^{14}C-methyl)glycine, called ^{14}C-glyphosate in this summary] was applied to sandy loam soil in pots at a rate of 4.48 kg a.s./ha. Primary crops of soybean, cabbage, wheat and beet, were planted 3 days after application. In parallel, unlabelled glyphosate was applied to identical pots for control purposes and kept in the same greenhouse to account for $^{14}CO_2$ fixation from degradation in soil. After harvesting the primary crop, different succeeding crops were planted at plant-back intervals of 30, 120 or 365 days. To simulate crop failure, some of the pots containing the primary crop received a second treatment of 4.48 kg a.s./ha and were replanted with the same crops as before (except for soybean where beet was replanted).

Table B.7.6.1.5-1: Overview of the different scenarios of crop rotation

Scenario type	Primary crop	Emergency crop (PBI 30 [1])	Four months rotation (PBI 120 [1])	One year rotation (PBI 365 [1])
A	Soybean	Beet	Beet	Cabbage
B	Cabbage	Cabbage	Wheat	Beet
C	Wheat	Wheat	Beet	Cabbage
D	Beet	Beet	Cabbage	Wheat

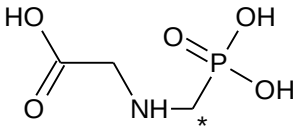
PBI Plant back interval in days (time interval between treatment and planting)
1 Days after soil treatment

Radioactive residues were extracted with water from respective plant materials. The extractability varied from 32 % TRR (beet, foliage, first rotation) to 76 % TRR (cabbage, third rotation). The extracts were analysed by ion-exchange column chromatography. Further ^{14}C-activity remained bound to the column. No further attempts were performed to resolublise these bound residues. In addition to the ^{14}C-activity that does not elute, many ^{14}C-products with elution patterns similar to glyphosate and AMPA were observed. No further investigations were conducted to identify those other ^{14}C-products.

Concerning radioactive residues found there was a reduction in the amount of uptake of ^{14}C-activity with time. There was no increase in the uptake of ^{14}C-activity in any of the rotation crops with the exception for emergency crops beet and cabbage, where a slight increase in TRRs was determined. The rotational crops from the 30 PBI scenario contained 0.002 – 0.018 mg/kg of glyphosate and 0.003 – 0.041 mg/kg AMPA, only for wheat residues were higher (0.046 mg/kg for glyphosate and 0.128 mg/kg for AMPA). Residues from respective plant materials for the 120 PBI decreased to <0.001 – 0.014 mg/kg for glyphosate and 0.001 – 0.010 mg/kg for AMPA. Residues from respective plant materials for the 365 PBI further decreased to <0.001 – 0.004 mg/kg for glyphosate and <0.001 – 0.004 mg/kg for AMPA.

I. Materials and Methods

A. Materials

1. Test material	N-(phosphono-^{14}C-methyl)glycine (namely ^{14}C-glyphosate within this summary)
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	No further information within the report
Chemical purity	98 – 99 %
Specific activity of the test substance applied:	0.42 MBq/mg (1.9 mCi/mmol)

2. Test material	
Soil:	Lintonia sandy loam (pH: 6.5; 0.6 % organic matter, 86.0 % sand, 11.0 % silt, and 1.8 % clay)
Crop:	Primary crop: Soybean, beet, wheat and cabbage (no information on variety) Rotational crops: Beet, cabbage and wheat (no information on variety)
Botanical name:	*Glycine max* *Beta vulgaris* var. *vulgaris* *Triticum aestivum* *Brassica oleracea* var. *capitata*
Crop part(s):	Soybean (foliage, pod), cabbage (whole plant), wheat (whole plant), beet (foliage, root)

B. Study design

1. In-life phase

The test substance contained ^{14}C-glyphosate with a specific activity of 0.42 MBq/mg (1.9 mCi/mmol). Detailed information on formulation of the test item is missing. ^{14}C-glyphosate was applied to the test pots (diameter of ~20 cm) at target rates of 4.48 kg a.s./ha on bare soil. The remaining pots were treated with an equivalent amount of unlabelled herbicide (^{12}C-glyphosate) to serve as controls. These control pots were maintained in the same greenhouse in order to differentiate between radioactivity taken up by the roots of the plants from the soil and photosynthetic fixation of $^{14}CO_2$ liberated by soil degradation of ^{14}C-glyphosate.

Three days after treatment, ^{14}C-treated and control pots were planted with wheat, beet, soybeans, or transplanted cabbage plants. When growth was assured, the wheat was thinned to 15 plants, cabbage to one, and beet and soybeans to two plants per pot. The primary crop plants were grown in the greenhouse, watered as needed from the bottom, and fertilised monthly (15 mL of a solution of 24.0 g of Rapid Grow in 4.5 L of water). After the harvest of the primary crops, the soil surface of the pots was lightly tilled by hand and planted with the rotational crops; beet replacing soybeans and wheat, cabbage replacing beet, and wheat replacing cabbage. One year after treatment, the soil surface was lightly tilled by hand and replanted with cabbage, beet, and wheat replacing the primary crops of soybean, wheat, cabbage, and beet, respectively. In the interim time between the plant back interval (PBI) 120 and PBI 365, the pots of soil were kept moist by watering and fertilising as needed.

For emergency crops a second treatment as described for the primary crops was conducted. Further information on timing of second application and formulation of test item for application is missing. Primary crops were harvested after 30 days and the pots replanted with emergency crops cabbage, beet and wheat. These crops are representative of the crops that would be planted after the failure of the initial crop. The crops grown for the first 30 days were not analysed.

Another scenario was established to expose wheat to $^{14}CO_2$. $^{14}CO_2$ was released from 3 mCi of ^{14}C-$NaCO_3$ contained in a vial in the glove bag by addition of an excess of H_3PO_4.

2. Sampling

The primary crops were harvested 90 days after planting, except soybean which was harvested 112 days after planting. Crops of the 2nd and 3rd rotation were harvested 120 days after planting with the exception of the 1 year rotational cabbage crop which were harvested 97 days after planting. Emergency crops were harvested 90 days after planting. Samples of soybean were separated into foliage and pod; samples of beet were separated into foliage and root. Details on sampling of cabbage and wheat are missing; it is assumed that the whole plants were sampled. The crop samples were rinsed to remove all adhering soil, weighed, frozen, lyophilised, weighed, and ground to 40 mesh in a Wiley mill. Information on storage of crop samples until analysis is missing.

3. Analytical procedures

Total radioactive residues (TRR) in all plant samples were determined by liquid scintillation counting (LSC) following combustion. Plant samples were combusted to $^{14}CO_2$ and trapped with phenethylamine based counting cocktail. The plants were lyophilised and ground to 40 mesh; 80 to 120 mg of plant material was placed directly into a gelatine capsule for combustion by Peterson Automatic Combustion Apparatus (PACA).

The lyophilised, ground, plant samples from each treatment were pooled and aliquots were extracted with of deionised water for 2 hours for each crop sample. The extracts were centrifuged, decanted, measured, and aliquoted for LSC. The extracts were analysed by chromatography on a column of AG 1-X8 (200 – 400 mesh) resin in the bicarbonate form prepared by washing the corresponding chloride form of resin with a solution of NH_4HCO_3 (1 M) followed by deionised water to give a neutral eluent. The entire water extract was applied to the column and eluted with solution of NH_4HCO_3 (0.2 M). For LSC analysis 5 mL fractions were collected.

Characterisation of glyphosate and AMPA in the water extracts was done by comparing of the elution volumes with a glyphosate and AMPA mixture and extracts of untreated crops spiked with glyphosate and AMPA (see elution volumes in the following table). An estimation of levels of glyphosate and AMPA was made on the basis of the ^{14}C-activity eluting in those areas where glyphosate and AMPA were shown to elute. The chromatograms of the standards showed the presence of approximately 1.0 % of [redacted], a known impurity of the ^{14}C-glyphosate preparation.

Table B.7.6.1.5-2: Comparison of column elution volumes of AMPA and glyphosate

Aqueous plant extract	Elution volume (mL)	
	AMPA	**Glyphosate**
Solvent	185 – 204	224 – 270
Soybean	l32 – 198	233 – 320
Wheat	122 – 176	215 – 303
Beet foliage	173 – 240	240 – 302
Beet root	170 – 240	240 – 300
Cabbage	139 – 192	192 – 259

II. Results and Discussion

A. Total radioactive residues (TRRs)

There was a reduction in the uptake of ^{14}C-activity with time. In no case was there an increase in the uptake of ^{14}C in any of the rotation crops with the exception of emergency crops of beet and cabbage, where a slight increase in TRRs was determined. TRRs in the primary crops ranged from 0.03 to 0.26 mg/kg for the control and from 0.13 to 3.65 mg/kg for the treated plant samples. These TRRs decreased after the first rotation to 0.05 to 0.23 mg/kg for the control and to 0.18 to 1.31 mg/kg for the treated plant samples. After the second rotation the TRRs further decreased to 0.01 to 0.27 mg/kg for the control samples and to 0.05 to 1.12 mg/kg for the treated plant samples. After the third rotation values for control samples were found to be 0.01 to 0.05 mg/kg and for treated plant samples to be 0.03 to 0.19 mg/kg.

Within the report, values for control samples were subtracted from corresponding treated values. These corrected values can be found within the following table (last column). For further calculation purposes within this summary, uncorrected values for treated samples were used without subtraction of corresponding control sample values.

Uptake by the treated wheat plants was exceptionally high. A possible explanation is that the pots contained no supports to keep the wheat plants from touching the treated soil. By allowing the wheat foliage to rest on the surface of the treated soil, some of the glyphosate or AMPA might enter the wheat plants through the foliage.

For unknown reasons crop growth was very poor during this study, resulting in a low wet weight. Watering by sub-irrigation with city water was believed to have caused a salt concentration on the surface of the pots causing toxic conditions for the plants. Watering from the top of the pots in the usual manner could have caused a dilution of pesticide and could have resulted in a lower uptake of ^{14}C.

Table B.7.6.1.5-3: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

Rotation	PBI (days)	Scenario type	Crop	Sampled commodity	Sampling (days after planting)	Sampling (DALT)	TRR (mg/kg)		
							control [1]	treated [1]	treated corrected for control [2]
Primary crop	- [3]	A	Soybean	Foliage	112	*115*	*0.15*	*0.21*	0.06
				Pod	112	*115*	*0.10*	*0.14*	0.04
		B	Cabbage	Whole plant	90	*93*	*0.03*	*0.13*	0.10
		C	Wheat	Whole plant	90	*93*	*0.26*	*3.65*	3.39
		D	Beet	Foliage	90	*93*	*0.10*	*0.46*	0.36
				Root	90	*93*	*0.06*	*0.31*	0.24
1st rotation (emergency crop) [4]	30	A	Beet	Foliage	90	*123* [5]	*0.05*	*0.32*	0.27
				Root	90	*123* [5]	*0.08*	*0.49*	0.41
		B	Cabbage	Whole plant	90	*123* [5]	*0.07*	*0.18*	0.11

Table B.7.6.1.5-3: Total radioactive residues in rotational crops planted after application of ^{14}C-glyphosate to bare soil

		C	Wheat	Whole plant	90	*123* [5]	*0.23*	*1.31*	1.08
		D	Beet	Foliage	90	*123* [5]	*0.05*	*0.20*	0.15
				Root	90	*123* [5]	*0.07*	*0.37*	*0.29* [6]
2nd rotation (4 months rotation)	120	A	Beet	Foliage	120	*240*	*0.02*	*0.05*	0.03
				Root	120	*240*	*0.02*	*0.08*	*0.06* [6]
		B	Wheat	Whole plant	120	*240*	*0.27*	*1.12*	0.86
		C	Beet	Foliage	120	*240*	*0.03*	*0.08*	0.05
				Root	120	*240*	*0.02*	*0.10*	0.08
		D	Cabbage	Whole plant	120	*240*	*0.01*	*0.08*	0.07
3rd rotation (1 year rotation)	365	A	Cabbage	Whole plant	97	*462*	*0.01*	*0.03*	0.02
		B	Beet	Foliage	120	*485*	*0.01*	*0.05*	0.04
				Root	120	*485*	*0.01*	*0.05*	0.04
		C	Cabbage	Whole plant	97	*462*	*0.02*	*0.06*	0.04
		D	Wheat	Whole plant	120	*485*	*0.05*	*0.19*	0.14

PBI Plant back interval (time between application onto bare soil and planting of crop)
DALT Days after last treatment (calculated as sum of days (given for PBI) + days (given for sampling days after planting) + 3 days (only for primary and emergency crop))
TRR Total radioactive residue
1 Calculated mean value of 2 replicates (4 replicates for first rotation)
2 Calculated within the report by subtraction of the control value from the respective treated value.
3 Primary crops were planted three days after treatment of test item onto bare soil.
4 Emergency crop after additional application of 4.48 kg a.s./ha. Information on exact time schedule is missing. After harvest of primary crop, primary plant parts were discarded without further analysis.
5 A second treatment was done to the pots before planting the emergency crop. The calculated DALT refers to the first treatment as information on timing of the second treatment is missing.
6 These values were recalculated as the mean values given in the report did not fit to the single values.
Italic figures were not part of the report, but correspond to values calculated upon figures given in the report.

B. Extraction and characterisation of residues

Plants containing ^{14}C-activity resulting from the soil treatment were analysed for extractability using water as the solvent for all matrices. The extractability for the treated samples varied from 32 % (beet, foliage, first rotation) to 76 % TRR (cabbage, third rotation, see Table B.7.6.1.5-4 - Table B.7.6.1.5-8). The extractability for the control samples varied from 29 % (wheat, first rotation) to 79 % TRR (cabbage, third rotation).
The extract was separated by an ion-exchange column (AG 1-X8 resin, HCO_3- form) into fractions representing glyphosate and AMPA and analysed by LSC.
For the control and treated samples, the recoveries of radioactivity after chromatography were between 14 and 88 %; further ^{14}C activity remained bound to the column. No further attempts were made to resolublise these bound residues. The chromatograms of the control crops served as comparison and showed the presence of ^{14}C-products resulting from $^{14}CO_2$ fixation which have an elution pattern similar to AMPA and glyphosate. No further information on these ^{14}C-products is given within the report.

Soybean, foliage and pods

For soybean, foliage and pods a TRR of 0.21 mg/kg and 0.14 mg/kg was found, respectively. After extraction with water 33 % and 37 % of the TRR were found in the water extracts. The chromatograms of the extracts of the control and treated soybean crops, showed ^{14}C-activity eluting in the glyphosate and AMPA regions. Detailed information on chromatographic results on these sample materials are not reported.

Cabbage

For cabbage a TRR of 0.13 mg/kg was found in the primary crop. The TRR increased slightly in the emergency crop (0.18 mg/kg) and then decreases to 0.08 mg/kg for PBI 120 days and finally decreased to 0.03 – 0.06 mg/kg for the PBI 365 days. After extraction with water between 48 % and 76 % of the TRR were found in the water extracts. The water extract contained 3.9 – 10.0 % (0.002 – 0.008 mg/kg) glyphosate and 1.7 – 6.7 % (0.002 – 0.005 mg/kg) AMPA. The chromatograms of the control rotational crops (PBI 120 and 365) showed the presence of ^{14}C-products resulting from $^{14}CO_2$ fixation which have an elution pattern similar to AMPA and glyphosate. No further information on these ^{14}C-products is given within the report.

Wheat

For wheat, whole plant a TRR of 3.65 mg/kg was found in the primary crop. The TRR decreased in the emergency crop (1.31 mg/kg) and then decreases to 1.12 mg/kg for PBI 120 days and finally decreased to 0.19 mg/kg for the PBI 365 days. After extraction with water between 48 % and 64 % of the TRR were found in the water extracts. The water extracts contained 0.5 – 9.9 % TRR (or <0.001 – 0.362 mg/kg) glyphosate and 0.3 – 9.8 % TRR (or <0.001 – 0.128 mg/kg) AMPA. The chromatograms displayed a broad range of ^{14}C-natural products, some of which eluted in the same fraction as AMPA or glyphosate. The found levels for AMPA or glyphosate were much higher than those seen in cabbage and beet. In this study it was difficult to grow wheat and to keep the plants from bending over and touching the soil. Harvested wheat was very dry resulting in a disproportionately high mg/kg value. The uptake of glyphosate was greater than AMPA with the exception of the emergency crop in which the uptake of AMPA was approximately three times the uptake of glyphosate. Comparison of the chromatographic traces shown indicates there were no unusual residues resulting from crop rotation.

Beet, foliage

For beet foliage a TRR of 0.46 mg/kg was found in the primary crop. The TRR decreased in the emergency crop (0.20 – 0.32 mg/kg) and then decreases to 0.05 – 0.08 mg/kg for PBI 120 days and finally decreased to 0.05 mg/kg for the PBI 365 days. After extraction with water between 32 % and 52 % of the TRR were found in the water extracts. The water extracts contained 1.0 – 2.0 % TRR (or <0.001 – 0.008 mg/kg) glyphosate and 1.3 – 4.6 % TRR (0.001 – 0.021 mg/kg) AMPA. The chromatographic analysis indicated there was a broad range of natural products arising from $^{14}CO_2$ fixation. The chromatograms of the emergency replant showed a narrow ^{14}C-peak eluting earlier than AMPA, and the corresponding control samples also showed ^{14}C-activity eluting in the same area. In all cases this unidentified peak corresponded to less than 7 % of the plant contained activity.

Beet, roots

For beet root a TRR of 0.31 mg/kg was found in the primary crop. The TRR increased in the emergency crop (0.37 – 0.49 mg/kg) and then decreases to 0.08 – 0.10 mg/kg for PBI 120 days and finally decreased to 0.05 mg/kg for the PBI 365 days. After extraction with water between 41 % and 58 % of the TRR were found in the water extracts. The water extracts contained 2.0 – 7.1 % TRR (or 0.002 – 0.022 mg/kg) glyphosate and 8.0 – 12.5 % TRR (or 0.004 – 0.041 mg/kg) AMPA. Within the chromatograms of the control samples there was a wide range of natural products containing ^{14}C-activity with the bulk of this activity eluting prior to the AMPA and glyphosate. The chromatographic patterns of the extracts of the treated samples were similar over the complete spectrum of different rotation scenarios. All of the extracts of the treated beet roots contained more activity in the region where AMPA eluted-than in the region where glyphosate eluted. The maximum level of glyphosate was 0.022 mg/kg, while the highest AMPA level seen was 0.041 mg/kg. There was no evidence of the occurrence of the formation of different degradation products as a function of time or the rotation of crops.

Table B.7.6.1.5-4: Extraction of the radioactive residues of glyphosate in soybean (foliage and pods) as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha

Experiment	**Soybean, foliage**		**Soybean, pods**	
PBI	Primary crop		Primary crop	
Sampling (DALT)	115		115	
Scenario type	A		A	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**
TRR	0.21	100	0.14	100
Extraction with water				
Water extract	*0.069*	33	*0.053*	38
Characterised	*0.069*	33	*0.053*	38
ERR	*0.069*	33	*0.053*	38
RRR (unextracted)	*0.141*	*67*	*0.087*	*62*
Total	0.21	100	0.14	100

Table B.7.6.1.5-4: Extraction of the radioactive residues of glyphosate in soybean (foliage and pods) as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha

Experiment	**Soybean, foliage**	**Soybean, pods**

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
n.a. Not analysed
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding.

Table B.7.6.1.5-5: Extraction of the radioactive residues of glyphosate in cabbage as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Experiment	**Cabbage**									
PBI	Primary crop		30 days		120 days		365 days		365 days	
Sampling (DALT)	93		123		240		462		462	
Scenario type	B		B		D		A		C	
Fraction	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.13	100	0.18	100	0.08	100	0.03	100	0.06	100
Extraction with water										
Water extract	*0.066*	51	*0.086*	48	*0.046*	58	*0.017*	57	*0.046*	76
Glyphosate	0.008	*6.2*	0.007	*3.9*	0.008	*10.0*	0.002	*6.7*	0.004	*6.7*
AMPA	0.005	*3.8*	0.003	*1.7*	0.005	*6.3*	0.002	*6.7*	0.002	*3.3*
Identified	*0.013*	*10.0*	*0.010*	*5.6*	*0.013*	*16.3*	*0.004*	*13.3*	*0.006*	*10.0*
Characterised	*0.053*	*41.0*	*0.076*	*42.4*	*0.033*	*41.8*	*0.013*	*43.7*	*0.040*	*66.0*
ERR	*0.066*	51	*0.086*	48	*0.046*	58	*0.017*	57	*0.046*	76
RRR (unextracted)	*0.064*	*49*	*0.094*	*52*	*0.034*	*42*	*0.013*	*43*	*0.014*	*24*
Total	0.13	100	0.18	100	0.08	100	0.03	100	0.06	100

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue (mg/kg values were calculated as sum of identified and characterised)
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
Identified was calculated as sum of glyphosate and AMPA. Characterised was calculated as water extract – identified.
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding.

Table B.7.6.1.5-6: Extraction of the radioactive residues of glyphosate in wheat as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Experiment	**Wheat**							
PBI	Primary crop		30 days		120 days		365 days	
Sampling (DALT)	93		123		240		485	
Scenario type	C		C		B		D	
Fraction	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	3.65	100	1.31	100	1.12	100	0.19	100
Extraction with water								
Water phase	*2.081*	57	*0.629*	48	*0.717*	64	*0.078*	41
Glyphosate	0.362	*9.9*	0.046	*3.5*	0.014	*1.3*	<0.001	*0.5*
AMPA	0.116	*3.2*	0.128	*9.8*	0.003	*0.3*	<0.001	*0.5*
Identified	*0.478*	*13.1*	*0.174*	*13.3*	*0.017*	*1.5*	*0.002*	*1.1*

Table B.7.6.1.5-6: Extraction of the radioactive residues of glyphosate in wheat as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Characterised	*1.603*	*43.9*	*0.455*	*34.7*	*0.700*	*62.5*	*0.076*	*39.9*
ERR	*2.081*	57	*0.629*	48	*0.717*	64	*0.078*	41
RRR (unextracted)	*1.570*	*43*	*0.681*	*52*	*0.403*	*36*	*0.112*	*59*
Total	3.65	100	1.31	100	1.12	100	0.19	100

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue (mg/kg values were calculated as sum of identified and characterised)
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
Identified was calculated as sum of glyphosate and AMPA. Characterised was calculated as water extract – identified.
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values below 0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.5-7: Extraction of the radioactive residues of glyphosate in beet, foliage as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Experiment	**Beet, foliage**											
PBI	Primary crop		30 days		30 days		120 days		120 days		365 days	
Sampling (DALT)	93		123		123		240		240		485	
Scenario type	D		A		D		A		C		B	
Fraction	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.46	100	0.32	100	0.20	100	0.05	100	0.08	100	0.05	100
Extraction with water												
Water extract	*0.216*	47	*0.102*	32	*0.082*	41	*0.019*	38	*0.041*	51	*0.026*	52
Glyphosate	0.008	*1.7*	0.005	*1.6*	0.002	*1.0*	<0.001	*2.0*	<0.001	*1.3*	<0.001	*2.0*
AMPA	0.021	*4.6*	0.006	*1.9*	0.004	*2.0*	0.001	*2.0*	0.001	*1.3*	0.002	*4.0*
Identified	*0.029*	*6.3*	*0.011*	*3.4*	*0.006*	*3.0*	*0.002*	*4.0*	*0.002*	*2.5*	*0.003*	*6.0*
Characterised	*0.187*	*40.7*	*0.091*	*28.6*	*0.076*	*38.0*	*0.017*	*34.0*	*0.039*	*48.5*	*0.023*	*46.0*
ERR	*0.216*	47	*0.102*	32	*0.082*	41	*0.019*	38	*0.041*	51	*0.026*	52
RRR (unextracted)	*0.244*	*53*	*0.218*	*68*	*0.118*	*59*	*0.031*	*62*	*0.039*	*49*	*0.024*	*48*
Total	0.46	100	0.32	100	0.2	100	0.05	100	0.08	100	0.05	100

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue (mg/kg values were calculated as sum of identified and characterised)
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
Identified was calculated as sum of glyphosate and AMPA. Characterised was calculated as water extract – identified.
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values below 0.001 mg/kg were set as 0.001 mg/kg.

Table B.7.6.1.5-8: Extraction of the radioactive residues of glyphosate in beet, root as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Experiment	**Beet, root**											
PBI	Primary crop		30 days		30 days		120 days		120 days		365 days	
Sampling (DALT)	93		123		123		240		240		485	
Scenario type	D		A		D		A		C		B	
Fraction	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
TRR	0.31	100	0.49	100	0.37	100	0.08	100	0.10	100	0.05	100
Extraction with water												

Table B.7.6.1.5-8: Extraction of the radioactive residues of glyphosate in beet, root as rotational crop planted after application of glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha (two applications for emergency crops, final dose rate 8.96 kg/ha)

Experiment	**Beet, root**											
Water extract	*0.174*	56	*0.265*	54	*0.170*	46	*0.032*	40	*0.041*	41	*0.029*	58
Glyphosate	0.022	*7.1*	0.018	*3.7*	0.012	*3.2*	0.002	*2.5*	0.002	*2.0*	0.002	*4.0*
AMPA	0.030	*9.7*	0.041	*8.4*	0.036	*9.7*	0.010	*12.5*	0.008	*8.0*	0.004	*8.0*
Identified	*0.052*	*16.8*	*0.059*	*12.0*	*0.048*	*13.0*	*0.012*	*15.0*	*0.010*	*10.0*	*0.006*	*12.0*
Characterised	*0.122*	*39.2*	*0.206*	*42.0*	*0.122*	*33.0*	*0.020*	*25.0*	*0.031*	*31.0*	*0.023*	*46.0*
ERR	*0.174*	56	*0.265*	54	*0.170*	46	*0.032*	40	*0.041*	41	*0.029*	58
RRR (unextracted)	*0.136*	*44*	*0.225*	*46*	*0.200*	*54*	*0.048*	*60*	*0.059*	*59*	*0.021*	*42*
Total	0.31	100	0.49	100	0.37	100	0.08	100	0.1	100	0.05	100

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue (mg/kg values were calculated as sum of identified and characterised)
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
Identified was calculated as sum of glyphosate and AMPA. Characterised was calculated as water extract – identified.
Values calculated upon dossier compilation are presented in italics. Minor deviations may occur due to rounding. Values below 0.001 mg/kg were set as 0.001 mg/kg.

C. Storage stability

Within the report information on storage duration and conditions is missing. The date of analysis is not indicated within the report.

D. Degradation pathway

Please refer to the overall pathway of glyphosate in rotational crops in Vol. 1, 2.7.7.

III. Conclusion

The metabolism of glyphosate was examined in rotational crops. Glyphosate radio-labelled with ^{14}C in the methyl position [N-(phosphono-^{14}C-methyl)glycine, called ^{14}C-glyphosate in this summary] was applied to sandy loam soil in pots at a rate of 4.48 kg a.s./ha. Primary crops of soybean, cabbage, wheat and beet, were planted 3 days after application. In parallel, unlabelled glyphosate was applied to identical pots for control purposes and kept in the same greenhouse to account for $^{14}CO_2$ fixation from degradation in soil. After harvesting the primary crop, different succeeding crops (cabbage, wheat and beet) were planted at plant back intervals of 30, 120 or 365 days. To simulate crop failure, some of the pots containing the primary crop received a second treatment of 4.48 kg a.s./ha after 30 days and were replanted with the same crops as before (except for soybeans where beet were replanted). Extraction of sample materials with water released 32 – 76 % TRR; no further attempts were performed to resolublise the bound residues. The low recoveries of radioactivity after chromatography may be reflected by the fact that applied radioactivity had been incorporated via $^{14}CO_2$ fixation into a variety of natural products which remained bound to the column. In addition to the uneluted ^{14}C-activity, many ^{14}C-products with elution patterns similar to glyphosate and AMPA were observed. No further investigations were conducted to identify those ^{14}C-products. Concerning radioactive residues found there was a reduction in the uptake of ^{14}C-activity with time. There was no increase in the uptake of ^{14}C in any of the rotation crops with the exception for emergency crops beet and cabbage, where a slight increase in TRRs was determined. The rotational crops from the 30 PBI scenario contained 0.002 – 0.046 mg/kg of glyphosate and 0.003 – 0.128 mg/kg AMPA. Residues from respective plant materials for the 120 PBI and 365 PBI decreased to <0.001 – 0.014 mg/kg for glyphosate and 0.001 – 0.010 mg/kg for AMPA.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study assessing the metabolic behaviour of glyphosate in rotational crops cabbage, wheat and beet has been previously evaluated at EU level. It was not performed under GLP. The study is deemed to largely comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Metabolism in Rotational Crops, 502 with major deficits: The radiochemical purity of the test item is not clearly specified and details on applications (formulation on test item and timing) are missing within the report. Information about timing of the second treatment (emergency crop) is missing. Developmental stages of the crop at harvesting are not reported. Harvest samples of wheat were not separated into grain and straw, no intermediate samples (green material) were collected; only results for the whole plant at harvest are available. Details about sampling of cabbage is missing, it is assumed that the whole plant was sampled. No information is provided on the storage stability for all major components of the total radioactive residues. Storage conditions and duration of plant samples is not given. Date of analysis is missing within the report. Extraction rates were low (32 – 76 %). The water extracts were only analysed by ion-exchange chromatography. No attempts were made to characterise the bound radioactivity. Analysis of primary crop soybean for glyphosate and AMPA was not conducted. Identification of glyphosate and AMPA was done by comparison with elution volumes of respective standards; no additional analytic method was established. Unextracted radioactive residues for each sample were not precisely quantified (not analysed by combustion/further extraction, only calculated from TRR - ERR).
The study is considered as not reliable for the assessment of the metabolic behaviour of glyphosate in rotational crops.

Assessment and conclusion by RMS:
As already summarized by the applicant in the box above, the study suffers from many shortcomings. Like for some other metabolism studies in the current dossier, no attempts have been made to investigate the unextracted residues, while their levels were above the trigger for further characterization. However, since it is even for some crops unclear what part of the crop has been sampled, extractability was in many cases low, crop growth was poor, and results on identification are considered not very reliable, the study is considered not acceptable.

B.7.6.1.6. Carrot, bean, pea, cabbage and corn

1. Information on the study

Data point:	CA 6.6.1/006
Report author	[redacted]
Report year	1976
Report title	Uptake and Metabolism of CP 67573 in representative vegetables and rotation crops
Report No	406
Document No	
Guidelines followed in study	Not specified
Deviations from current test guideline	A review of this study indicates the following deviations from OECD Guideline for the Metabolism in Rotational Crops, 502: • Not a typical rotational crop study regarding number of rotations and rotational intervals, only one rotation was conducted (PHI 29-101). • Information about application method and formulation of test item is missing. • Developmental stages of the crops at application and harvesting are not reported, but could be roughly estimated based on planting and sampling dates. • Physical facility and environmental conditions not described • No information on the storage stability. • No description of conditions and length of storage of samples. • Relevant amounts of residues remained in the extracts (>0.01 mg/kg, >10 % TRR) that were not further investigated. • Relevant amounts of non-extractable residues were not characterised / not investigated.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015)

GLP/Officially recognised testing facilities	No, GLP was not compulsory at the time the study was performed
Acceptability/Reliability:	Conclusion applicant: valid (Category 2a) Conclusion RMS: supportive only

2. Full summary of the study according to OECD format

Executive Summary

The metabolism of glyphosate was examined in rotational crops. The uptake of glyphosate and/or its metabolites from soils into representative vegetables: carrots (root type), cabbage (leafy type), string beans and peas (both legumes) was investigated. In addition to the vegetables mentioned previously, sweet corn was used as one of the rotation crops. Plants were grown on two different soils, a sandy loam (Norfolk soil) and a silt loam (Ray soil). At the maximum plant growth of the primary crops, N-(phosphono-^{14}C-methyl)glycine (namely ^{14}C-glyphosate within this following summary) was applied to the bare soil at a rate of 4.48 kg a.s./ha. The primary crops were sampled 4 - 11 weeks after treatment. Rotational crops (same as primary plants plus sweet corn) were planted within a 1 - 23 day interval after harvest of the primary crops (PBI 29 - 79 / 101 days) and harvested 6.5 to 17.5 weeks after planting. In addition, soil samples were taken after harvest of the primary and of the rotational crops from each plot. The fate of glyphosate and its metabolites in soil in primary and in rotational crops was investigated.

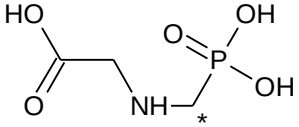
The radioactive residues were quantified by LSC following combustion. In primary crops the residues were higher for plants grown on Ray soil (silt loam; up to 1.07 mg/kg) than for plants grown on Norfolk soil (sandy loam; up to 0.22 mg/kg). The radioactive residues detected in rotational crops grown on the two different soils were comparable (all between about 0.040 and 0.280 mg/kg. The extractabilities of plant material with water were between 55.7 – 92.2 % TRR, except for some matrices of corn, carrots and bean leaves, from which lower amounts (23.8 – 51.6 % TRR) were extracted.

For crops grown on the sandy loam soil, glyphosate was the major component detected in the plant extracts of primary and rotational crops. Glyphosate was detected at up to 0.137 mg/kg (primary crops) and up to 0.128 mg/kg (rotational crops). AMPA was less abundant in these extracts and was detected between 0.002 and 0.044 mg/kg. Components that were characterised upon their elution behaviour and are designated as “neutrals”, “others” or “indeterminates”, were detected at up to 0.037 mg/kg in the extracts of primary and rotational crops from Norfolk soil.
In plant extracts from primary crops grown on the silt loam soil, the amounts of AMPA (found at up to 0.041 mg/kg), were generally about twice as high as the concentrations of glyphosate. Glyphosate was not present in the extracts of rotational crops and AMPA was found at only low amounts (up to 0.004 mg/kg). The major part of radioactive residues were neutrals and / or indeterminates, representing up to 0.140 mg/kg.

Analyses of the soil samples revealed that glyphosate was relatively stable in the sandy loam soil. About 82 % of the applied radioactivity was still left after 4 weeks and 27 % after 31 weeks. In contrast, in the silt loam soil about 70 % of the applied radioactivity dissipated within 4 - 7 weeks and after about 25 weeks ≤10 % of the applied radioactivity was detected. The main component in all extracts of the sandy loam soil was unchanged glyphosate (67 - 93 %), AMPA was detected between 4 - 23 %. For the silt loam soil, taken after 4 - 7 weeks about 20 % glyphosate and about 70 % AMPA were identified in the soil extracts, while after 24 weeks no glyphosate and about 40 % AMPA were detected.

I. Materials and Methods

A. Materials

1. Test material	N-(phosphono-^{14}C-methyl)glycine
Chemical structure:	 * Position of radiolabel
Radiochemical purity:	>99.9 % after purification (by AG-50W-X8 chromatography)
Specific activityof the test substance applied:	Batch I: 1.98 MBq/mg (9.07 mCi/mmol) Batch II: 1.76 MBq/mg (8.03 mCi/mmol) Batch III: 0.41 MBq/mg (1.87 mCi/mmol)

2. Test material	
Soil:	Ray silt loam (pH: 8.1; cation exchange capacity: 10.4 %; sand: 4.6 %; silt: 84.2 %; clay: 10.0 %, organic matter: 1.2 %) Norfolk sandy loam (pH: 5.7; sand: 86.0 %; silt: 11 %; clay: 2.3 %, organic matter: 1.0 %)
Crop:	Carrot (Nantes) Cabbage (Wisconsin Golden Acres cabbage) Peas (Alaska peas) String beans (bush type; Burpees' Stringless and Tendergreen varieties) Sweet corn (variety DeKalb XL-45)
Botanical name:	*Daucus carota* subsp. *sativus* *Brassica oleracea* var. *Capitata* *Pisum sativum* *Phaseolus vulgaris* *Zea mays*
Crop part(s):	String bean and pea (pods and leaves), carrot (roots and leaves), cabbage (head and leaves), corn (leaf, kernels, cob)

B. Study design

1. In-life phase

Three batches of ^{14}C-glyphosate were used in the study, having specific activities of 1.98 MBq/mg (9.07 mCi/mmol; batch I), 1.76 MBq/mg (8.03 mCi/mmol; batch II) and 0.41 MBq/mg (1.87 mCi/mmol; batch III).
For the experiments with peas, string beans and cabbage in Norfolk sandy loam soil, the treatment solution was a mixture of 80 % of batch III and 20 % of batch I. For experiments with carrots in sandy loam soil, a mixture of 55.7 % batch III, 24.7 % batch II and 19.6 % of non-radioactive glyphosate was used. For the treatment of Ray silt loam soil (growing of carrots), the mixture was comprised of 10.7 % batch I and 89.3 % batch III. For experiments with string beans, silt loam soil was treated entirely with batch III.

The soils of the test plots were treated on the surface with the ^{14}C-glyphosate in 0.1 M NH_4HCO_3 solution at target rates of 4.48 kg a.s./ha (4lbs/A) on bare soil which was equivalent to applying 7.8 mg ^{14}C-glyphosate per pot and 22.7 mg per bucket. Detailed information about application method and formulation of test item is missing within the study report.
For controls, similar crops were planted in corresponding containers in order to check for $^{14}CO_2$ fixation from the soil metabolism of the applied radiolabelled glyphosate and grown side by side with the treated crops. All plants were nourished with the modified Hoagland's solution about every 5 - 7 days.

The plants were grown in 15 cm (6 inch) diameter pots and/or 30.5 cm (12 inch) diameter buckets using sandy loam and silt loam soils. The soil of the test plots of the main crops (primary crops) were treated 16 - 96 days after planting, while the maximum growth of the plants was taking place. Treatments were carried out 18 days after planting the peas, 16 days for string beans, 81 days for carrots, and 53 - 60 days for cabbage for sandy loam soil and 15 days after planting the string beans and 81 -96 days after planting carrots for silt loam soil. The primary crops were harvested 4 - 11 weeks after treatment. Rotational crops were planted l - 23 days after harvesting the main crops. Planting of the primary and rotational crops was according to the following design:

Table B.7.6.1.6-1: Overview of the different scenarios of crop rotation

Plot No.	**Primary crop**	**Rotational crops**
Norfolk soil (sandy loam)		
A	Pea	Carrot
		Cabbage
B	String bean	Corn, sweet
C	Carrot	Cabbage
		String bean
D	Cabbage	Carrot
		Pea

Table B.7.6.1.6-1: Overview of the different scenarios of crop rotation

Plot No.	Primary crop	Rotational crops
Ray soil (silt loam)		
E	String bean	Carrot
		Cabbage
F	Carrot	-

2. Sampling

Sampling of plants: The plants were cut off about 2.5 cm above the soil level and any visible soil and dirt were wiped off. String bean and pea pods were sampled separately from their respective leaves. Carrots were pulled out from the soil and washed in a series of three distilled water baths. In addition, the residual soils were removed with the aid of a toothbrush and water rinse.
Wet weights of each sample were taken. Cabbage and carrots were sliced. Samples were then frozen and lyophilised (freeze-dried) after freezing. After drying, the dry weights were determined and the samples were ground to 40 mesh size in a Wiley mill. Aliquots were combusted to determine the total ^{14}C-content.

Sampling of soils: Triplicate samples were taken from each pot and 5 - 7 samples from each bucket. Samples were taken with the aid of a cork borers, dug 8 - 9 cm deep into the soil. Each sample was placed in a tared vial, frozen and lyophilised.

In the following table the time intervals/ sampling times of treatment and harvest of primary crops, rotational crops and soil are given.

Table B.7.6.1.6-2: Overview of the different scenarios of crop rotation

Rotation	Plot No.	Crop	Age of crop at treatment (days)	PBI (days)		Crop sampling (DALT)	Soil sampling (weeks [1])
				min	max		
Norfolk soil (sandy loam)							
Primary crop	A	Pea	18	-	-	28	4
	B	String bean	16	-	-	46	6.5
	C	Carrot	81	-	-	50	7
	D	Cabbage	53-60	-	-	78	11
Rotational crop	A	Carrot [2]	-	*29*	*51*	98	18
		Cabbage [2]	-	*29*	*51*	99	18
	B	Corn, sweet	-	*47*	*69*	70	16.5
						110	22
	C	Cabbage	-	*51*	*73*	45	13.5
		String bean	-	*51*	*73*	122	27
	D	Carrot	-	*79*	*101*	45	17.5
		Pea	-	*79*	*101*	122	31
Ray soil (silt loam)							
Primary crop	E	String bean	15	-	-	30	4
	F	Carrot	81-96	-	-	51	7
Rotational crop	E	Carrot	-	*52*	*74*	122	24.5
		Cabbage	-	*52*	*74*	122	24.5
	F	-	-	-	-	-	-

Table B.7.6.1.6-2: Overview of the different scenarios of crop rotation

Rotation	Plot No.	Crop	Age of crop at treatment (days)	PBI (days)		Crop sampling (DALT)	Soil sampling (weeks [1])
				min	max		

PBI Plant back interval in days (time interval between treatment and planting): PBI was calculated upon the statement in the report, that rotational crops were planted l - 23 days after harvesting the primary crops: DALT of primary crop + 1 day (min) or + 23 days (max). A more detailed calculation is not possible due to missing details within the report.
DALT Days after last application (called "duration of treatment" within the report)
1 Soil was sampled in different intervals after treatment, given in weeks.
2 Crops were transplanted to the treated soil at an age of 47 days of growing.
Italic figures were not part of the report, but correspond to values calculated upon figures given in the report.

3. Analytical procedures

Total radioactive residues (TRR) in the plant and soil samples were determined by combustion and LSC. To determine the radioactivities in soils and plant samples, the Petersen Automatic Combustion Apparatus (PACA) was used. This apparatus quantitatively oxidises carbon ^{14}C-containing materials to $^{14}CO_2$. For combustions, two homogeneous aliquots from the dried samples were weighed after determining the soil dry weights. The total ^{14}C-content of each pot/bucket was then calculated from the combustion results.

For extracting radioactive residues from vegetables, the ground plant samples were extracted for 2 hours with water. The remaining solids were removed by centrifugation. The extracts were assayed by liquid scintillation counting (LSC). Water extracts from vegetable samples were analyzed by AG-50W-X8 and AG-1-X8 column chromatography. In the analysis of crops from the silt loam soil, AG-50 column chromatography was not applicable. A large amount of so called "neutral materials" eluting in front of glyphosate overshadowed the glyphosate present such that partitioning between these two entities was not possible. Therefore analysis was carried out by HVE (high voltage electrophoresis) and AG-1-X8 (HCO_3^-) chromatography in these cases.

Soil samples were extracted for 2 hours with 0.5 M NH_4OH. Aliquots of the supernatant were concentrated under vacuum and analyzed by LSC and by AG-50W-X8 column chromatography as well as by HVE separately. Radioactive spots on TLC plates or paper electrophoretograms were located and quantified using the Beta Camera.

The assignments of glyphosate and AMPA was verified by analysis of reference items.

To verify the identity of glyphosate and its metabolite AMPA, the radioactive components were isolated from selected plant samples (peas, carrots and cabbage from the sandy loam soil and string beans from the silt loam soil). Different ion exchange and size exclusion chromatographic resins (AG-50W-X4, AG-1-X8, AG-50W-X8, Bio-Gel P-2) were used for purification of ^{14}C-glyphosate and ^{14}C-AMPA.
Depending on the purity of the compound being isolated, the sample was either derivatised or purified further by HVE, another AG-1-X8 column and finally AG-50W-X8 chromatography.
The purified samples were derivatised to give either trimethyl N-trifluoroacetyl glyphosate or dimethyl N-trifluoroacetyl AMPA followed by determination using GC with phosphorous specific detection (PFD). In addition, detection was by flame ionisation detection coupled with radioactive detection (FID/RAD) as well as GC-MS for metabolite identification. The identification was verified by comparison of the analyses performed with (derivatised) reference compounds.

II. Results and Discussion

A. Total radioactive residues (TRRs)

The results of the soil uptake experiment are summarised in the table below. The values are given in % of the applied radioactivity (% AR) and in mg/kg of the total radioactive residues (TRR).

Regarding the primary crops, uptake from sandy loam soil ranged from 0.051 to 0.27 % of the applied radioactivity for treated plants. Control plants showed an uptake of 0.006 to 0.02 %. The uptake by rotational crops from sandy loam soil ranged from 0.023 to 0.26 % of the applied radioactivity. Comparatively high values were found in some of the control plants, reaching from 0.002 to 0.11 %.
Uptake from treated silt loam soil was found to be higher than in the sandy loam soil. The uptake ranged from 0.05 % for bean pods to 1.05 % for the bean leaves. Uptake of the control plants was 0.006 to 0.07 %. Rotational crops in the silt loam soil showed low residues (0.047 to 0.074 %); the control plants were all <0.01 % of the applied radioactivity.

After harvest, total radioactive residues (TRR) in the primary crop samples ranged from 0.080 to 1.070 mg/kg. The highest residue levels were detected in string bean leaves (1.07 mg/kg), carrot leaves (0.494 mg/kg) and carrot root (0.31 mg/kg) grown on silt loam soil. For crops grown on sandy loam soil, highest values were found in pea tops (0.22 mg/kg). Generally, residue levels were lower in the analysed plants from the sandy loam soil (see table B.7.6.1.6-3).

For rotational crops, the highest residue levels were detected in pea pods (0.28 mg/kg) and in pea leaves (0.19 mg/kg) grown on sandy loam soil. All other TRR values were between 0.039 mg/kg and 0.094 mg/kg for plants grown on either soil.

Table B.7.6.1.6-3: Radioactivity found in primary crops and rotational crops after application of ^{14}C-glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha

Rotation	PBI (days)	Plot No.	Crop	Sampled commodity	Sampling (DALT)	Treated plants		Control plants (% AR)
						% AR	TRR (mg/kg)	
Norfolk soil (sandy loam)								
Primary crop	-	A	Pea	Tops	28	0.051	0.22	0.006
		B	String bean	Tops	46	0.13	0.13 [2]	0.02
				Pods		0.07		n.a.
		C	Carrot	Leaves	50	0.21	0.17	0.01
				Root		0.15	0.11	0.018
		D	Cabbage	Head	78	0.04	0.08 [1]	0.02 [1]
				Leaves		0.27		
Rotational crop	29-101	A	Carrot	Leaves	98	0.14	0.086	n.a.
				Root		0.16	0.094	n.a.
			Cabbage	Cabbage [1]	99	0.26	0.056	0.11
		B	Sweet corn	Leaves	70	0.14	0.04	0.12
				Leaves	110	0.11	0.09	0.11
				Kernel	110	0.016	0.06	0.022
				Cob	110	0.023	0.05	0.043
		C	Cabbage	Cabbage [1]	45	0.20	0.045	0.03
			String bean	Leaves	122	0.044	0.08	0.012
				Pods		0.016	0.04	0.003
		D	Carrot	Leaves	45	0.06	0.08	0.02
				Root		0.09	0.05	0.04
			Pea	Leaves	122	0.07	0.19	0.008
				Pods		0.046	0.28	0.004
Ray soil (silt loam)								
Primary crop	-	E	String bean	Leaves	30	1.05	1.07	0.07
				Pods		0.05	0.19	0.006
		F	Carrot	Leaves	51	0.516	0.494	0.039
				Root		0.57	0.31	0.027
Rotational crop	52-74	E	Carrot	Leaves	122	0.038	0.061	0.005
				Root		0.047	0.039	0.007
			Cabbage	Cabbage [1]	122	0.074	0.051	0.006
		F	-	-	-	-	-	-

Table B.7.6.1.6-3: Radioactivity found in primary crops and rotational crops after application of ^{14}C-glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha

PBI Plant back interval in days (time interval between treatment and planting): PBI was calculated upon the statement in the report, that rotational crops were planted 1 - 23 days after harvesting the primary crops: DALT of primary crop + 1 day (min) or + 23 days (max). A more detailed calculation is not possible due to missing details within the report.
DALT Days after last application (called "duration of treatment" within the report)
AR Applied radioactivity
TRR Total radioactive residue
n.a. Not analysed
1 Sampled commodity was designated as "cabbage"; information about separation of head and leaves is missing.
2 Sampled commodity was designated as "string bean"; information about separation of tops and pods is missing.

In Norfolk soil 81.86 % of the applied radioactivity was still left after 4 weeks and 27.07 % after 31 weeks. Ray soil showed a faster biodegradability and about 70 % of the applied radioactivity dissipated in 4 - 7 weeks. At the end of the experiment ≤10 % of the applied were detected in Ray soil. For further details see the following table.

Table B.7.6.1.6-4: Radioactivity found in soil after application of ^{14}C-glyphosate to bare soil at a dose rate of 4.48 kg ^{14}C-glyphosate/ha

Rotation	Plot No.	Crop	Soil sampling (weeks [1])	% AR [2]
Norfolk soil (sandy loam)				
Primary crop	A	Pea	4	81.86
	B	String bean	6.5	71.66
	C	Carrot	7	67.8
	D	Cabbage	11	52.59
Rotational crop	A	Carrot	18	58.11
		Cabbage	18	62.82
	B	Sweet corn	16.5	40.36
			22	53.04
	C	Cabbage	13.5	28.65
		String bean	27	61.80
	D	Carrot	31	27.07
		Peas	17.5	57.43
Ray soil (silt loam)				
Primary crop	E	String bean	4	30.8
	F	Carrot	7	29.4
Rotational crop	E	Carrot	24.5	6.43
		Cabbage	24.5	10.00
	F	-	-	-

AR Applied radioactivity
1 Soil was sampled in different intervals after treatment, given in weeks.
2 % AR values were calculated within the report as mean values of several samples per pot/bucket; each taken sample was measured twice.

B. Extraction and characterisation of residues

The mean extractabilities of plant samples and the composition of radioactive components in these extracts are shown in Table B.7.6.1.6-5 to Table B.7.6.1.6-9.

Sandy loam soil: The extractabilities with water of plants grown on the Norfolk sandy loam soil were good (all between 57.5 and 92.2 % TRR). Only the extractabilities of some corn and carrot matrices were somewhat lower (39.9 to 59.4 % TRR). The values are depicted in Table B.7.6.1.6-5 to Table B.7.6.1.6-7.

Upon chromatography, two components, namely glyphosate and AMPA, were identified.
Glyphosate was the predominant compound in extracts from all matrices of the primary crops (pea tops, string beans, carrots and cabbage) from Norfolk soil, ranging from 0.026 to 0.137 mg/kg (30.2 to 62.1 % TRR). AMPA was detected in the range from 0.005 to 0.013mg/kg representing 3.2 to 7.0 % TRR. Further components that were designated as "neutrals", "others" and "indeterminates" were characterised by their chromatographic properties and amounted to up to 0.021 mg/kg (neutrals), 0.010 mg/kg (others) and up to 0.029 mg/kg (indeterminates). The RRR in primary crops was between 0.017 mg/kg (7.8 % TRR, pea tops) and 0.073 mg/kg (43.0 % TRR, carrot leaves).

Glyphosate was also the most abundant compound in rotational crops grown on Norfolk soil, accounting for 0.016 to 0.128 mg/kg (19.6 to 46.4 % TRR), with the exception of corn, carrot (after cabbage) and cabbage (after carrots). In these matrices, comparable amounts of glyphosate and AMPA were detected (0.002 to 0.008 mg/kg or 2.9 to 12.0 % TRR). In the latter matrices, components designated as "neutrals" represented the majority of radioactive residues. (0.005 to 0.022 mg/kg, 12.8 to 43.2 % TRR). Altogether, components in the extract designated as "neutrals", "others" and "indeterminates", that were not identified but characterised by their chromatographic properties were detected at up to 0.026 mg/kg (neutrals) up to 0.013 mg/kg (others) and 0.037 mg/kg (indeterminates). The RRR in rotational crops was between 0.004 mg/kg or 9.7 % TRR (bean pods) and 0.055 mg/kg or 58.6 % TRR (carrots). No attempts were done to further solubilise this bound radioactivity.

Silt loam soil: The extractabilities with water of plants grown on the Ray silt loam soil were moderate (51.3 – 74.6 % TRR), with the exception of string bean leaves (primary crop) and carrot leaves (rotational crop), that had poor extractabilities with water (23.8 % and 35.8 %). The individual values are shown in Table B.7.6.1.6-8 to Table B.7.6.1.6-9.

In the analysis of extracts from Ray soil, AG-50 column chromatography was not applicable. The large amount of neutral materials, eluting in front of glyphosate overshadowed the amount of glyphosate present. Analysis was therefore carried out by HVE and AG-1 chromatography in these cases. Since uptake by rotation crops grown in Ray soil was very low, analytical problems were formidable, and only the AG-1 column chromatography was applicable.

In the extracts of primary crops grown on Ray soil (string beans and carrots), the amounts of glyphosate ranged from 0.008 to 0.047 mg/kg showing significantly lower percentages (l.0 to 9.0 % TRR) than plant extracts from the Norfolk soil (compare section above). AMPA was more prominent than glyphosate, amounting to 0.017 to 0.041 mg/kg (1.9 to 22.5 % TRR). Altogether, characterised components in the extract designated as "neutrals", "others" and "indeterminates" were detected at amounts up to 0.140 mg/kg (neutrals), up to 0.037 mg/kg (others) and up to 0.130 mg/kg (indeterminates). The highest amount of neutrals (45.5 % TRR) was detected in the extract of carrots. The radioactive residues that were not extracted with water (RRR) were comparatively high and were detected between 0.080 mg/kg and 0.805 mg/kg (representing between 25.4 and 76.2 % TRR). No attempts were done to further identify this not extracted radioactivity.
In the extracts from rotational crops from Ray soil (cabbage and carrot) no glyphosate was detected, while AMPA ranged from 0.001 to 0.004 mg/kg (2.4 to 9.0 % TRR).
Components in the extracts designated as "neutrals", "others" and "indeterminates", that were not identified but characterised were detected at up to 0.019 mg/kg (neutrals) and up to 0.007 mg/kg (others and indeterminates). The residues not extracted with water (RRR) ranged between 0.013 and 0.430 mg/kg or 34.1 to 64.2 % TRR. No attempts were done to further identify this not extracted radioactivity.

Table B.7.6.1.6-5: Distribution of radioactive residues of glyphosate and its metabolites in primary crop (pea) and rotational crops (cabbage, carrot) after application of glyphosate to Norfolk sandy loam soil

Scenario type A	**Primary crop**		**Rotational crops**					
	Pea tops		**Cabbage**		**Carrot, root**		**Carrot, leaves**	
DALT	**28**		**99**		**98**		**98**	
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	100.0	**0.22**	**100.0**	**0.056**	**100.0**	**0.094**	**100.0**	**0.086**
Extraction with water								
Aqueous extract	92.2	0.203	91.8	0.051	41.4	0.039	45.7	0.039
Glyphosate	*62.1*	0.137	*46.4*	0.026	*19.6*	0.018	*21.1*	0.018
AMPA	*5.9*	0.013	*4.5*	0.002	*5.6*	0.005	*2.3*	0.002
Total identified	***68.0***	***0.150***	***50.9***	***0.028***	***25.2***	***0.024***	***23.4***	***0.020***
Neutrals	*6.2*	*0.014*	*15.9*	*0.009*	*12.5*	*0.012*	*14.0*	*0.012*
Others	*4.7*	*0.010*	*8.4*	*0.005*	*3.8*	*0.004*	*4.6*	*0.004*
Indeterminate	*13.4*	*0.029*	*16.7*	*0.009*	*n.d.*	*n.d.*	*3.3*	*0.003*
Total characterised	***24.2***	***0.053***	***40.9***	***0.023***	***16.2***	***0.015***	***21.8***	***0.020***
ERR	**92.2**	**0.203**	**91.8**	**0.051**	**41.4**	**0.039**	**45.7**	**0.039**

Table B.7.6.1.6-5: Distribution of radioactive residues of glyphosate and its metabolites in primary crop (pea) and rotational crops (cabbage, carrot) after application of glyphosate to Norfolk sandy loam soil

Scenario type A	**Primary crop**		**Rotational crops**					
	Pea tops		**Cabbage**		**Carrot, root**		**Carrot, leaves**	
DALT	**28**		**99**		**98**		**98**	
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	100.0	**0.22**	**100.0**	**0.056**	**100.0**	**0.094**	**100.0**	**0.086**
RRR	***7.8***	***0.017***	***8.2***	***0.005***	***58.6***	***0.055***	***54.3***	***0.047***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
n.d. Not detected
Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Remark: Data presented for pea tops originate from a small scale experiment. Results of the large scale experiment (using AG-50 column) were comparable (see report, table 9).
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.
Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in *italics.*

Table B.7.6.1.6-6: Distribution of radioactive residues of glyphosate and its metabolites in primary crops (string bean) and rotational crop (corn) after application of glyphosate to Norfolk sandy loam soil

Scenario type B	**Primary crop**		**Rotational crops**							
	String beans		**Corn first harvest**		**Corn second harvest**		**Corn kernel**		**Corn cob**	
DALT	**46**		**70**		**110**		**110**		**110**	
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	100.0	**0.13**	**100.0**	**0.04**	**100.00**	**0.09**	**100.0**	**0.06**	**100.0**	**0.05**
Extraction with water										
Aqueous extract	**87.5**	**0.110**	**48.0**	**0.020**	**59.4**	**0.053**	**39.9**	**0.024**	**51.5**	**0.026**
Glyphosate	*54.2*	0.068	*12.0*	0.005	*5.6*	0.005	--	--	*3.2*	0.002
AMPA	*6.0*	0.007	*11.1*	0.003	*9.0*	0.008	--	--	*9.0*	0.005
Total identified	***60.1***	***0.075***	***23.2***	***0.008***	***14.5***	***0.013***	--	--	***12.3***	***0.007***
Neutrals	*12.4*	*0.016*	*12.8*	*0.005*	*23.3*	*0.021*	*17.6*	*0.011*	*26.6*	*0.013*
Others	*7.2*	*0.009*	*12.0*	*0.005*	*7.6*	*0.007*	--	--	*10.8*	*0.005*
Indeterminate	*7.8*	*0.010*	*0.0*	*0.000*	*14.0*	*0.012*	--	--	*1.9*	*0.001*
Total characterised	***27.4***	***0.034***	***24.8***	***0.010***	***44.9***	***0.040***	***17.6***	***0.011***	***39.2***	***0.020***
ERR	**87.5**	**0.110**	**48.0**	**0.020**	**59.4**	**0.053**	**39.9**	**0.024**	**51.5**	**0.026**
RRR	***12.5***	***0.020***	***52.0***	***0.020***	***40.6***	***0.037***	***60.1***	***0.036***	***48.5***	***0.024***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue
Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Remark: Data presented for string beans originate from a small scale experiment. Results of the large scale experiment (using AG-50 column) were comparable (see report, table 9).
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.

Table B.7.6.1.6-6: Distribution of radioactive residues of glyphosate and its metabolites in primary crops (string bean) and rotational crop (corn) after application of glyphosate to Norfolk sandy loam soil

Scenario type B	Primary crop		Rotational crops							
	String beans		Corn first harvest		Corn second harvest		Corn kernel		Corn cob	
DALT	46		70		110		110		110	
	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg
	100.0	0.13	100.0	0.04	100.00	0.09	100.0	0.06	100.0	0.05

Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in italics.

Table B.7.6.1.6-7: Distribution of radioactive residues of glyphosate and its metabolites in primary crops (carrot) and rotational crops (string bean and cabbage) after application of glyphosate to Norfolk sandy loam soil

Scenario type C	Primary crop				Rotational crops					
	Carrot		Carrot leaves		String bean leaves		String bean pod		Cabbage	
DALT	50				122				45	
	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg
	100.0	0.11	100.0	0.17	100.0	0.08	100.0	0.04	100.0	0.045
Extraction with water										
Aqueous extract	77.2	0.085	57.0	0.097	66.2	0.053	90.3	0.036	57.5	0.026
Glyphosate	*34.5*	0.038	*30.2*	0.051	*23.8*	0.019	*40.1*	0.016	*8.3*	0.004
AMPA	*5.0*	0.006	*3.2*	0.005	*3.5*	0.003	*7.9*	0.003	*6.7*	0.003
Total identified	***39.5***	***0.044***	***33.3***	***0.056***	***27.3***	***0.022***	***48.0***	***0.019***	***15.0***	***0.007***
Neutrals	*17.8*	*0.020*	*12.5*	*0.021*	*14.4*	*0.012*	*20.0*	*0.008*	*24.0*	*0.011*
Others	*5.0*	*0.006*	*3.6*	*0.006*	*5.0*	*0.004*	*8.9*	*0.004*	*9.3*	*0.004*
Indeterminate	*14.8*	*0.016*	*7.6*	*0.013*	*19.6*	*0.016*	*13.4*	*0.005*	*9.3*	*0.004*
Total characterised	***37.7***	***0.041***	***23.7***	***0.040***	***38.9***	***0.031***	***42.3***	***0.017***	***42.5***	***0.019***
ERR	**77.2**	**0.085**	**57.0**	**0.097**	**66.2**	**0.053**	**90.3**	**0.036**	**57.5**	**0.026**
RRR	***22.8***	***0.025***	***43.0***	***0.073***	***33.8***	***0.027***	***9.7***	***0.004***	***42.5***	***0.019***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Remark: Data presented for carrot originate from a small scale experiment. Results of the large scale experiment (using AG-50 column) were comparable (see report, table 9).
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.
Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in *italics.*

Table B.7.6.1.6-8: Distribution of radioactive residues of glyphosate and its metabolites in primary crop (cabbage) and rotational crops (pea and carrot) after application of glyphosate to Norfolk sandy loam soil

Scenario type D	Primary crop				Rotational crops							
	Cabbage		Cabbage head		Pea leaves		Pea pods		Carrot		Carrot leaves	
DALT	78				122				45			
	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg	% TRR	mg/ kg

	100.0	**0.08**	**100**	n.d.	**100.0**	**0.19**	**100.0**	**0.28**	**100.0**	**0.05**	**100.0**	**0.08**
Extraction with water												
Aqueous extract	**69.1**	**0.055**	**65.9**	n.d.	**88.7**	**0.168**	**88.5**	**0.248**	**73.4**	**0.037**	**51.6**	**0.042**
Glyphosate	*32.1*	0.026	*39.2*	**n.d.**	*40.4*	0.076	*45.8*	0.128	*6.9*	0.003	*7.7*	0.006
AMPA	*7.0*	0.006	*2.9*	n.d.	*11.1*	0.021	*15.6*	0.044	*7.6*	0.004	*2.9*	0.002
Total identified	***39.0***	***0.032***	***42.1***	*n.d.*	***51.4***	***0.097***	***61.4***	***0.172***	***14.5***	***0.007***	***10.6***	***0.008***
Neutrals	*17.0*	*0.014*	*18.5*	*n.d.*	*13.6*	*0.026*	*9.0*	*0.025*	*43.2*	*0.022*	*17.9*	*0.015*
Others	*4.2*	*0.003*	*2.0*	*n.d.*	*5.5*	*0.010*	*4.8*	*0.013*	*8.5*	*0.004*	*8.6*	*0.007*
Indeterminate	*8.8*	*0.007*	*2.9*	*n.d.*	*18.2*	*0.034*	*13.3*	*0.037*	*7.2*	*0.004*	*14.5*	*0.012*
Total characterised	***30.1***	***0.024***	***23.4***	*n.d.*	***37.3***	***0.071***	***27.1***	***0.076***	***58.9***	***0.030***	***41.0***	***0.033***
ERR	**69.1**	**0.055**	**65.9**	n.d.	**88.7**	**0.168**	**88.5**	**0.248**	**73.4**	**0.037**	**51.6**	**0.042**
RRR	***30.9***	***0.025***	***34.1***	*n.d.*	***11.3***	***0.022***	***11.5***	***0.032***	***26.6***	***0.013***	***48.4***	***0.038***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
n.d. Not determined
Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Remark: Data presented for cabbage originate from a small scale experiment. Results of the large scale experiment (using AG-50 column) were comparable (see report, table 9).
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.
Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in *italics*.

Table B.7.6.1.6-9: Distribution of radioactive residues of glyphosate and its metabolites in primary crops (string bean) and rotational crops (cabbage and carrot) after application of glyphosate to Ray silt loam soil

Scenario type E	**Primary crop**				**Rotational crops**					
	String bean leaves		**String bean pod**		**Cabbage**		**Carrot**		**Carrot leaves**	
DALT	**30**				**122**		**122**			
	% TRR	**mg/ kg**	**% TRR**	**mg/ kg**	**% TRR**	**mg/ kg**	**% TRR**	**mg/ kg**	**% TRR**	**mg/ kg**
	100.0	**1.07**	**100.0**	**0.19**	**100.0**	**0.051**	**100.0**	**0.039**	**100.0**	**0.061**
Extraction with water										
Aqueous extract	23.8	0.262	57.1	0.104	55.7	0.028	65.9	0.026 [1]	35.8	0.018
Glyphosate	*1.0*	0.012	*7.9*	0.014	*n.d.*	n.d.	*n.d.*	n.d.	*n.d.*	n.d.
AMPA	*1.9*	0.020	*22.5*	0.041	*8.4*	0.004	*9.0*	0.003	*2.4*	0.001
Total identified	***2.9***	***0.032***	***30.4***	***0.055***	***8.4***	***0.004***	***9.0***	***0.003***	***2.4***	***0.001***
Neutrals	*6.1*	*0.068*	*20.2*	*0.037*	*20.3*	*0.010*	*48.2*	*0.019*	*22.5*	*0.011*
Others	*3.4*	*0.037*	*5.5*	*0.010*	*13.4*	*0.007*	*8.7*	*0.003*	*10.9*	*0.005*
Indeterminate	*11.3*	*0.125*	*1.0*	*0.002*	*13.6*	*0.007*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Total characterised	***20.9***	***0.230***	***26.7***	***0.049***	***47.3***	***0.024***	***56.9***	***0.022***	***33.4***	***0.017***
ERR	**23.8**	**0.262**	**57.1**	**0.104**	**55.7**	**0.028**	**65.9**	**0.026**	**35.8**	**0.018**
RRR	***76.2***	***0.808***	***42.9***	***0.086***	***44.3***	***0.023***	***34.1***	***0.013***	***64.2***	***0.043***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
[1] This value was recalculated because the value 0.020 mg/kg as given in the report does not fit to 65.9 % TRR.
n.d. Not detected

Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.
Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in *italics.*

Table B.7.6.1.6-10: Distribution of radioactive residues of glyphosate and its metabolites in primary crops (carrots) after application of glyphosate to Ray silt loam soil

Scenario type F	**Primary crop**			
	Carrot		**Carrot leaves**	
DALT	**51**			
	% TRR	**mg/kg**	**% TRR**	**mg/kg**
	100.0	**0.31**	**100.0**	**0.494**
Extraction with water				
Aqueous extract	74.6	0.230	51.3	0.269
Glyphosate	*2.5*	0.008	*9.0*	*0.047* [1]
AMPA	*5.6*	0.017	*3.3*	0.017
Total identified	***8.1***	***0.025***	***12.4***	***0.065***
Neutrals	*45.5*	*0.140*	*8.1*	*0.043*
Others	*2.8*	*0.009*	*6.0*	*0.031*
Indeterminate	*18.2*	*0.056*	*24.8*	*0.130*
Total characterised	***66.5***	***0.205***	***38.9***	***0.204***
ERR	**74.6**	**0.230**	**51.3**	**0.269**
RRR	***25.4***	***0.080***	***48.7***	***0.225***

DALT Days after last treatment
TRR Total radioactive residue
ERR Extractable radioactive residue
RRR Residual radioactive residue (calculated by subtraction: TRR – ERR)
[1] Value out of the report (0.005 mg/kg) did not fit to the value given in % TRR, therefore this value was recalculated.
Remark: Values in % TRR were recalculated during dossier compilation, since the given values were based on a 100 % value of the extract. Input values in % of radioactivity in the extract were taken from table 7 of the report and used for the recalculation of % TRR. Additionally mg/kg values of "neutrals", "others" and "indeterminate" were calculated. Minor deviations to values in % TRR given in table 10 of the report may occur due to rounding.
Total identified was calculated as sum of glyphosate and AMPA. Total characterised was calculated as aqueous extract – identified.
Other fractions were only characterised by their chromatographic behaviour. They were designated as "neutrals", others" and "indeterminate".
Values calculated upon dossier compilation are presented in *italics.*

Soil samples were extracted with NH_4OH-solution. The extractabilities and the composition of radioactive components in the extracts are shown in Table B.7.6.1.6-11 and Table B.7.6.1.6-12.

Generally, the extractability of soil samples was high (in all cases ≥73.6 %), except for soil samples taken after harvest of rotational crops from Ray soil. From these samples, only 25.4 and 28.8 % were extracted.
Analyses showed that the predominant compound in Norfolk soil extracts was still the parent. It represented above 84.0 % of the of the radioactive residues in the extracts of soil taken within 11 weeks and more than 66.5 % in samples taken within 31 weeks. AMPA was detected between 4.4 and 22.5 %.
In Ray soil the predominant compound in the extracts was AMPA (68.0 and 74.5 %) after 4-7 weeks, while the parent compound glyphosate represented about 20 %. After 24.5 weeks nearly no glyphosate, was detected (up to 2.0 %) in the extracts of two soil samples, AMPA was found at 36.6 and 37.1 %. In the soil extract scenario E (cabbage) besides glyphosate and AMPA, the metabolite N-methylaminomethylphosphonic acid was detected at 6.6 %.

Table B.7.6.1.6-11: Extraction of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil (after harvest of primary crops)

	Norfolk sandy loam soil				Ray silt loam soil	
Scenario Type	A	B	C	D	E	F
Primary crop	Pea	String bean	Carrot	Cabbage	String bean	Carrot
DALT (weeks)	4	6.5	7	11	4	7
	% AR	% AR	% AR	% AR	% AR	% AR
Radioactivity left in soil [1]	81.86	71.66	67.8	52.59	30.8	29.4
Extraction with 0.5 M NH_4OH						
Aqueous extract [2]	n.a.	91.8	96.5	98.9	74.1	73.6
Glyphosate [3]	n.a.	92.5	92.9	84.5	18.9	19.2
AMPA [3]	n.a.	4.4	5.0	13.0	74.5	68.0
Identified [4]	n.a.	*96.9*	*97.9*	*97.5*	*93.4*	*87.2*

DALT Days after last treatment given in weeks
AR Applied radioactivity
n.a. not analysed
1 Given values refer to % applied radioactivity.
2 These values correspond to mean values calculated within the report for experiments with different analytical methods used (AG-50, AG-1, HVE, TLC, and FID/RAD).
3 Given values refer to % of the compound in the extract.
4 Identified was calculated as sum of glyphosate and AMPA.
Values calculated upon dossier compilation are presented in *italics.*

Table B.7.6.1.6-12: Extraction of the radioactive residues of glyphosate in soil after application of glyphosate to bare soil (after harvest of rotational crops)

	Norfolk sandy loam soil							Ray silt loam soil	
Scenario Type	A	B		C		D		E	
Rotational crop	Carrot/ cabbage	Corn	Corn	Cabbage	String bean	Carrot	Pea	Carrot	Cabbage
DALT (weeks)	18	16.5	22	27	13.5	31	17.5	24.5	24.5
	% AR	% AR	% AR	% AR	% AR	% AR	% AR	% AR	% AR
Radioactivity left in soil [1]	59.95/ 62.82[4]	40.36	53.04	28.65	61.80	27.07	57.43	6.43	10.00
Extraction with 0.5 M NH_4OH									
Aqueous extract	97.2	124.0	90.8	81.1	90.7	94.0	100.5	25.4	28.8
Glyphosate [2]	90.8	78.9	66.5	67.0	79.5	68.0	67.8	0.0	2.0
AMPA [2]	6.7	11.2	15.1	8.5	8.0	8.1	22.5	37.1	36.6
CP 70948 [5]	-	-	-	-	-	-	-	-	6.6
Identified [3]	*97.5*	*90.1*	*81.6*	*75.5*	*87.5*	*76.1*	*90.3*	*37.1*	*45.2*

DALT Days after last treatment given in weeks
AR Applied radioactivity
1 Given values refer to % of the applied radioactivity.
2 Given values refer to % of the compound in the extract.
3 Identified was calculated as sum of glyphosate and AMPA; for ray soil the metabolite CP 70948 was summed up, too.
4 For the scenario type A only results for one soil sample were presented, no information which pots were sampled.
5 CP 70948 = N-methylaminomethylphosphonic acid
Values calculated upon dossier compilation are presented in *italics.*

C. Storage stability

Storage intervals for frozen samples and extracts are not reported. No information on storage stability is reported.

D. Degradation pathway

Please refer to the overall pathway of glyphosate in rotational crops in Vol. 1, 2.7.7.

III. Conclusion

Plants were grown on two different soils, a sandy loam (Norfolk soil) and a silt loam (Ray soil). For Norfolk soil the primary crops were string beans, peas, carrots and cabbage and for Ray soil string beans and carrots. At the maximum growth of the primary crops, ^{14}C-glyphosate was applied to the bare soil at 4.48 kg a.s./ha. The plants were sampled 4 - 11 weeks after treatment. After harvesting of the primary crops, rotational crops (same as primary plants, and sweet corn) were planted within a 1 - 23 day interval (PBI 29 - 79 / 101 days) and harvested 45 to 122 days after treatment

(DALT). In addition, soil samples were taken after harvest of the primary crops as well as after harvest of the rotational crops from each plot. The recovered radioactivity and its composition in soil, in primary crops and in rotational crops was determined.

The uptake of glyphosate by the plants reflects the amount of glyphosate and/or metabolites present in the soils. Glyphosate appears to be relatively stable in the sandy loam soil (Norfolk soil) with $t_{1/2}$ of 17 - 19 weeks in contrast to $t_{1/2}$ of 3 - 4 weeks in the silt loam soil (Ray soil).

The residue levels in the investigated commodities of primary crops from Norfolk soil were lower (0.08 - 0.22 mg/kg) than those from primary crops grown on Ray soil (0.19 - 1.07 mg/kg). The amounts of radioactive residues in rotational crops were comparable for plants from both soils and ranged between (0.04 - 0.094 mg/kg), except for pea leaves and pots, where radioactive residues up to 0.28 mg/kg were detected.
Generally, 55.7 – 92.2 % of the TRR were extractable with water from the plant material. Somewhat lower amounts (23.8 – 51.6 % TRR) were extracted from leaves of string beans and carrots of primary crops from Ray soil and from carrot leaves and corn (kernel and cob) of the rotational crops from both soils. The remaining residues after solvent extraction (RRR) amounted to 0.004 - 0.808 mg/kg (7.8 – 76.2 % TRR) and were not investigated.

Glyphosate was the major component detected in the plant extracts of primary crops (0.026 - 0.137 mg/kg) and rotational crops (0.003 - 0.128 mg/kg) from Norfolk soil. AMPA was less abundant in these extracts (0.002 - 0.044 mg/kg). Components that were characterised according to their elution behaviour (designated as "neutrals", "others" or "indeterminates") amounted to 0.007 - 0.037 mg/kg.
In plant extracts of primary crops from Ray soil, AMPA was found at higher amounts than glyphosate (0.017 - 0.041 mg/kg), except for carrot leaves. Glyphosate amounted to 0.008 - 0.046 mg/kg. Glyphosate was not detected in the extracts of rotational crops from Ray soil, while AMPA was found at low amounts (up to 0.004 mg/kg). In the extracts of primary and rotational crops of Ray soil, high amounts of neutrals and / or indeterminates were found, representing up to 0.140 mg/kg.

The higher uptake from silt loam (Ray soil) was seen as a result of rapid degradation of ^{14}C-glyphosate to $^{14}CO_2$ which was fixed by the plants, resulting in the high amount of neutral materials and non-extractable components which were proposed to represent incorporation of ^{14}C into natural products.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study assessing the metabolic behavior of glyphosate in two different soils, in primary crops (beans, peas, carrots and cabbage) and rotational crops (same as primary crops plus corn) has been previously evaluated at EU level. It was not performed under GLP (as in 1976 GLP was not yet established at the test facility) but is considered to be scientifically valid.
The study is deemed to largely comply with current requirements as laid down in Reg. (EU) No 283/2013 and OECD Guideline for the Metabolism in Rotational Crops, 502 with some deficits.
The study is not a typical rotational crop study as it included analysis of primary crops and only one rotation was conducted; nevertheless it contains supportive data on the uptake of glyphosate/metabolites by primary crops and rotational crops from two different soils.
Information about the application method and formulation of test item is missing. Developmental stages of the crops at application and harvesting are not reported, but could be roughly estimated based on planting and sampling dates.
No information on storage duration of plant samples and aqueous plant extracts is given in the study report.
Relevant amounts of non-extractable residues were not characterised / not investigated (residual radioactive residues were 11.3 - 76.2 % TRR (0.013 - 0.808 mg/kg)).
Relevant amounts of residues in the extracts (11.3 - 45.5 % TRR (0.012 - 0.140 mg/kg)) were not investigated.

Despite the shortcomings, the present study is considered scientifically valid and supportive of the whole package of studies on the metabolism of glyphosate in primary and rotational crops.

Assessment and conclusion by RMS:
The confined rotational crop study has some shortcomings, as already summed up by the applicant. In particular, the observation that the residual radioactive residues were often not further investigated, while the levels were higher than 0.01 mg/kg or 0.05 mg/kg, is considered an important deviation. Similarly, relevant levels of extractable residues should have been further investigated. In addition, storage stability has not been addressed. On the other

hand, the study provides useful information on the metabolism of glyphosate in rotational crops. Altogether, the study is considered supportive only.

B.7.6.2. Magnitude of residues in rotational crops

No rotational field trials have been submitted, since they are considered as non-relevant by the applicant. Further considerations on this topic can be found in Vol.1, 2.7.7.

B.7.7. OTHER STUDIES

B.7.7.1. Effect on the residue level in pollen and bee products

Based on the decision-making scheme presented in the Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey (SANTE/11956/2016 rev. 9), the effect of the defended uses on the residue level in honey needs to be addressed since applications on non-target plants (in-field weeds and adjacent plants) are intended which might take place during the flowering period from April to September. Next to a tunnel residue trial (B.7.7.1.1), the applicant provided monitoring data (B.7.7.1.2), as well as literature data belonging to Category A (B.7.7.1.3), which will be summarised in the following sections.

B.7.7.1.1. Tunnel residue trial

1. Information on the study

Data point:	CA 6.10.1/001
Report author	[redacted]
Report year	2020
Report title	Determination of residues of glyphosate in honey after one application in *Phacelia tanacetifolia* at 4 sites in Germany 2019
Report No	S19-04329
Document No	-
Guidelines followed in study	OECD Guideline for the Testing of Chemicals on Crop Field Trial (TG 509 published in September 2009) EC (2018) Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey (SANTE/11956/2016 rev. 9) Commission Regulation (EU) 283/2013 and 284/2013 implementing Regulation (EC) 1107/2009 (Oct. 2009)
Deviations from current test guideline	According to SANTE/11956/2016 rev. 9, for one trial no replicate sample could be taken due to the low amount of food stores available in the hives. Additional deviations are listed and discussed below in "assessment and conclusion by RMS".
Previous evaluation	New study for AIR5
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 1 **Conclusion AGG:** The study is considered to be acceptable.

2. Full summary of the study according to OECD format

Executive Summary

The objective of the study was to collect honey samples from *Phacelia tanacetifolia* (known by the common names lacy phacelia, blue tansy or purple tansy) after one application of MON52276 under semi-field conditions in order to measure the residues of glyphosate. MON52276 is a soluble concentrate formulation with a nominal content of 360 g/L of glyphosate as its isopropylammonium salt.
The study included 4 field trials in Germany during the 2019 season. The Phacelia fields were treated once, at a target rate of 2.16 kg glyphosate per hectare. Samples of honey were taken from the bee combs for analysis 6-8 days after application. Residues of glyphosate and AMPA in honey ranged from <0.025 mg/kg to 6.9 mg/kg and from <0.025 mg/kg to 0.028 mg/kg, respectively.

I. Materials and Methods

A. Materials

1. Test material	
Description:	MON52276 (Roundup BIO, Glyphosate 360 g/L)
Active ingredient(s):	Glyphosate (formulated as IPA salt)
CAS number:	38641-94-0
Content of a.s. nominal:	41.5 % w/v 360 g/L
Content of a.s. analysed:	31.1 %
Formulation type:	SL

Test commodities				
Trial:	Crop:	Botanical name:	Variety:	Crop parts:
S19-03987-01	Phacelia	*Phacelia tanacetifolia*	Balo	Honey (ripe; after comb closure; water content < 20%)
S19-03987-02	Phacelia	*Phacelia tanacetifolia*	Balo	Honey (ripe; after comb closure; water content < 20%)
S19-03987-03	Phacelia	*Phacelia tanacetifolia*	Stala	Honey (ripe; after comb closure; water content < 20%)
S19-03987-04	Phacelia	*Phacelia tanacetifolia*	Stala	Honey (ripe; after comb closure; water content < 20%)

B. Methods

1. Field phase

Four semi-field trials were conducted to collect honey samples from Phacelia during 2019 in Germany (S19-03987-01, S19-03987-02, S19-03987-03 and S19-03987-04). One application of MON52276 (360 g/L glyphosate) was performed to Phacelia at BBCH 63-65 at a target rate of 6 L product/ha, corresponding to a target rate of 2.16 kg a.s./ha. The volume of water used to prepare the spray solution was in the range of 392-413 L/ha. The main application parameters are outlined in the table below.

Trial no.	Application code	Growth stage (BBCH)	Application rate kg a.s./ha	Water volume L/ha
S19-04329-01	T	64-65	2.202	408
S19-04329-02	T	64-65	2.232	413
S19-04329-03	T	63-65	2.182	404
S19-04329-04	T	63-65	2.118	392

On each trial site, one tunnel (5 m x 40 m) confining the bees was established on the control and the treated plot. One bee hive was set up per tunnel for the control and treated plot each. Application was performed with boom sprayer equipped with flat fan nozzles, which were duly calibrated before use. The actual applied amount was calculated by measuring the remaining spray solution after application.
Honeybee colonies *(Apis mellifera* L.) were used as sampling device and for honey production (i.e. collection of nectar and processing to honey). Approx. 2 weeks before set-up all hives were equipped with a queen exclusion chamber to decrease brood production and decrease the amount of nectar needed by bees for feeding the brood. Thus, the chance of getting honey for sampling is increased. For all hives, a colony assessment was performed prior set-up (0 to 5 days before setup of hives) and 3 to 5 days after sampling of honey. For the colony assessment, the following parameters were recorded: strength of the colony, presence of a healthy queen (i.e. presence of eggs or presence of queen cells), visual assessment (number of cells containing pollen, nectar, and brood), as well as the assessment of the flight activity.
Shortly before set-up of hives in the tunnels, 3 empty combs were marked and placed in the brood body. The details are given in the table below.

Trial no.	Set-up of colonies[1]	No of empty combs added[2]
S19-04329-01	28.06.2019 / 1 DBA / BBCH 64-65	3 in C / 3 in T
S19-04329-02	14.07.2019 / 1 DBA / BBCH 64-65	3 in C / 3 in T
S19-04329-03	17.072019 / 1 DBA / BBCH 63-65	3 in C / 3 in T
S19-04329-04	02.09.2019 / 1 DBA / BBCH 63-65	3 in C / 3 in T

1 DBA = days before application. BBCH was recorded on the day after set-up.
2 C = control tunnel; T = treated tunnel

Since glyphosate is a herbicide, the treated Phacelia quickly decayed and became unattractive to the bees, which is also indicated by a lowered flight activity. Therefore, 3-7 days after application the colonies were all moved to a single monitoring site where they were allowed to freely fly in the surroundings. The vegetation and agricultural fields in a 3 km radius around the monitoring site were recorded and mainly consisted of forest and grassland with small patches of wild flowers. Some arable crops such as peas and corn were also present but not flowering anymore. The area thus provided sufficient food sources for the bees to forage on, but without intensive agriculture and no flowering main crops in the near surroundings, which could be attractive to honeybees.
Honey was collected 2-5 days later, once mature (i.e. at comb closure and sugar content ≥ 80 %). Honey was collected from initially empty combs which were introduced in the hive the evening before the application performed the following day.

2. Sampling

Honey was sampled as separate sample from each tunnel of each trial. Honey was collected from initially empty combs which were introduced in the hive the evening before the application. Honey was collected once mature after comb closure for subsequent residue analysis. Untreated control specimens were taken before treated specimens. Each field specimen (original field sample, as well as field retain sample) was taken from several spots across the combs. Honey was collected by gently pushing a spoon into the walls of storage cells, allowing the honey to flow onto the spoon, or with a syringe or with a plastic pipette, extracting the honey from single cells. Sampling was done by collecting honey from several combs or spots within a comb. The details are given in the table below.
The combs introduced in the hives on the evening before application were weighted on three occasions: once before being introduced into the hives, once before relocation to the monitoring site, and once before sampling. By doing to, the percentage of honey that was produced during the exposure phase (confinement in tunnels) could be determined. In trials S19-04329-01, -02, -03, and -04, the percentage of honey sampled for analysis that was produced during the exposure phase was 70%, 83%, 59% and 24%, respectively.

Trial	Commodity	DALA[1]	Subsample[2]	Quantity (g)	Sugar content (%)	Date of sampling
S19-04329-01	Honey (ripe; after comb closure)	6	A	58.36	80.4	05.07.2019
			R	20.27		
S19-04329-02	Honey (ripe; after comb closure)	8	A	99.12	81.0	23.07.2019
			R	67.94		
S19-04329-03	Honey (ripe; after comb closure)	8	A	65.13	81.1	26.07.2019
			R	24.22		
S19-04329-04	Honey (ripe; after comb closure)	8	A	5.98[3]	82.0	11.09.2019
			R	0.0		

1 Days after last application.
2 A = analysed sample; R = retain sample
3 Not enough food stores were present in the hive in order to collect at least 10 g in the A-samples nor to collect R-samples.

3. Analytical phase

Residue analysis was conducted according to Monsanto method ME-2220-01. The residues of glyphosate and AMPA were extracted from the samples by high-speed blending using 0.1 % formic acid in water. Following centrifugation and filtration the analytes were determined by LC-MS/MS using internal standards. The limit of quantitation (LOQ) for glyphosate and AMPA was 0.025 mg/kg with a limit of detection (LOD) of 0.0075 mg/kg for each analyte expressed as itself.

Treated and untreated specimens were maintained deep frozen and adequately separated during storage and shipment. The maximum sample storage interval from sampling to extraction was 83 days, and the maximum interval from extraction to analysis was 2 days. Samples were stored frozen at ≤ -18 °C at the analytical facility prior to analysis. The method validation was done with a set of recoveries at the LOQ (5 x 0.025 mg/kg for each analyte) and 10x LOQ (5 x 0.25 mg/kg for each analyte) level. Additional concurrent recoveries were performed at 10x LOQ (1 x 0.25 mg/kg for each analyte) and 320x LOQ (3 x 8.0 mg/kg for each analyte). The results are summarised in the table below.

Table B.7.7.1.1-1: Recovery results

Matrix	Analyte	Fortification level (mg/kg)	Recovery[1]				
			Range (%)	Mean (%)	Standard deviation (%)	Relative standard deviation (%)	Number analyses (n)
Honey	Glyphosate	Quantification transition 168 > 63 m/z					
		0.025	108, 109, 112, 101, 101	106	5.0	4.7	5
		0.5	91, 96, 89, 98, 91, 96	94	3.6	3.9	6
		8.0	91, 91, 90	91	0.58	0.64	3
		Overall	**89-112**	**97**	**7.7**	**7.9**	**14**
	AMPA	Quantification transition 110 > 63 m/z					
		0.05	100, 96, 101, 98, 92	97	3.6	3.7	5
		0.5	98, 98, 96, 103, 99, 102	99	2.7	2.7	6
		8.0	83, 82, 100	88	10	11	3
		Overall	**82-103**	**96**	**6.5**	**6.7**	**14**

1 Residues of glyphosate and AMPA in blank matrix were below the limit of detection (< 0.0075 mg/kg).

II. Results and Discussion

The test item was applied and specimens were generated and analysed according to the study objectives. The results of the analyses, therefore, allow to evaluate the residue behaviour of glyphosate and its metabolite AMPA after usage of MON52276 when applied to Phacelia plant. Residues of glyphosate and AMPA in honey ranged from <0.025 mg/kg to 6.9 mg/kg and from <0.025 mg/kg to 0.028 mg/kg, respectively. No residues of glyphosate and AMPA above the LOQ (0.025 mg/kg) were found in any untreated specimens of honey samples. Detailed residue levels are shown in the table below.

Table B.7.7.1.1-2: Residue levels of glyphosate and AMPA in honey after one application of MON52276 (360 g/L glyphosate)

Trial No. / Location / EU zone / Year	Crop/ Variety	Growth stage[1] (BBCH)	Commodity	Residue found[2,3] (mg/kg)		DALA[4] (days)
				Glypho-sate	AMPA	
S19-04329-01 / [redacted], Baden-Württemberg, Germany/ NEU / 2019	*Phacelia tanacetifolia* / Balo	64-65	Honey	6.9	0.028	6
S19-04329-02 / [redacted], Baden-Württemberg, Germany/ NEU / 2019	*Phacelia tanacetifolia* / Balo	64-65	Honey	0.87	<0.025 (n.d.)	8

Table B.7.7.1.1-2: Residue levels of glyphosate and AMPA in honey after one application of MON52276 (360 g/L glyphosate)

Trial No. / Location / EU zone / Year	Crop/ Variety	Growth stage[1] (BBCH)	Commodity	Residue found[2,3] (mg/kg)		DALA[4] (days)
				Glypho-sate	AMPA	
S19-04329-03 / [redacted], Baden-Württemberg, Germany/ NEU / 2019	*Phacelia tanacetifolia* / Stala	63-65	Honey	3.2	<0.025 (n.d.)	8
S19-04329-04 / [redacted] Baden-Württemberg, Germany/ NEU / 2019	*Phacelia tanacetifolia* / Stala	63-65	Honey	<0.025 (n.d.)	<0.025 (n.d.)	8

1 Growth stage at harvest
2 LOQ (limit of quantification): 0.025 mg/kg
3 n.d. (not detected): <0.0075 mg/kg
4 Days after last application

III. Conclusion

Residues of glyphosate and AMPA in honey, sampled 6-8 days after application of glyphosate at the rate of 2.12-2.23 kg a.s./ha. ranged from <0.025 mg/kg to 6.9 mg/kg and from <0.025 mg/kg to 0.028 mg/kg, respectively. No residues of glyphosate and AMPA above the LOQ (0.025 mg/kg) were found in any untreated specimens of honey samples.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The study was previously not evaluated at EU level. It was performed under GLP and is considered to be reliable and acceptable. The study is deemed to comply with current requirements as laid down in Reg. (EU) No 283/2013, OECD Guideline for the Testing of Chemicals, 509 and SANTE/11956/2016 rev. 9. It adequately supports the use for glyphosate.

Assessment and conclusion by RMS:
The objective of the study was to determine the residue levels of glyphosate and AMPA in honey collected by honey bees from *Phacelia tanacetifolia* (known by the common names lacy phacelia, blue tansy or purple tansy) at four trial sites in Germany during the growing season 2019. A single foliar application was performed at a target rate of 2.16 kg/ha. The application rate covers the single and yearly application rates of the defended uses, except for the application rate for the ground-directed post-emergence use in orchards and vines (2 x 1.44 kg/ha (max. 2.88 kg/ha per year), interval 28 days). The RMS assumes, however, that honey will be harvested more than once per month and therefore, the single application rate of 1.44 kg/ha is considered more relevant and this application rate is covered by the target rate of the current study.

The test procedure was in accordance with the technical guidelines (SANTE/11956/2016 rev. 9) for tunnel residue trials with a few exceptions:

- Applications should be timed before noon to ensure a maximum amount of hours of honey collection during the first day. The application in trial S19-04329-02, however, was performed between 13:22 and 13:38. It is difficult to estimate the effect of this deviation on the residue level at harvest, especially when considering that the levels of glyphosate were the lowest in this trial (with the exception of trial S19-04329-04 in which a fairly low amount of honey was sampled). Nevertheless, the trial is considered for evaluation.
- At least 100 g of honey should be sampled per trial site, or as close as possible to this, whereas the analysed samples from trials S19-04329-01 to -03 contained 58.36-99.12 g of honey, and the analysed sample from trial S19-04329-04 only contained 5.98 g of honey. With regard to trial S19-04329-04, the study author states that "*this deviation may question the validity of the trial but it is also important to note that the same could happen in practice.*" Whereas the amount of honey sampled in trials S19-04329-01 to -03 are considered sufficient by the RMS, the sampled amount in trial S19-04329-04 is considered too low, also taking into account that residue levels were below the LOQ exactly in trial S19-04329-04. It is not possible to state whether this is a co-incidence or whether the low specimen weight contributes to this result. In conclusion, the results from trial S19-04329-04 are not taken into account for evaluation.

The health assessment of the colonies was conducted in accordance with the technical guidelines (SANTE/11956/2016 rev. 9) and results between the different beehives were comparable. It is noted, however, that the results of the colony assessment of the control hives were not available.

The performance of the analytical method was sufficiently demonstrated and the method is considered acceptable. It is noted, however, that additional information regarding the extraction efficiency is needed for confirmation. Specimens were stored in accordance with the demonstrated period of storage stability (6 months in honey for glyphosate and AMPA, respectively). No residues above the LOQ were detected in control specimens. The following residues are selected for evaluation:

Honey
Glyphosate: 0.87, 3.2, 6.9 mg/kg
AMPA: 2x <0.025, 0.028 mg/kg

B.7.7.1.2. Monitoring data

Assessment and conclusion by RMS:
The applicant provided the summary of monitoring data as shown below, but did not provide a box with the "assessment and conclusion by applicant". Considering the information dealt with, it is considered acceptable that the applicant did not provide its assessment.
The summary as provided below is considered acceptable by the RMS and the evaluation of the data will be performed in Volume 1, Section 2.7.10.

According to the Technical Guidelines SANTE/11956/2016 rev. 9 of 14 September 2018 it is possible to set temporary MRLs in honey on the basis of monitoring data.

The detailed results of the EU pesticide residue monitoring were downloaded from the Zenodo website. The search gives a list of references with pesticide residue monitoring data for individual EU and EEA Member States. For each

State the monitoring data for 2016 and 2017 were provided. At the time of when this evaluation was conducted, the detailed monitoring data for 2018 were not published yet.

For each year, the monitoring data from all States were grouped to obtain a single data set of EU monitoring data (one for 2016 and one for 2017). The residue data for parent glyphosate and AMPA in honey were extracted from these EU monitoring databases for 2016 and 2017 and grouped with all the glyphosate and AMPA residues in honey from the 2016 and 2017 monitoring.

The dataset includes a total of 618 analytical results, which were provided by Germany (n = 512), Austria (n = 104) and the Netherlands (n = 2). A total of 406 samples were analysed for parent glyphosate. Out of these, 212 samples were also analysed for AMPA. There are no duplicate results in the table (sample analysed more than once). The LOQs were variable and ranged between 0.01 and 0.05 mg/kg for parent glyphosate (except for one sample for which the reported LOQ was 0.14 mg/kg) and between 0.01 and 0.03 mg/kg for AMPA. Measurable residues of glyphosate (i.e. residues ≥ LOQ) were found in 42 samples and these residues ranged between 0.01 mg/kg and 0.61 mg/kg. The remaining 364 samples showed residues of glyphosate < LOQ. The residues of AMPA were always < LOQ (n = 212). It is noted that results from AMPA are not shown in the table below since these are not relevant for MRL setting.

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
0010ED3D84B350ECF74D	Germany	2017	0.03	-	0.03
0139B97862BA5A133890	Germany	2016	0.02	-	0.02
01B1A9E9326C9FCDE411	Germany	2016	0.03	-	0.03
01B39E812B5FB3261DC9	Germany	2016	0.05	-	0.05
0503A2D246277166756C	Germany	2016	0.02	-	0.02
057C97A7C7F6F2952058	Germany	2017	0.03	-	0.03
05C6160133CF5F6DAB87	Germany	2017	0.03	-	0.03
0633B4DFC18BCBD64290	Austria	2017	0.01	-	0.01
06C32351DB221A730F26	Germany	2017	0.03	-	0.03
0717DDA152B12B92B311	Germany	2016	0.02	-	0.02
07C84539F8F83FAE7146	Austria	2017	0.01	-	0.01
0887729C9A2AB715FB2F	Germany	2017	0.03	-	0.03
097FDE14EBBAAEEC62D6	Germany	2017	0.03	-	0.03
0D4FE46805C2637FF9B1	Germany	2016	0.02	-	0.02
0E12C4BF67F647DB3A62	Germany	2016	0.02	0.039	0.039
0F6059D1E9FFFDB5A6FE	Germany	2016	0.02	-	0.02
0FC307488DD4E5DB847A	Germany	2016	0.03	-	0.03
101EF1A53C9A42809065	Austria	2017	0.01	-	0.01
10D6CE6863BD0D413F81	Germany	2016	0.02	-	0.02
11759E9F1EA6144BA097	Germany	2017	0.02	-	0.02
11943BFBE808F91EAA5C	Germany	2016	0.01	0.04	0.04
11F8D8A76B8B5B12F541	Austria	2017	0.01	-	0.01
12193F6A7928C444C735	Germany	2016	0.02	-	0.02
12CFBDC62782BF4FD3C2	Austria	2017	0.01	-	0.01
12EA985B7A3319C65D22	Germany	2016	0.045	-	0.045
12FC6919E5CD60595B0F	Germany	2017	0.03	-	0.03
15407597C3646034426B	Austria	2017	0.01	-	0.01
16D1A64965D1356D8A52	Germany	2017	0.02	-	0.02
188483C2611E27D6DD8A	Germany	2017	0.03	-	0.03
18CBF05125D206688A56	Germany	2016	0.02	-	0.02
18CE6F2E873044814C07	Germany	2016	0.02	0.091	0.091
1A4AA6A322C37D80AC06	Germany	2016	0.02	-	0.02
1AB5B63CB7C4DFD1DFD1	Germany	2016	0.05	-	0.05
1B7CB38A2EFE60B3D76F	Germany	2016	0.02	-	0.02
1CC90E11ABCB676FE3DA	Germany	2017	0.025	-	0.025
1DEFE2D9459BFE12E150	Germany	2016	0.02	-	0.02

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
1DFADE970058F772BF37	Germany	2016	0.01	-	0.01
1E201548E13A920CB49E	Germany	2017	0.03	-	0.03
1E85298720AE8F17C375	Germany	2016	0.02	-	0.02
1F705A6DB45B56859ABD	Germany	2016	0.02	-	0.02
1F93306032396BED9DDF	Germany	2016	0.03	-	0.03
1FB0734D1518C8EB8BCE	Germany	2017	0.03	-	0.03
1FDC0C1FDF56820DB16D	Germany	2016	0.02	-	0.02
1FFBC8FF62766BA50AB1	Germany	2017	0.03	-	0.03
20F0B5CD960405C4B79A	Germany	2017	0.03	0.051	0.051
214222EB6A951D1006BA	Germany	2017	0.03	-	0.03
23ABCDF6E0AEF4719392	Germany	2017	0.02	-	0.02
2581CBFE79C09DE183FF	Germany	2017	0.03	-	0.03
25CD1BFE952C39C45286	Germany	2016	0.02	-	0.02
25F6862ED207250A2773	Germany	2016	0.02	-	0.02
260CD96BEB85DEC45563	Germany	2016	0.02	-	0.02
2646B98CAC2FF9C5CA3E	Germany	2016	0.02	-	0.02
2740B1FE8E07DE01DF75	Austria	2017	0.01	-	0.01
27B44AA63B641BD37AEF	Austria	2017	0.01	-	0.01
28A4789EBF15D16B60DE	Germany	2016	0.02	-	0.02
2A23AE492E0622B69A8C	Germany	2016	0.01	0.013	0.013
2A2BBBC787A4AE7ED848	Germany	2016	0.03	-	0.03
2A6BFD3D4295B356F79B	Germany	2017	0.03	-	0.03
2AA7FA4EC495B1A6AB2F	Germany	2016	0.02	-	0.02
2AB2C24ED88E08B00775	Austria	2017	0.01	-	0.01
2AB5141C16E6C86B9B36	Germany	2017	0.02	-	0.02
2C33CF35A2EDD8B6B779	Germany	2016	0.05	-	0.05
2CDD41800D588146C083	Germany	2017	0.03	-	0.03
2EEADF8C7E215C858A59	Germany	2016	0.02	-	0.02
30C3C2489A8E5C4CA0D9	Germany	2017	0.03	-	0.03
30C71A645F5A30C81780	Germany	2016	0.02	-	0.02
3191CBB51C0254AB128B	Germany	2017	0.03	-	0.03
321D2553924907BD9DF2	Germany	2016	0.02	-	0.02
32214A96B487A91DA34E	Germany	2017	0.03	-	0.03
3289C64E15640DF73954	Germany	2016	0.02	-	0.02
33C8D2BBF4495ED96154	Germany	2017	0.03	-	0.03
342A760D3A4CE0DA1AFA	Austria	2017	0.01	-	0.01
34414278279F114B54FD	Germany	2016	0.02	-	0.02
3473529106345353AF55	Germany	2016	0.02	-	0.02
388249F2BDA5FCB552F3	Germany	2016	0.02	-	0.02
38C25CC208381E225204	Germany	2016	0.02	-	0.02
38F68E544DC89304F3E8	Germany	2016	0.02	-	0.02
393193522867AC20A19B	Germany	2016	0.05	0.61	0.61
39E7E78714FFB3976937	Germany	2017	0.03	-	0.03
3A47D2A16AC24D44FF61	Austria	2017	0.01	-	0.01
3B720900FBD7FF145596	Germany	2017	0.03	0.042	0.042
3C2875B84DDE93100B4E	Germany	2017	0.03	-	0.03
3C313C0DE51C180890C7	Germany	2016	0.03	-	0.03
41760C123C2EA0E4510F	Germany	2016	0.02	0.029	0.029
423DACAA846163EA81CF	Germany	2016	0.01	-	0.01
4242EA0EF05C605DDBE2	Germany	2016	0.02	-	0.02
4282828ABDABC787CFAF	Germany	2016	0.02	-	0.02
42CC9DF136D74AC5B792	Austria	2017	0.01	-	0.01

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
44020748C94D04CADD7E	Germany	2017	0.03	-	0.03
44AC8C8359C94865988D	Austria	2017	0.01	-	0.01
44CCD06B79EA3ADB4EDF	Germany	2016	0.02	0.15	0.15
4529C978DCB4E26D9EED	Germany	2016	0.02	-	0.02
45CE0A626FC46E639429	Germany	2016	0.02	-	0.02
46A746BE890A09A2AF21	Germany	2016	0.02	-	0.02
46CBA2F1C0BCE89B2143	Germany	2016	0.03	-	0.03
47AAC86ABDDDB87F093C	Germany	2016	0.01	-	0.01
486975036EFAEEAF179C	Germany	2017	0.03	-	0.03
4878F99BD7F07629350F	Germany	2017	0.03	-	0.03
487AC1D0AFC731CA986C	Austria	2017	0.01	-	0.01
48AAEFE4D7CCF96FF500	Germany	2016	0.03	-	0.03
48BFBF166B927A33C17D	Germany	2016	0.05	-	0.05
495BA8FE03CD26C08E47	Germany	2016	0.02	-	0.02
4A73A4A9D00407844CD0	Austria	2017	0.01	0.039	0.039
4B3FD64CF7C523DBA5E0	Germany	2016	0.03	-	0.03
4B7DEB85CCA5096E0118	Austria	2017	0.01	-	0.01
4D020978622AFA18B985	Germany	2016	0.02	-	0.02
4D3C7CD8B668E76932AF	Germany	2017	0.03	-	0.03
4DB6B6CB7BC747C1B119	Germany	2017	0.03	-	0.03
4DE2B90E8D9AD8E06756	Germany	2016	0.02	-	0.02
4DF9AAE785BA401CD4EF	Germany	2016	0.01	-	0.01
4E6E38E37B09EF0B80E5	Germany	2017	0.02	-	0.02
4F83B45B6EC13E3EEAC0	Germany	2016	0.03	-	0.03
4FE15900E93329E73C1D	Germany	2016	0.02	-	0.02
5054E648EDB7D3740644	Germany	2016	0.02	-	0.02
513EA638CA004626143B	Germany	2017	0.03	-	0.03
51795F0563C9DE14B587	Netherlands	2016	0.01	0.013	0.013
52F290B09E3513B05144	Germany	2016	0.02	-	0.02
54A0E676967DF6ED4B55	Germany	2016	0.02	-	0.02
59BD36F5CD277DB7202C	Germany	2017	0.03	-	0.03
5A2AABD2C17D892D535F	Germany	2016	0.03	-	0.03
5BC029017EBFD93C62E3	Germany	2017	0.03	-	0.03
5C78CA52BE27D0A871EC	Germany	2017	0.025	-	0.025
5CBCF8F8F66E1EC4B7D3	Germany	2016	0.03	-	0.03
5CC7B8B02966E78285CD	Germany	2016	0.02	-	0.02
5D9E6817321530CAC0A7	Germany	2017	0.03	-	0.03
5F0D71DFD634A1AEA34E	Germany	2016	0.03	-	0.03
5F19CAB8B080C9C0C237	Germany	2016	0.02	-	0.02
5F6A274B5D8ED470AA1F	Germany	2017	0.02	0.03	0.03
605ABB7E3E6C418E3D0F	Germany	2016	0.02	-	0.02
6066D5410103FB4386FB	Germany	2017	0.02	-	0.02
60B66A6CD7E000FCB80F	Germany	2017	0.03	-	0.03
61B0EDB0F314231E779B	Germany	2016	0.02	-	0.02
6219B8DCA44B5E25A35A	Germany	2016	0.045	-	0.045
62217BE09E16AA467886	Germany	2017	0.03	-	0.03
62387794DEEFB01F8AC5	Germany	2016	0.045	-	0.045
6243009C246101281CE8	Germany	2016	0.02	-	0.02
62DE55243930B2D55C12	Germany	2017	0.025	-	0.025
637CBB069F3D4F20EBB2	Germany	2016	0.02	-	0.02
63C68F8C0BF2B39EB09C	Germany	2016	0.02	-	0.02
643A89CE323EF5D96C56	Germany	2016	0.02	-	0.02

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
64426E1786100DFB4888	Germany	2016	0.03	-	0.03
64D28634DAD724369545	Germany	2016	0.02	-	0.02
64D55C5728DC6506A10E	Germany	2016	0.02	-	0.02
64F1AC5A9205672004D6	Germany	2016	0.02	-	0.02
658866EAFF21B81A9BA4	Germany	2017	0.02	-	0.02
65A3D15F13EDB36A6A1F	Germany	2016	0.02	-	0.02
65A56F427442A3254001	Germany	2017	0.03	-	0.03
65EDEA3058473329EF4E	Germany	2016	0.02	-	0.02
66002C08C86459DC70F5	Austria	2017	0.01	-	0.01
667257925AFDDFFBD9A0	Germany	2016	0.03	-	0.03
6781060F25763395DCA9	Germany	2017	0.03	0.059	0.059
68E2D1E508A54156880F	Austria	2017	0.01	-	0.01
690402318DD79EB45D6B	Germany	2017	0.03	-	0.03
6AA1126BFDA72AD35C6A	Germany	2016	0.02	-	0.02
6AEA0B3013DBBB9AD4B0	Germany	2016	0.02	0.049	0.049
6B7455B698C56B950332	Germany	2016	0.05	-	0.05
6DD754DB8A72216E9628	Germany	2016	0.02	-	0.02
6E0D7E8F55011C6F6884	Germany	2016	0.01	-	0.01
6E281500C78CADCF81EB	Germany	2017	0.03	-	0.03
6E4DCBF4A853B782AFB9	Germany	2017	0.02	0.028	0.028
6EEB7B270A390B63A884	Germany	2016	0.02	-	0.02
6FB36DEDDFE9FF1B07D5	Germany	2017	0.03	-	0.03
6FCB7C0A20C806F807D0	Germany	2016	0.02	-	0.02
70E092E686211202B76A	Germany	2016	0.02	-	0.02
71F9C40A55C1B8C27DCE	Germany	2017	0.025	0.037	0.037
72028AA18DB700B7479C	Germany	2016	0.02	-	0.02
7385E46D5C48B705A884	Austria	2017	0.01	-	0.01
73DA08BC994B498166F8	Germany	2016	0.02	-	0.02
7408243461552D27BD8A	Germany	2016	0.05	-	0.05
746F904E020CE7F977C7	Germany	2017	0.03	-	0.03
748C07B17EC1EC926F60	Germany	2017	0.03	-	0.03
74EE34AF14AF18731007	Germany	2016	0.01	0.05	0.05
75C835AC94B1A874EC77	Germany	2017	0.03	-	0.03
77883D04E27FAFFFBE3	Germany	2016	0.03	-	0.03
77BC5B40E088F5B2C14C	Germany	2017	0.03	-	0.03
791D7294497EAF736B88	Germany	2017	0.03	-	0.03
7A97ED6B2CFD0CB09576	Germany	2016	0.02	-	0.02
7AB317EC4C8E147B6F34	Germany	2016	0.02	-	0.02
7AF617F2746972E24F98	Germany	2016	0.02	-	0.02
7B073031A2C7B9AE004F	Germany	2016	0.045	0.05	0.05
7B1CFD9517F01C5462D9	Germany	2016	0.02	-	0.02
7C2BA778A06B200645DB	Austria	2017	0.01	-	0.01
7C686718B15A83364FCE	Germany	2016	0.02	-	0.02
7CC0A6DE017923926360	Germany	2017	0.025	-	0.025
7CF1B73EAF8EAB46F0D6	Germany	2017	0.03	-	0.03
7E5B4C26A9891A7A1963	Austria	2017	0.01	-	0.01
7EBF9A9C78DE4200101C	Germany	2016	0.05	-	0.05
7F0ACF5986386F80DB24	Germany	2017	0.03	-	0.03
7F8D2C4FB16595E24DB2	Germany	2017	0.03	-	0.03
7F990F82279647A4809A	Germany	2016	0.03	0.126	0.126
7FE34A18FA42FF2926FB	Germany	2017	0.03	0.046	0.046
7FFD7589381A7C36920B	Austria	2017	0.01	-	0.01

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
82919908D160246B643F	Germany	2016	0.02	-	0.02
82CA0D9CED533D7C885A	Germany	2016	0.03	-	0.03
837A8F46739971107D3B	Germany	2016	0.03	-	0.03
839E1517A8A59791C3EE	Germany	2017	0.03	-	0.03
846D8D3BA11481B67991	Germany	2017	0.03	-	0.03
84E760EEAB4F104EE3DC	Germany	2016	0.03	-	0.03
852778757F48751D8283	Germany	2017	0.03	-	0.03
855810F24EA90D0880F5	Austria	2017	0.01	-	0.01
85F192460FA7D05E3EF6	Austria	2017	0.01	-	0.01
86169E970E6C34770300	Germany	2016	0.02	-	0.02
86EB68CD1256D6D08739	Germany	2017	0.02	-	0.02
877DE720BD4DC5273712	Germany	2016	0.03	0.031	0.031
878D7E5E57F5A72426D6	Germany	2017	0.025	-	0.025
881B6F0D2252C73591CB	Austria	2017	0.01	-	0.01
88CF10FA329617BE76E0	Germany	2017	0.03	-	0.03
88E5BBDEDA780177D926	Germany	2016	0.02	-	0.02
892B0203DEEE9A2DD84D	Germany	2017	0.03	-	0.03
8946EDA67A062F5A347F	Germany	2016	0.01	-	0.01
89937F111AF5CED421AC	Germany	2017	0.02	-	0.02
8A821ABF479E66E54FA0	Germany	2017	0.02	-	0.02
8AE56183FCACBCA987D2	Germany	2017	0.03	-	0.03
8B46DF802EA714C223F5	Germany	2017	0.02	-	0.02
8CC15A0BBD8CEB0B03F6	Germany	2016	0.02	-	0.02
8DAD3995A288FF698F55	Austria	2017	0.01	-	0.01
8DCDAE0B9F5BDADD731F	Germany	2017	0.03	0.03	0.03
8E9470BF16C8CD0BC89C	Austria	2017	0.01	-	0.01
8F8DE319E61CEF845564	Germany	2016	0.02	-	0.02
9012CA697981F2A9A7D3	Austria	2017	0.01	0.01	0.01
90ADC7A926E55FFC38EF	Netherlands	2016	0.01	0.022	0.022
90D4DD17562036C7F563	Germany	2016	0.02	-	0.02
90DF2B617D0310B4AD8B	Germany	2016	0.14	-	0.14
90F9C2FE1F7BAEFFE8B4	Germany	2016	0.02	-	0.02
9287FDD6D32321E8D3AA	Germany	2017	0.03	-	0.03
92EE01423B11081FEFB3	Germany	2016	0.02	-	0.02
93C764B99CF2F37D3E72	Germany	2016	0.02	-	0.02
93CA47D9B2100DE89A54	Germany	2016	0.02	-	0.02
954E769D06495BA182AD	Germany	2016	0.03	-	0.03
95A50E491F8077509CFF	Germany	2016	0.01	-	0.01
95D24B73862C787DC13A	Germany	2017	0.03	-	0.03
95ED3E9F7FFC0115FE57	Germany	2016	0.02	-	0.02
961551D857666A6B66A8	Germany	2016	0.02	-	0.02
96922889702907E7919A	Germany	2016	0.02	-	0.02
96F6D0C0CC5DFCD31C20	Germany	2016	0.05	-	0.05
97A8BFA408FC5B301596	Germany	2017	0.03	-	0.03
97AD960AAE4EF0304890	Germany	2016	0.05	-	0.05
97B71DF07283BFCDDCCA	Germany	2017	0.03	-	0.03
98231E2F6D9D7AAD9B98	Germany	2017	0.03	-	0.03
9964AECFE8B2E828AA35	Germany	2017	0.03	-	0.03
99842C278092E0104EFB	Germany	2016	0.02	-	0.02
9A0D2843BEEC87415E2B	Germany	2017	0.03	0.311	0.311
9A6221E9B9A7DD927D7C	Germany	2017	0.025	-	0.025
9A93D43A4F95629ABE81	Germany	2017	0.03	-	0.03

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
9AB19FFCA6F4C3932FC4	Germany	2017	0.02	-	0.02
9B49313EB40080ADF356	Germany	2016	0.05	-	0.05
9B770194405680A0D76B	Germany	2016	0.02	-	0.02
9C5EAFBA15AA8D5A2EBB	Germany	2016	0.02	-	0.02
9EF21FCD9303483E33FA	Austria	2017	0.01	-	0.01
9F05D4166763FBCF8095	Germany	2016	0.02	-	0.02
A000208E3D07C779C7FE	Germany	2016	0.02	-	0.02
A0695F8162886F2801E1	Germany	2017	0.01	-	0.01
A157B37DD3B41572AAB5	Germany	2017	0.03	-	0.03
A1F135E9640C92FBB15C	Austria	2017	0.01	-	0.01
A2013A9A0321483F7C55	Germany	2016	0.02	-	0.02
A25D28E66AE0B51F0B6B	Germany	2016	0.02	-	0.02
A29D34B2BF87772601DF	Germany	2016	0.02	-	0.02
A325137A7789F3E47B09	Germany	2017	0.03	-	0.03
A339EBF43CCEA2D2C346	Germany	2017	0.03	-	0.03
A37869A69CF3EBEE119C	Austria	2017	0.01	-	0.01
A3D2F40FC8E589723C73	Germany	2017	0.03	-	0.03
A44A8DEC050F2D478BDA	Germany	2016	0.02	-	0.02
A45AF66A9A9D7CAA1EF9	Germany	2016	0.02	0.292	0.292
A45E457268AE1E02A21F	Germany	2016	0.03	-	0.03
A59D1D7BED4504B489AC	Germany	2016	0.02	-	0.02
A5C922F50F56C65FBB67	Germany	2016	0.01	-	0.01
A72519BCF55A13D14C8A	Germany	2016	0.02	-	0.02
A7486C8A00117A0465FB	Germany	2016	0.02	-	0.02
A81C688CE444C3785C58	Germany	2016	0.03	-	0.03
A920D3838E5205CEED0B	Germany	2017	0.03	0.058	0.058
A9C5887600B5F3E8DEA2	Germany	2017	0.03	-	0.03
A9E73876895057652367	Germany	2017	0.025	0.033	0.033
AAC30AD4E617AAEE884B	Germany	2016	0.02	-	0.02
AB29B109B10D3734310C	Germany	2016	0.02	-	0.02
AB570BA7752BCA2D1693	Austria	2017	0.01	-	0.01
AD5DA660200DD26F65DE	Austria	2017	0.01	0.034	0.034
AD6A7B83E3FCB7579677	Germany	2016	0.02	-	0.02
ADA9DA84F2464166CB4A	Germany	2017	0.03	-	0.03
ADCA1D77EEDEA8265243	Germany	2016	0.01	-	0.01
ADF00296009BC18D4675	Germany	2016	0.03	-	0.03
ADF7EAE1A4ECEE680256	Germany	2016	0.02	-	0.02
AF4B389238385E64BFE3	Germany	2016	0.02	-	0.02
AF8AD3DE1DC339807E5A	Germany	2016	0.01	-	0.01
AFA4A9E051C237454E4B	Austria	2017	0.01	-	0.01
AFFB00C2536FCF43CEFA	Germany	2017	0.03	-	0.03
B0E14E2221220E5E7539	Austria	2017	0.01	-	0.01
B17C7439B96CC06FD1A7	Germany	2016	0.02	-	0.02
B1975E497178DF460D6E	Germany	2017	0.03	-	0.03
B363422F69706107DD08	Germany	2016	0.02	-	0.02
B3753717657628146F90	Germany	2017	0.03	-	0.03
B3FE5F1619947FD45CDD	Germany	2017	0.02	-	0.02
B42144744411C4C83C6C	Germany	2016	0.02	-	0.02
B64A4E5388A08EE72831	Germany	2016	0.02	-	0.02
B68679EE3A8DC13B10DA	Germany	2016	0.03	-	0.03
B7FBFACE636DE4CD49C6	Germany	2017	0.03	-	0.03
B822FCEBA5BCA7FEFE3D	Germany	2016	0.02	-	0.02

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
B868F3D46C4CD577BA4A	Germany	2016	0.02	-	0.02
B898683CAD6FE28C1403	Germany	2016	0.02	-	0.02
B93879A14E3EFC1AE541	Germany	2017	0.03	-	0.03
BAEE814948E33DDE10A0	Germany	2017	0.02	-	0.02
BB6C0DD739666471C56D	Germany	2017	0.03	-	0.03
BB760C7CCADA32BB431A	Germany	2017	0.03	-	0.03
BB80E95621623AD63458	Germany	2016	0.02	-	0.02
BDE0B73281472655E22C	Germany	2016	0.02	-	0.02
BE089A03791315608770	Germany	2016	0.02	-	0.02
BEB90B122ED9B0BBA34E	Germany	2017	0.03	-	0.03
BEFB4B93051DBD2438FC	Germany	2017	0.025	-	0.025
BF04CA6ED38FD9D23881	Germany	2016	0.03	-	0.03
BF9F5F7FBADBEA16A0BB	Germany	2016	0.045	-	0.045
C1EE3741CDF2ED3F69C7	Germany	2016	0.05	-	0.05
C20156F798B7F354D580	Germany	2016	0.01	0.015	0.015
C26D440A42F1170CE04C	Germany	2016	0.02	-	0.02
C2F24DF38FEF17D4DA2B	Germany	2017	0.02	0.023	0.023
C367435C05506E0D830D	Germany	2017	0.03	0.031	0.031
C39F9455B0255D2F1C17	Austria	2017	0.01	-	0.01
C3A8DCE57204E9DA311A	Germany	2016	0.03	-	0.03
C5D1FBE239926E08B835	Germany	2016	0.02	-	0.02
C68DFF922A94923A9BA1	Germany	2017	0.03	-	0.03
C84CF939C8D6F8AB33AA	Germany	2016	0.01	-	0.01
C8C2E1CEB4D1543838CF	Germany	2016	0.03	-	0.03
C8F2755E1C71DB9F17F6	Austria	2017	0.01	-	0.01
C99D668DD376CBA3CABC	Austria	2017	0.01	-	0.01
CA00C043F01A351D96C4	Germany	2016	0.045	-	0.045
CA91122F45DA93989F2E	Germany	2016	0.045	-	0.045
CAC41083439CD59E7156	Germany	2016	0.02	-	0.02
CC25AE5423AFEB7EC89F	Austria	2017	0.01	-	0.01
CC2A2F023F610FECE56E	Germany	2017	0.025	-	0.025
CD3E0739BB7186FA5532	Germany	2016	0.02	-	0.02
CDD0F99BE95E9D7FDEBF	Germany	2016	0.02	-	0.02
CEC1B2EF316FD564D5DC	Germany	2016	0.05	-	0.05
CEE198D5A0D0E14CCC1A	Germany	2017	0.03	-	0.03
D0736FD96750CC0A7598	Germany	2016	0.02	-	0.02
D3D29BD805141B05D060	Germany	2017	0.03	-	0.03
D49BC68BDC78A091CDA3	Austria	2017	0.01	0.03	0.03
D5166581735E53BDBA8E	Germany	2016	0.02	-	0.02
D556BE14786DE73E2003	Germany	2016	0.01	0.019	0.019
D61B3B904EE1BD9AE9F3	Germany	2016	0.02	-	0.02
D80331C0851A1D4FCB1A	Germany	2017	0.02	-	0.02
D9917CF5BC392DC998A4	Germany	2017	0.025	-	0.025
D9A047B3A0B233CCD7E0	Germany	2016	0.02	-	0.02
DC2C50F94F5C3EB5C584	Germany	2016	0.05	-	0.05
DC38BABCAA1913D6437E	Germany	2017	0.02	0.033	0.033
DC509DF3112A0561C57C	Germany	2016	0.045	-	0.045
DCFA16220FEFA4845608	Germany	2016	0.02	-	0.02
DD3E6F215FF02BABF2AC	Germany	2016	0.02	-	0.02
DE2F17301202AE9AF32E	Germany	2017	0.03	-	0.03
DF33C6138094C8C2DFC2	Germany	2017	0.03	-	0.03
DF7243519491F1181D1F	Germany	2017	0.03	-	0.03

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
DF8557DFDD6F148C40C3	Germany	2016	0.02	-	0.02
E2BF651257CBADF20ED0	Austria	2017	0.01	-	0.01
E33FE7559CB6FFEA670E	Austria	2017	0.01	-	0.01
E3B33D9160E1B8F4DB87	Germany	2017	0.025	-	0.025
E581F039DF143AAEA426	Germany	2016	0.05	-	0.05
E5C63D90CBE6AA2B1F80	Germany	2017	0.03	-	0.03
E5EF8A339197FEA5ECA9	Germany	2017	0.03	-	0.03
E633B924DDB649EA419A	Germany	2017	0.025	-	0.025
E654C5D87E32DD7F48AD	Germany	2016	0.02	-	0.02
E66A3DE4E2B1C258A4B3	Germany	2016	0.045	-	0.045
E6B184DEA62165EBF504	Germany	2016	0.02	-	0.02
E73157D382C23458E23C	Germany	2016	0.02	-	0.02
E7486E8A9BC2E2AE020B	Germany	2016	0.02	-	0.02
E810BE286CEE91929046	Germany	2017	0.02	0.025	0.025
E8DE46276D310F03A6BC	Germany	2016	0.045	-	0.045
E982457A41D556D26D0E	Austria	2017	0.01	0.41	0.41
EA29D5D3A2EE8197E523	Germany	2016	0.02	-	0.02
EA65D60DC17A6538EF0F	Germany	2017	0.03	-	0.03
EA6F3BFAD79EA0F734D4	Germany	2017	0.03	-	0.03
EA8A9339449D8B962895	Germany	2016	0.03	-	0.03
EB2F50944825B563EAAE	Germany	2017	0.025	0.064	0.064
EE0B1BBB97B0F1039BF9	Germany	2016	0.02	-	0.02
EF3CA64C715700C3ECA6	Germany	2017	0.03	-	0.03
EF85A2C4C178429F88B6	Germany	2016	0.03	-	0.03
EFADDA98DEFF9CAE58F8	Germany	2017	0.03	-	0.03
F0087893E88FA02195A0	Germany	2017	0.03	-	0.03
F0431E8CD382485CD547	Germany	2016	0.02	-	0.02
F12F2AF558BE5B2F5C92	Germany	2016	0.02	-	0.02
F1B508B77AC9289D02C3	Austria	2017	0.01	-	0.01
F23E30FAFF38138EB1B4	Germany	2017	0.03	-	0.03
F363151E1BD978A959D0	Germany	2016	0.02	-	0.02
F3667D783AB7ADCDCEFE	Germany	2016	0.02	-	0.02
F38F7D4DCA8E06800571	Germany	2016	0.02	-	0.02
F3BD3D55F4F18ED9185A	Austria	2017	0.01	-	0.01
F421BE2D2835630E6A3F	Germany	2016	0.02	-	0.02
F4DE407DC9CC9F0A0814	Germany	2017	0.025	0.025	0.025
F4F2F82EB796855208AB	Germany	2016	0.02	-	0.02
F57B6F34570EA2B74759	Austria	2017	0.01	-	0.01
F615D99119B67D45F3D2	Germany	2016	0.02	-	0.02
F62AD440B1C6DB155635	Germany	2016	0.02	0.59	0.59
F64403227BA2B94BDD15	Germany	2016	0.02	-	0.02
F6682ECD61DAFE127725	Germany	2017	0.03	0.078	0.078
F67C565C212495E9B53A	Austria	2017	0.01	-	0.01
F6A3F8A8B425ADC1D0F0	Austria	2017	0.01	-	0.01
F7CF571768FBC5F1CF11	Germany	2016	0.02	-	0.02
F8550393D66EC2DD2E83	Germany	2016	0.02	-	0.02
F870F98259404A3F9A54	Germany	2017	0.03	0.128	0.128
F8E03379888F179A694D	Germany	2016	0.02	-	0.02
F9BE9B523D4C2502B44F	Austria	2017	0.01	-	0.01
F9D7FE4DCCDBDCAC8A7C	Germany	2017	0.03	-	0.03
FAC2237E071EB2421EBA	Germany	2016	0.02	-	0.02
FBF17D16F61505DA65EC	Germany	2017	0.03	-	0.03

Table B.7.7.1.2-1: Monitoring data of glyphosate in honey for the years 2016 and 2017

Lab Sample Code	Country	Year	LOQ	Residues above LOQ	Residue level for MRL calculation
FC3B945F03225273931D	Germany	2016	0.03	-	0.03
FC8C4970BC9A0BAE1B84	Austria	2017	0.01	-	0.01
FCF467699E0ABA1F91F4	Austria	2017	0.01	-	0.01
FD635ED7BABF88BD40BA	Germany	2016	0.03	-	0.03
FDB5C5E0A7443C968820	Germany	2017	0.03	-	0.03
FEA36AAA7CF9DABEE139	Austria	2017	0.01	-	0.01

B.7.7.1.3. Public literature

B.7.7.1.3.1. Reference 1

1. Information on the study

Data point	CA 6.10.1/003
Report author	El Agrebi, N. *et al.*
Report year	2020
Report title	Honeybee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey
Document No.	DOI 10.1016/j.scitotenv.2019.135312 E-ISSN 1879-1026
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

In order to assess bee and human exposure to residues of glyphosate-based herbicide (GBH) and its main degradation products aminomethylphosphonic acid (AMPA) and to characterise the risk posed by these substances, we analysed 3 different bee matrices; beebread (N = 81), wax (N = 100) and 10-paired samples of wax/honey collected in 2016/2017 from 379 Belgian apiaries. A high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS) was used as analytical method. Limit of quantification and detection (LOQ and LOD) for GBH residues and AMPA in the 3 matrices was respectively of 10 ng/g (0.01 mg/kg) and 1 ng/g (0.001 mg/kg). In beebread, 81.5 % of the samples showed a residue concentration > LOQ and 9.9 % of the samples a residue concentration < LOQ (detection without quantification); no significant difference in detection rate was found between the north and the south of the country. Glyphosate was detected in beeswax less frequently than in beebread (i.e. 26 % > LOQ versus 81.5 % > LOQ). The maximum GBH residues and AMPA concentration found in beebread (respectively 700 ng/g and 250 ng/g) led to sub-lethal exposure to bees. The Hazard Quotient (HQ) for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds for bees. For human health, the highest exposure to GBH residues in pollen corresponded to 0.312 % and 0.187 % of the ADI and of the ARfD respectively and, to 0.002 % and to 0.001 % for beeswax. No transfer of glyphosate from wax to honey was detected. Considering our results and the available regulatory data on the glyphosate molecule considered solely, not including the adjuvants in GBH formulation, the consumption of these three contaminated matrices would not be a food safety issue. Nonetheless, caution should be taken in the interpretation of the results as new studies indicate possible glyphosate/GBH residues toxicity below regulatory limits and at chronic sub-lethal doses.

Materials and Methods

Study areas

Three different bee matrices were sampled for the analysis of GBH residues and AMPA: (i) beebread (N = 179), (ii)

wax from the brood chamber (N = 100) and additionally (iii) a combination of wax from the honey super and corresponding extracted honey (N = 10). We used 379 non-professional apiary sites located in Belgium, including 2,997 colonies of *Apis mellifera*. For beebread and wax sampling, apiaries were selected (193 for beebread, 186 for wax and honey) from the Federal Agency for the Safety of the Food Chain (FASFC) apiaries database that included 4,949 registered beekeepers in 2015. The apiaries were stratified by province (N = 20/province and 10 provinces in Belgium) and randomly distributed in Flanders (northern Belgium) and Wallonia (southern Belgium). All sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication (Ravoet *et al.*, 2015). Quantum GIS (QGIS Development Team, 2009; http://qgis.osgeo.org) was used to create the maps in **Figure 1** and **Figure 2**.

The risks posed by formulated products in the present study are restricted to the active ingredient glyphosate plus AMPA and the total risk of commercial products utilised by farmers is not the subject of this study.

Beebread collection

Beebread sampling (N = 179) was carried out by FASFC beekeepers and apiary technicians (Healthy Bee national monitoring program) between September and October 2016 from 193 apiaries including 865 colonies, out of 75 municipalities covering the entire Belgian territory (**Figure 1**). The samples were provided with a protocol defining sampling collection details and were personally instructed by expert beekeepers to improve the harmonisation of the procedure across apiaries. At each apiary, one hive was sampled randomly by cutting a comb portion of 8 by 8 cm filled with beebread. The coded samples were kept in hermetic plastic bags and stored at -20°C the same day in order to be processed. A cool-box was used for shipment of samples from FASFC to Liège University to ensure that samples were maintained frozen (Tosi *et al.*, 2018) until processing.

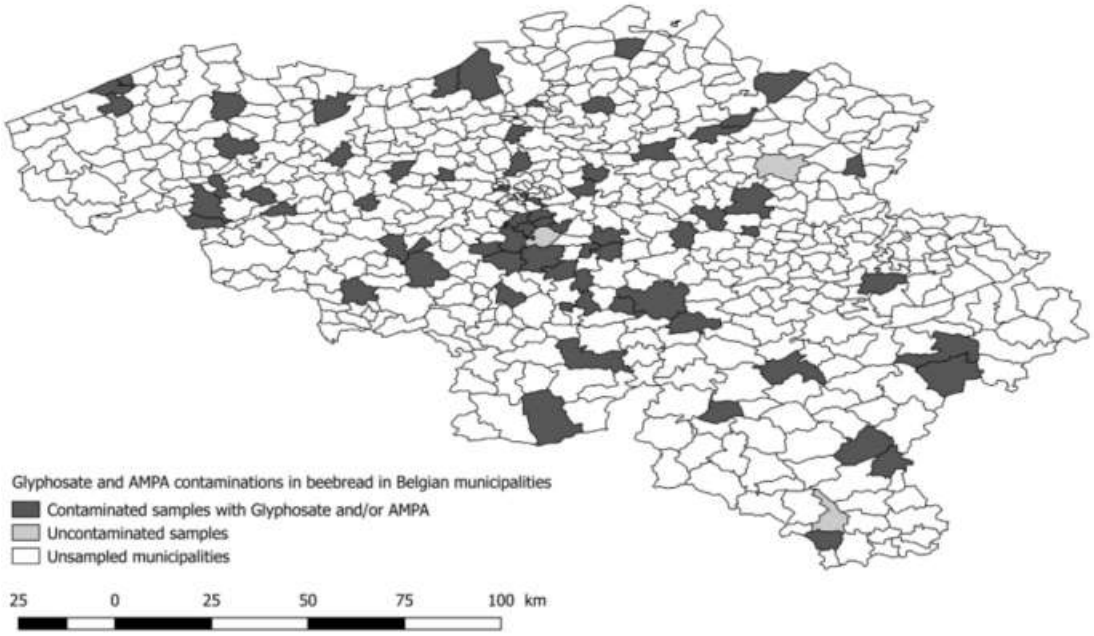


Figure 1: Glyphosate residues and AMPA contaminations in beebread across Belgium, in 2016.

Beebread extraction

For analyse purpose, 20 g of beebread were extracted manually from each comb sample using a disposable surgical blade (1 blade per sample). Cleaned beebread samples were stored in a 60 mL marked sterile polycarbonate containers with screw cap. Only 81 samples of beebread could be extracted from the 179 comb samples in adequate amounts for analysis.

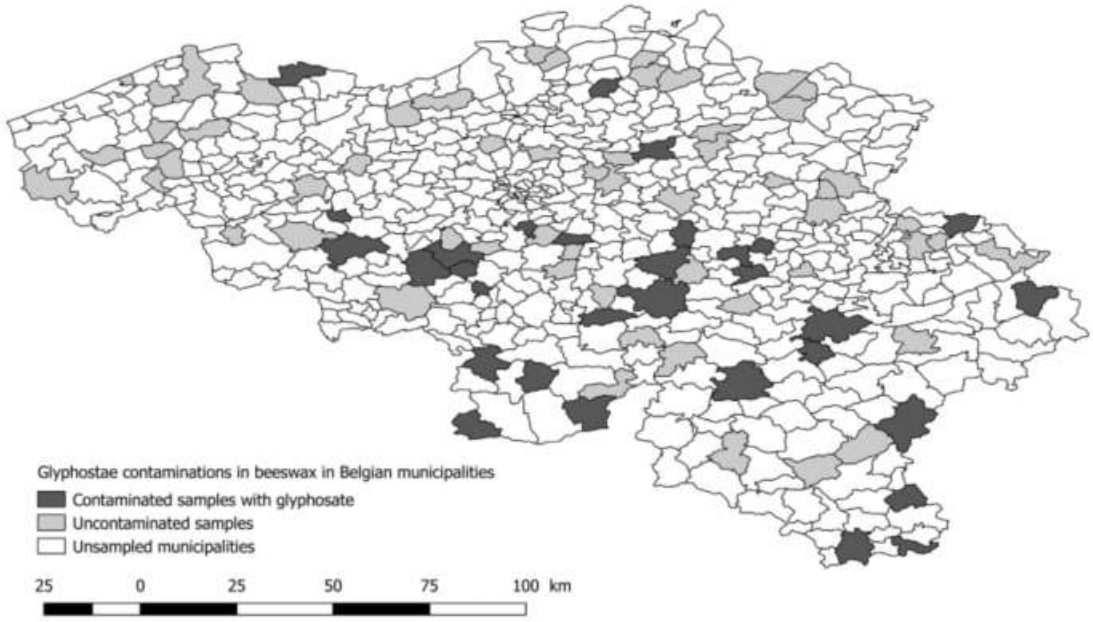


Figure 2: Glyphosate residues and AMPA contaminations in beeswax across Belgium, in 2016.

Wax collection

Twenty grams (20 g) of wax from the brood chamber were sampled during spring 2016. Together with sampling, wax renewal rates were registered in a questionnaire (<50 % and ≥50 %). The coded samples were kept in hermetic plastic bags and stored the same day at -20°C until analysis. Financial limitations allowed us to randomly select only 100 wax samples out of the 186 original samples (2132 hives). These 100 samples were equally distributed between Flanders and Wallonia in 89 municipalities (**Figure 2**).

Honey/wax sampling

After wax analysis, out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber, 10 beekeepers were randomly selected. Among these beekeepers, samples of 20 g of wax and of 50 g of honey harvested in summer 2017 were extracted both from the honey super (pairwise samples). The coded wax samples were kept in hermetic plastic bags, honey in polypropylene disposable containers and shipped the same day to the laboratory. Sampling and analysis of honey for GBH residues and AMPA were performed in September 2017 in the same laboratory and according to a similar method as for beebread and beeswax. Concentrations of GBH residues measured in honey were compared to the Maximum Residue Level (MRL) for human consumption (50 ng/g) (Regulation (EC) No 396/2005).

Glyphosate-based herbicide residues and AMPA detection

The GBH residues and AMPA analyses were carried out between May and June 2017 (September 2017 for the 10-paired samples of wax/honey) by the Phytocontrol laboratory (France) ISO 17,025 accredited under the number No 1–1904 for the analysis of bee products by the French competent authority. The analysis method used for the targeted matrices (beebread, beeswax, and honey) was a high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS). The analytes were extracted using an aqueous solution followed by a simple clean up with a C18 solid-phase extraction (SPE) cartridge, and then glyphosate and AMPA were derivatised using 9-fluorenyl-methoxycarbonyl (FMOC-Cl) in borate buffer. For beeswax, an additional hexane treatment was used in order to defat the extract. The derivatives of glyphosate and AMPA were separated on a C18 column (105 x 4.6 mm; 5 µm) with gradient elution with the mobile phase of acetonitrile and 5 mmol/L ammonium acetate (pH 9), and finally detected with negative ion electrospray ionisation-mass spectrometry (ESI-MS) in multiple reaction monitoring (MRM) mode (drying gas flow at 15 mL/min, nebulizing gas flow at 3 L/min). Limits of quantification (LOQ) for both glyphosate and AMPA in the 3 matrices were 10 ng/g, while limits of detection (LOD) were 1 ng/g. Matrix effects were compensated by the addition of ^{13}C labeled glyphosate (used as internal standard) to the sample prior extraction, as well as in spiked samples used to set up the calibration curve. Three levels of spiking, including the LOQ, were performed on several matrices of different categories, which were analysed in condition of repeatability and intermediate fidelity. The mean spiked recoveries of glyphosate and AMPA at 3 spiked levels ranged from 72.2 % to 112.9 % with the relative standard deviations (RSD, n = 5) of 0.1 % – 4.5 %. The tolerance interval was plotted

with a beta probability of 80 %, which represents the proportion of future values that the routine method will produce over the entire field of application. This allows to ensure that the molecule of glyphosate is extracted correctly and to correct any matrix effects.

Exposure assessment and risk characterisation to honeybee health

We estimated the Hazard Quotient (HQ) for honeybees using the method described by (Stoner and Eitzer, 2013). The HQ is calculated as the exposure divided by the toxicity expressed, in this study, as the maximum residue concentration (ng/g or ppb) in beebread samples divided by the oral acute LD 50 (mg/bee) and multiplyed by 100. An adult bee that consumed 100 mg pollen with an HQ of 1000 would have consumed approximately 10 % of the LD_{50} for the pesticide during this development stage (=10 days as nurse bee) (Calatayud-Vernich *et al.*, 2018). Assuming that 10 % of the LD_{50} should never be exceeded (Atkins *et al.*, 1981), the HQ value of 1000 would correspond to the limit of concern for bee health (Stoner *et al.*, 2013; Traynor *et al.*, 2016). For beeswax, we used a contact HQ of 5000 as threshold safety value, since residue concentrations are significantly higher in wax, and contact exposure routes are poorly understood in this matrix (Traynor *et al.*, 2016).

Then, we also assessed the risk posed by GBH residues and AMPA in beebread to honeybee health through the assessment of the honeybee exposure to these compounds through beebread consumption. To estimate the beebread consumption, we used published pollen consumption values. A nurse bee consumes between 13 and 120 mg of pollen during its first 10 days of life (OECD, 1998; Rortais *et al.*, 2005) with a mean value equal to 65 mg (Chauzat and Faucon, 2007). As a worst-case scenario, we took into account the maximum consumption level of 12 mg of pollen per day. Then, we multiplied this highest level of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the oral acute LD_{50} of these compounds.

Until very recently, risk assessment procedures did not implement yet the side-effects of pesticides on developing brood and the chronic effects in general (OECD, 2017). We could only assess the acute risk for adult bees since the possible toxicity of GBH residues on bee larvae is currently not sufficiently characterised.

Risk to consumer's health

For human health, GBH residues toxicity has been redefined in 2015 (European Food Safety Authority, 2015); an acceptable daily intake (ADI) for consumers has been set to 0.3 mg/kg body weight/day and the acute reference dose (ARfD) at 0.5 mg/kg body weight/day. Concerning AMPA residues, only the ADI value is available (0.3 mg/kg body weight/day). ADI is the quantity of a chemical that can be ingested daily for a lifetime causing no harm (on the basis of all known facts) (Renwick, 2002). ARfD is the quantity of a chemical that can be ingested by a person at a single time causing no harm. MRL is the maximum concentration of pesticide residue legally permitted in or on food commodities or animal feeds (Food and Authority, 2017).

Then, we assessed the risk posed by GBH residues and AMPA in beebread and beeswax to consumer's health through the assessment of the consumer exposure to these compounds through pollen and beeswax consumption. Thus, we assumed that beebread contamination levels correspond to pollen contamination levels. To estimate the pollen and beeswax consumption, we used published consumption data. According to EFSA (EFSA, 2007), the 95th percentile of the daily consumption of beeswax corresponds to 1.29 g/person, which is 0.022 g/kg b.w. for a 60 kg individual. Concerning the daily consumption of pollen, the highest 95th percentile value recorded in the EFSA Comprehensive European Food Consumption Database (EFSA, 2018) corresponds to 69.55 g/person, that is 1.35 g/kg b.w. for a 52 kg individual, in France (according to the second version of the FoodEx food classification system). Then, as a worst-case scenario, we multiplied these high levels of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the reference toxicological values of these compounds (above mentioned) to characterise the risk.

Statistical analysis

Yearly wax renewal rates were divided into 2 categories: <50 % and ≥50 % of wax frames changed per year in the brood chamber. A Fisher's exact test was used to compare the annual renewal rate of wax frames between regions (Flanders versus Wallonia).

A Fisher's exact test was used for each pairwise comparison of frequency of detection of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (beebread versus beeswax). A two-sample Wilcoxon rank-sum (Mann-Whitney) (i.e. non-parametric test) test was used for each pairwise comparison of concentration of GBH residues and AMPA depending on the region/country and the matrix for GBH residues only (bee-bread versus beeswax).

A logistic regression (odds ratio's (OR) with 95 % confidence intervals (95 % CI)) was used to test a possible risk factor of GBH residues detection in beeswax and regions (Stata SE 14.1®, Stata-Corp LP, College Station, TX, USA).

For all tests, a level of significance of 5 % was used and divided, if needed, by the number of comparisons performed for the Bonferroni correction.

Results

Glyphosate-based herbicide residues and AMPA in beebread

In beebread, a high detection of GBH residues was registered (91.4 % of positive samples overall) and AMPA (25.9 % positive samples) in both Belgian regions. Glyphosate LOQ value (10 ng/g) was lower than the glyphosate median lethal doses LD_{50} for bees (10^6 ng/g). No significant difference of contamination prevalence in beebread between regions was confirmed by a one-tailed Fisher's exact test (1 degree of freedom; α =0.05) (N = 81; p > 0.20) (**Table 1**). GBH residues and AMPA were not detected in only 6 samples (7.4 %), coming from 3 of the 75 sampled municipalities (**Figure 1**). Only 2 samples contained AMPA without GBH residue.

Table 1: Glyphosate and AMPA detection, residue levels and hazard quotient to bees in beebread, beeswax and honey samples in Flanders (North Belgium), Wallonia (South Belgium) and Belgium.

Matrix	Region	Sampling period	Nb. analysed samples	Nb. samples > LOQ	Nb. samples < LOQ	Nb. samples detected	% samples > LOQ	% samples < LOQ	% samples detected	Multi-test for detection	Average [] ng g^{-1}	S.D. [] ng g^{-1}	Multi-test for []	Max [] ng g^{-1}	Median [] ng g^{-1}	Max HQ
Beebread	**GBH**															
	Flanders	Fall 2016	39	34	3	37	87.2%	7.7%	94.9%	aa	58.44	133.28	aa	700	23	7
	Wallonia		42	32	5	37	76.2%	11.9%	88.1%	aa	52.41	39.70	aa	160	49.5	1.6
	Belgium		81	66	8	74	81.5%	9.9%	91.4%	aa	55.52	98.89	aa	700	26	7
	AMPA															
	Flanders	Fall 2016	39	5	3	8	12.8%	7.69%	20.5%	a-	39.8	25.16	a-	77	38	0.8
	Wallonia		42	10	3	13	23.8%	7.14%	30.9%	a-	80.8	78.09	a-	250	58.5	2.5
	Belgium		81	15	6	21	18.5%	7.4%	25.9%	a-	67.13	67.09	a-	250	44	2.5
	GBH															
Beeswax	Flanders	Spring	48	3	1	4	6.3%	2.08%	8.3%	ab	28.33	22.90	aa	54	21	0.5
	Wallonia	2016	52	23	5	28	44.2%	9.62%	53.8%	bb	66.43	84.01	aa	320	40	3.2
	Belgium		100	26	6	32	26%	6%	32%	cb	62.04	80.05	aa	320	36	3.2
Honey	Flanders	Summer	2	0	1	1	0%	50%	50%	/	/	/	/	/	/	/
	Wallonia	2017	8	1	0	1	12.5%	0%	13%	/	11	/	/	11	11	/
	Belgium		10	1	1	2	10%	10%	20%	/	11	/	/	11	11	/

GBH: Glyphosate based herbicide, Nb.: number; > LOQ: detection with quantification, <LOQ: detection without quantification, []: concentration; AMPA: aminomethylphosphonic acid; HQ beebread (oral) threshold value = 1000; HQ wax (contact) threshold value = 5000, + detection is the sum of samples > LOQ and < LOD; S.D.: standard deviation; /: not determined. Multi-testing: a Fisher's exact test and a two-sample Wilcoxon rank-sum (Mann-Whitney) test were respectively used for each pairwise comparison of frequency of detection and mean concentration of the compounds. Different letters were used for significant differences. The first position letter corresponds to the comparison of regions for a same matrix; the second position letter corresponds to the comparison of breebread and beeswax for the mean concentration of GBH. A level of significance of 5% was used, divided by the number of tests performed for the Bonferroni correction.

Exposure assessment and risk characterisation of GBH residues in beebread for honey bees

Based on the honeybee oral acute LD_{50} (48 h) of glyphosate (100 mg/bee = moderate toxicity for adult bees) (Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate 2015; Lewis *et al.*, 2016) and on the maximum concentration of GBH residues detected in beebread (700 ng/g), the estimated maximum HQ (oral) of GBH residues for beebread found in Belgium is equal to 7 (=700/100). Because the honeybee oral acute LD_{50} (48 h) of AMPA is currently unknown in published data, it was impossible to estimate its corresponding HQ.

Considering the maximum consumption level of 12 mg of pollen per day (Rortais *et al.*, 2005) (worst-case) and the maximum concentration of GBH residues detected in beebread (700 ng/g), this would correspond to a dose of 84 ng of GBH residues ingested per nurse bee over 10 days (0.012 g x 700 ng/g x 10 days). This exposure level corresponds to about 0.08 % of the oral glyphosate LD_{50}. As mentioned, in the open literature, no oral acute LD_{50} (48 h) for AMPA is available. To assess the risk of AMPA to bees, we used, therefore, the parent compound glyphosate LD_{50} (Traynor *et al.*, 2016). AMPA detection in beebread (250 ng/g) would correspond to about 0.03 % of the oral glyphosate LD_{50}. Cumulatively, GBH and AMPA maximal concentration would correspond to about 0.12 % of oral glyphosate LD_{50}.

Glyphosate-based herbicide residues and AMPA in beeswax

GBH residues were found in 32 % of Belgian beeswax samples (N = 100, T1). A significantly higher GBH residues prevalence was found in Wallonia (53.8 % positive sample, **Figure 2**), as compared to Flanders (8.3 % positive samples, one-tailed Fisher's exact test (1 degree of freedom; α = 0.05), p < 0.001); confirmed by a logistic regression comparing contaminations in both regions (with Flanders as a reference): OR = 18.4, 95 % CI = 4.66–72.60, p < 0.001). A two-sample Wilcoxon rank-sum (Mann-Whitney) test showed that the average GBH residue concentration observed in Wallonia is not significantly higher than in Flanders (p = 0.33) (**Table 1**).

Exposure assessment of GBH residues in beeswax

No trace of AMPA has been detected in beeswax. HQ (contact) of beeswax for the maximum GBH residues concentration in Belgium is equal to 3.2 (= 320/100).

Wax renewal rate in Flanders and Wallonia

Beekeepers should renew the wax foundation of their bee colonies periodically. This improves bee health reducing the disease and chemical load of beeswax and allowing bees to rear their brood in a freshly built environment.

Flemish beekeepers had a significant higher wax renewal rate (≥ 50 % per year) as compared to Walloon ones (N = 98, one-tailed Fisher's exact test (1 degree of freedom; α =0.05) , p = 0.017) (data not shown).

Risk assessment for the consumer of contaminated beebread and beeswax

As shown in **Table 1**, GBH residues contaminated significantly more frequently beebread (87.2 % >LOQ) than beeswax (26 % >LOQ) (one-tailed Fisher's exact test (1 degree of freedom; a =0.05), N = 181; p < 0.001) but the average concentration found in beebread (55.52 ng/g) and wax (51.3 ng/g) were statistically comparable (two-sample Wilcoxon rank-sum (Mann-Whitney) test; p > 0.05).

A high consumption level (95th percentile) of the most contaminated pollen and beeswax by GBH residues, according to our results, leads to an exposure of respectively 0.936 and 0.007 mg GBH residues/kg b.w./day through beeswax and pollen consumption. Concerning AMPA, the highest exposure corresponds to 0.334 mg AMPA/kg b.w./day through pollen consumption).

Transfer of GBH residues and AMPA from beeswax to honey

We wondered if a transfer of GBH residues and AMPA from beeswax to honey was possible. Thus, to further test this hypothesis, we concomitantly collected both wax and honey from the bee colony honey supers of 10 apiaries out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber. We found 1 out of 10 wax samples (10 %) contaminated with GBH residues (concentration: 48 ng/g). In honey, 2 out of 10 samples were contaminated by GBH residues (20 %; 11 ng/g for the first sample and a detection lower than the quantification limit [LOQ] < 10 ng/g for the second sample). These 3 positive GBH residues samples came from different bee colonies. No trace of AMPA was detected in any of the matrices. The highest GBH residues concentration detected in honey was about 5 times lower than the MRL (50 ng/g).

Discussion

Beebread

Our study showed an extended presence of GBH residues in beebread (81.5 % positive samples at the national level) in both Belgian regions. AMPA was found in 18.5 % of beebread samples at the national level. Only 2 samples contained AMPA without GBH residue. The LOQ values for glyphosate and AMPA are of 10 ng/g, which makes the analysis method very sensitive. Simultaneous AMPA/GBH residues detection in beebread could be explained by the GBH residues degradation in the matrix or by their simultaneous occurrence in the environment. In soil, the primary pathway degradation of glyphosate residues is microbial action, which yields AMPA and glyoxylic acid (Roberts *et al.*, 1999). The maximum GBH residues concentration found (700 ng/g) led to sublethal exposure (not acutely toxic to bees), corresponding to a dose of 84 ng/bee (0.08 % of its LD_{50}), ingested over the first 10 days of life of a nurse bee. AMPA dose in beebread also corresponded to a sub-lethal exposure (to about 0.03 % of oral glyphosate LD_{50}) alone or cumulated with GBH residues (about 0.12 % of oral glyphosate LD_{50}). However, while the LD_{50} is measured as a one-time dose, bees could be exposed to GBH residues contaminated beebread for a longer period, when re-contamination occurs, since glyphosate degradation time DT_{50} ranges between 1.0 and 67.7 days. Therefore, the use of the LD_{50} as a single benchmark could underestimate the exposure risk to bees.

Bee and bee colony health is significantly impaired by doses that are lower than those we found through sub-lethal effects. Helmer *et al.* (Helmer *et al.*, 2015) orally exposed bees to sub-lethal field-realistic doses of GBH residues (1.25, 2.50, and 5.00 ng/bee) and showed a significant decrease (p < 0.05; n = 40) of beta-carotene and protein levels in their bodies after 10 days. Our results confirm Helmer's field-realistic doses (lower than 700 ppb, corresponding to 84 ng/bee). Other studies (Herbert *et al.*, 2014), showed that adult *A. mellifera* workers exposed orally to 2.5 and 5 mg/L of GBH residues (field-realistic doses equivalent) presented reduced sucrose sensitivity leading to loss and difficulty in establishing associative memories, which, in turn, could cause inefficient collection of nectar and pollen for the colony and, finally, compromise its survival. Oral exposure to GBH residues concentrations (2.5, 5.0, and 10.0 mg/L, corresponding to a dose of 0.125, 0.25, and 0.5 µg/bee) affects honeybee cognitive abilities, with potential long-term negative consequences for colony foraging success (Balbuena *et al.*, 2015). Exposures to 5 and 10 mg/L of GBH residues (dose of 0.25 and 0.5 µg/bee) perturb the gut microbiota of honeybees. Bee gut symbionts influence bee development, nutrition, and defence against natural enemies (Motta *et al.*, 2018). Perturbations of these gut communities may affect bee susceptibility to environmental stressors, including poor nutrition (Tosi *et al.*, 2017) and pathogens (Motta *et al.*, 2018). Moreover, in evaluating the effect of Roundup® on the royal jelly-producing glands, Faita *et al.* (2018) showed that exposure to GBH residues resulted in the alteration of these glands that can trigger damage to the development and survival of bee colonies.

Regarding AMPA, no trace was found in honey and beeswax. In beebread, the maximum AMPA concentration was 250 ng/g. Because no information on AMPA toxicity to bees is available yet in the open literature, we were not able to assess its risks to bees. Nevertheless, Blot *et al.* (2019) confirmed that glyphosate have sub-lethal effects on the

honeybee microbiota, while AMPA did not induce any significant change.

Beeswax

Measured GBH residues concentrations should not cause acute lethal effects since the estimated HQ for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds (1000 and 5000, respectively). Since beebread can be stored in the hive for months after collection in the field, glyphosate degradation have likely reduced its concentration over time. Furthermore, bees typically collect multiple chemicals simultaneously (Tosi *et al.*, 2018). Because bees are bio-indicators of environmental health and pollution, residues found in bee products provide valuable information on environmental punctual contamination or accumulation which, nevertheless, might be underestimated (i.e. residue degradation, dilution of highly-concentrated samples, technical limitations such as LOD) or overestimated (i.e. accumulation of contaminated pollen) (Tosi *et al.*, 2018).

Due to glyphosate high water solubility and a very low octanol/water partition coefficient (Log P (= Log Kow) at pH 7 and at 20°C = –3.2), GBH residues were expected to be found only in beebread but not in wax (a very hydrophobic matrix). Beeswax samples contamination rate was of 26 % at the national level. The addition of surfactant in the formulation of end-use pesticide products is at the origin of the phenomenon allowing glyphosate, which is water-soluble, to penetrate lipid-based structures (Shokri *et al.*, 2001). Nevertheless, the risk assessment for honey bees and the consumer has been evaluated for glyphosate molecule solely without the concomitant formulation ingredients and adjuvants, nor other possibly concurring pesticides (Tosi *et al.*, 2018). The use of the glyphosate/AMPA molecule solely does not render the combined toxic effects of the formulation constituents nor the synergetic potential effects of pesticide combinations.

Wallonia had both a higher GBH residue detection rate (53.8 %) and a significantly lower rate of wax foundation renewal rate, as compared to Flanders ($p = 0.017$). This supports our hypothesis that the beekeeping management practice of renewing wax foundation can protect bees from the accumulation of pesticide residues inside the hive. No trace of AMPA could be detected in beeswax, probably because the matrix is not suitable for microorganism growth due to its rich hydrophobic protective properties (Fratini *et al.*, 2016), resulting in no degradation of glyphosate in AMPA. Beeswax's conservative properties for pesticide residues combined with the beekeeping practice of wax recycling (Perugini *et al.*, 2018), may be at the origin of the unequal detection of GBH residues in Flanders and Wallonia. This result highlights the importance of replacing at least 50 % of wax frames per year, the current recommendation being the yearly replacement of 25 to 33 % of the wax from the brood chamber (ITSAP, 2017; Vergaert, 2017).

For human health, the highest exposure to GBH residues in pollen corresponds to 0.312 % and 0.187 % respectively of the ADI and of the ARfD, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 700 ng of GBH residues/g). The exposure to GBH residues through the beeswax consumption (1.29 g/day/person of contaminated beeswax with 320 ng of GBH residues/g) corresponds to only 0.002 % and 0.001 % respectively of the ADI and of the ARfD. Concerning AMPA, the highest exposure to this compound corresponds to 0.111 % of the ADI, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 250 ng of AMPA/g).

Honey

The honey analysis resulted in a maximum GBH residues concentration of 11 ng/g, not exceeding the EU MRL (50 ng/g) for honey and theoretically meaning no risk for the consumer. In a survey on GBH residues in honey samples originating from different countries (Brazil, Canada, China, Germany, Greece, Hungary, India, Korea, Mexico, Uruguay, New Zealand, Spain, Taiwan, Ukraine, Vietnam and USA), GBH residues were found in fifty nine percent (59 %) of analysed samples, with concentrations ranging between 17 and 163 ng/g (mean = 64 ng/g) (Rubio *et al.*, 2014).

Our concomitant analyses of wax and honey in samples (N = 10) from honey supers resulted in one wax sample being contaminated (48 ng/g). The low contamination in honey supers suggests that GBH residues are mostly stored in the brood chamber, where pollen and nectar are stored and where most bee activity occurs. This preliminary study showed no transfer from wax to honey. Because our results on the concomitant honey/wax contamination are based on limited data (N = 10), they should be confirmed with further studies.

For human health, considering our results and the assumptions we made with the available regulatory data, the consumption of these three contaminated food matrices (pollen, beeswax, and honey) would not be a food safety issue, nonetheless, caution should be taken in the interpretation the results as new studies confirmed glyphosate toxicity below regulatory limits (Mesnage *et al.*, 2015), and the genotoxicity of AMPA (Mañas *et al.*, 2009).

Bees are major pollinators in agricultural systems. Beebread, beeswax, and honey pesticide residue contamination can impact the viability of a colony when larvae develop on highly contaminated beeswax and feed with contaminated food (Orantes-Bermejo *et al.*, 2010). Even a low concentration of pesticide residues can have amplified toxic effects

on animals, including bees, through interactions with other chemicals (Zhu *et al.*, 2017) or environmental stressors. The pesticide risk to bees can synergistically amplify the adverse effect of non-chemical stressors too and conversely, nutritional stress can synergistically increase the toxicity of pesticides (Tosi *et al.*, 2017).

Conclusions

Our study gives a glimpse of bees and human exposures to GBH residues. At this stage, glyphosate is analysed alone, even though it is never used in this form but only as part of a mixture with adjuvants in commercial formulations. Clarifications and further research are needed to estimate the risk of the herbicide alone and in formulations (i.e. with the adjuvants), especially at levels below the regulatory safe limits and over longer durations. More studies are needed to assess synergies with other pesticides, and longer term exposures at sub-lethal doses. More transparency is needed regarding the commercial formulation products.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article describes a survey of pesticide residues (glyphosate/AMPA) in various bee-related matrices (beebread, wax, honey) from Belgium. While the representativeness of the sampling procedures may be questioned and although the results of the analytical method validations are not provided in a high level of details, the results are considered reliable. A considerable number of samples of beebread/pollen (n = 82) and beeswax (n = 100) were analysed for parent glyphosate and its metabolite AMPA. However, according to the guideline SANTE/11956/2016 rev. 9 the intake of pollen and wax by consumers is negligible and, therefore, it is not a regulatory requirement to investigate the residue levels in these commodities. The publication also provides analytical results for 10 honey samples. Only one of these samples was found to contain residues of parent glyphosate above the LOQ of 0.010 mg/kg (at 0.011 mg/kg). None of the honey samples showed detectable residues of AMPA (i.e. these residues were < 0.001 mg/kg). Since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data, these results are deemed relevant.

The publication concludes that, based on the observed residue levels, the intake of pollen, beeswax and honey by consumers does not cause any health issue. While this conclusion is certainly correct some of the details of the risk assessment are questionable. For instance, the considered ADI of 0.3 mg/kg bw/day for parent glyphosate is obsolete (and was already obsolete at the time when the publication was issued). Furthermore, the long-term residue intakes were calculated based on maximum residue levels and high percentile consumption figures, which does not correspond to the standard approach.

The publication also includes extensive considerations on bee safety, which, however, are not relevant to this section of the dossier and, therefore, are not discussed here.

Assessment and conclusion by RMS:
The publication describes the monitoring of pollen, wax, and honey from Belgian apiaries for residues of glyphosate and AMPA. As already stated by the applicant and in contrast to honey, the intake of pollen and wax is negligible and therefore the results are less relevant. Out of ten honey samples, only one contained residues of glyphosate above the LOQ (0.011 mg/kg) which is below the existing MRL of 0.05* mg/kg. The residue levels fit well within the available monitoring data obtained by official monitoring laboratories (see Volume 3, B.7.7.1.2). Furthermore, as already stated, the risk assessment conducted in the publication is questionable since toxicological endpoints as well as assumed consumer intake levels are not in line with the data usually considered within the regulatory framework.

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered relevant for the MRL calculation for two reasons: (i) the data were not obtained by official monitoring laboratories; and (ii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

B.7.7.1.3.2. Reference 2

1. Information on the study

Data point	CA 6.10.1/004

Report author	Thompson, T.S. *et al.*
Report year	2019
Report title	Determination of glyphosate, AMPA, and glufosinate in honey by online solid-phase extraction-liquid chromatography-tandem mass spectrometry
Document No.	DOI 10.1080/19440049.2019.1577993 E-ISSN 1944-0057
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

A simple method was developed for the simultaneous determination of glyphosate, its main degradation product (aminomethylphosphonic acid), and glufosinate in honey. Aqueous honey solutions were derivatised offline prior to direct analysis of the target analytes using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Using the developed procedure, accuracies ranging from 95.2 % to 105.3 % were observed for all analytes at fortification levels of 5, 50, and 150 µg/kg with intra-day precisions ranging from 1.6 % to 7.2 %. The limit of quantitation (LOQ) was 1 µg/kg for each analyte. Two hundred honey samples were analysed for the three analytes with AMPA and glyphosate being most frequently detected (99.0 % and 98.5 % of samples tested, respectively). The concentrations of glyphosate were found to range from < 1 to 49.8 µg/kg while those of its degradation product ranged from < 1 to 50.1 µg/kg. The ratio of glyphosate to AMPA was found to vary significantly amongst the samples where both analytes were present above the LOQ. Glufosinate was detected in 125 of 200 samples up to a maximum concentration of 33.0 µg/kg.

Materials and Methods

Reagents and standards

Reagent water (>18 MΩ resistivity) was produced using a Barnstead NANOPure reverse osmosis system. Acetonitrile (ACN; HPLC grade) was purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Ammonium carbonate (ACS reagent grade), sodium carbonate (ACS reagent grade), 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), and neat reference materials of glyphosate, AMPA, and glufosinate ammonium were obtained from Sigma Aldrich Canada (Oakville, ON, Canada). Isotopically labelled forms of the analytes, specifically $^{13}C_2$,^{15}N-glyphosate, ^{13}C,^{15}N-AMPA, and D_3-glufosinate hydrochloride, were purchased from Toronto Research Chemicals (North York, ON, Canada).

Individual stock standard solutions of glyphosate, AMPA, and glufosinate were prepared by dissolving 10 mg of each analyte in 10 mL of reagent water). A mixed working spike solution containing 1 µg/mL of each analyte in water was prepared from the stock standard solutions. A second working spike solution containing 0.1 µg/mL of each analyte was prepared by diluting the 1 µg/mL solution ten-fold with water. Stock standards of the isotopically labelled internal standards were likewise prepared in water but at a concentration of 100 µg/mL. A working solution containing 1 µg/mL of each internal standard compound was prepared by mixing 0.1 mL of each stock standard solution and diluting to a final volume of 10 mL.

A 0.1 M solution of sodium carbonate, used to adjust the pH of the honey solutions prior to derivatisation, was prepared in reagent water. A 0.05 % (w/v) solution of FMOC-Cl in ACN was prepared fresh for use in derivatising the analytes and their corresponding internal standards.

Sample preparation

Two gram portions of individual honey samples were weighed into 15-mL polypropylene centrifuge tubes (VWR Canada, Edmonton, AB, Canada). The samples were fortified with 50 µL of the working internal standard solution and allowed to sit for 10 min prior to the addition of 5 mL of reagent water. The centrifuge tubes were capped and mixed on a mechanical shaker until the honey was completely dissolved.

Due to difficulties encountered in obtaining a honey sample which did not contain traces of glyphosate, calibration

standards were prepared in reagent water. To compensate for the final volume of the honey solution obtained by dissolving 2 g of honey in 5 mL of water, the volume of reagent water added to each 15-mL centrifuge tube was 6.5 mL. A series of 9 calibration standards were prepared by spiking the reagent water aliquots with equivalent analyte concentrations of 0, 1, 5, 10, 20, 50, 75, 100, and 200 µg/kg. Each calibration standard was also spiked with 50 µL of the working internal standard solution. Replicate spiked honey samples for method validation were prepared by fortifying portions of a honey sample which was found to be free of all three analytes at the LOQ values (1 µg/kg) of the proposed method. The levels of fortification for the spiked replicates were chosen at equivalents of 5, 50, and 150 µg/kg).

Prior to analysis by LC-MS/MS, 0.5 mL aliquots of all honey solutions and calibration standards were pipetted into a 2-mL polypropylene microvial to which 0.5 mL of 0.1 M sodium carbonate solution was added. The tubes were capped and mixed by inverting several times. A 0.2 mL portion of the FMOC-Cl in ACN solution was added to each microvial which was then recapped and mixed using a high-speed orbital shaker (Bead Ruptor 12, Omni International Inc., Kennesaw, GA, USA) for two 90 s cycles at maximum speed. Next, the micro-vials were mixed for an additional 60 min using a rocking bed mixer. After derivatisation, the honey mixtures were filtered using 25 mm nylon filters (0.25 µm pore size) directly into polypropylene LC vials (Chromatographic Specialties, Brockville, ON, Canada).

Instrumental analysis

The configuration of the online SPE-LC-MS/MS setup is illustrated in **Figure 1**. The Shimadzu liquid chromatograph system included a SIL30AC autosampler, two LC30AD solvent delivery pumps, and a CBM20A module controller. A six-port, two-position, electronically actuated switching valve (Rheodyne MXT715, Scientific Products and Equipment, Oshawa, ON, Canada) was used to incorporate the online SPE cartridge within the LC-MS/MS system via contact closure through the LC module controller. An Oasis HLB extraction cartridge, 20 × 3.9 mm with 5 µm particles (Waters Ltd., Mississauga, ON, Canada) was employed for the online SPE step. The extraction cartridge was protected by a 4 × 2 mm i.d. RP-1 polymeric guard cartridge (Phenomenex, Torrance, CA, USA). The analytical column was an Agilent Zorbax Extend-C18 column (50 × 2.1 mm, 1.8 µm) preceded by a guard column with similar stationary phase material (5 × 3.0 mm). A binary gradient elution programme employing 10 mM ammonium carbonate in water and ACN as the two mobile phases was used for the online SPE step and the final chromatographic separation. The parameters for the gradient elution programme including the switch positioning of the six-port valve are listed in **Table 1**. The LC was re-equilibrated at initial conditions for 4 min prior to the next injection. The injection volume for all analyses was 50 µL.

A Sciex 4500 quadrupole tandem mass spectrometer was interfaced to the LC using an electrospray ionisation (ESI) probe. The MS/MS was operated in the negative ESI mode with the following general parameters: probe temperature = 700°C; ion spray voltage = −3.5 kV; curtain gas = 20 units; source gases 1 and 2 at 70 units each; collisionally activated dissociation (CAD) gas value = 8 units. All analyses were performed using multiple reaction monitoring (MRM) with the analyte-specific parameters provided in **Table 2**. The dwell time for each MRM transition was 50 ms. A programmable six-port switching valve incorporated into the MS/MS was used to divert flow from the analytical column to the MS/MS only from 6.5 to 9 min during the LC gradient elution programme in order to minimise contamination of the MS ion source.

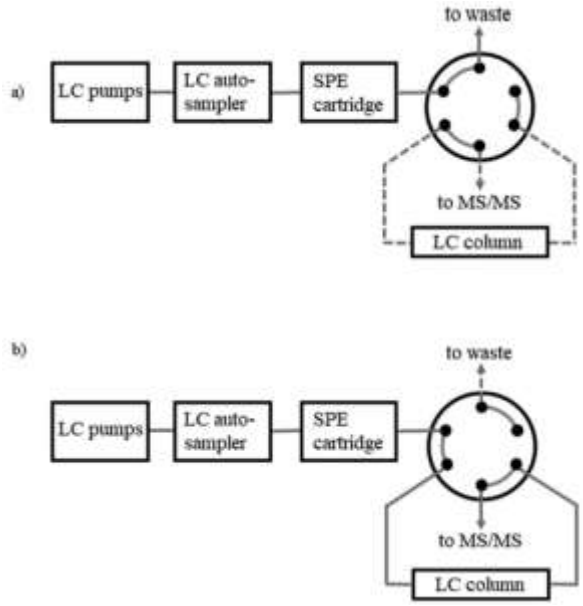


Figure 1: Schematic diagram of online solid-phase extraction coupled to LC-MS/MS showing solvent flow with switching valve in (a) position #1 for flushing bulk matrix to waste and (b) position #2 for elution and chromatographic separation of analytes prior to MS/MS detection.

Table 1: LC gradient elution program and six-port switching valve position.

Time (min)	%A (10 mM $(NH_4)_2CO_3$)	%B (ACN)	Flow rate (mL min^{-1})	Valve position
0	100	0	1.00	to waste
2.9	100	0	1.00	to waste
3.0	100	0	0.35	to waste
5.5	*73.6**	*26.4**	0.35	to analytical column
12.0	5	95	0.35	to analytical column
12.5	5	95	0.35	to analytical column
13.0	5	95	0.60	to analytical column
15.5	5	95	0.60	to analytical column
16.00	5	95	0.35	to analytical column
17.00	98	2	0.35	to analytical column
19.00	98	2	0.35	to waste

*Estimated composition based on programmed gradient from 100% A at 3.0 min to 5% A at 12.0 min.

Table 2: MRM parameters for analytes and corresponding internal standards.

Compound	Precursor > product ions*	DP (V)	CE (eV)
glyphosate	***390 > 168***	−40	−16
	390 > 150	−40	−34
$^{13}C_2$,^{15}N-glyphosate	***393 > 170***	−40	−16
AMPA	***332 > 110***	−40	−16
	332 > 136	−40	−20
^{13}C,^{15}N-AMPA	***334 > 112***	−40	−16
glufosinate	***402 > 180***	−45	−14
	402 > 206	−45	−20
D_3-glufosinate	***405 > 183***	−45	−14

*Transition in bolded italics used for quantitation.

Results and Discussion

Considerations for proposed analytical method

There were two main considerations which dictated the direction taken for the development of the method to determine trace residues of glyphosate, AMPA, and glufosinate in honey. The first consideration was the desired LOQ which was established based on the maximum residue limit (MRL) for glyphosate and glufosinate in honey. While Health Canada has not established an MRL for either glyphosate or glufosinate in honey, the EU has set the maximum

acceptable concentration at 50 µg/kg for each compound (European Union: Pesticides database 2016). It was decided that the targeted LOQ value should not exceed one-tenth of this MRL value (in other words be 5 µg/kg or lower). The main reason for this targeted LOQ was to have a method which would permit its application to a general survey to establish baseline residue levels rather than determine compliance with existing MRL values.

The second consideration was the necessity to isolate the analytes from the honey matrix which is comprised mainly of the monosaccharides fructose and glucose as well as lower amounts of disaccharides and various other carbohydrates (Bell 2007). On the basis of weight, water typically accounts for less than 20 % of the honey matrix with the majority of the remaining components consisting of simple sugars. The challenge of separating the highly polar analytes of interest from the relatively large quantities of highly polar carbohydrates prior to MS/MS analysis was a significant factor in the development of the proposed testing method.

One of the major advantages of LC over GC is the amenability of the former for the determination of analytes with polar functional groups without the necessity of performing derivatisation. There are, however, still two inherent benefits to performing derivatisation of highly polar analytes such as glyphosate, AMPA, and glufosinate prior to analysis by LC-MS based techniques. Derivatisation of highly polar analytes can result in increased retention using reversed phase stationary phases and increased sensitivity in electrospray ionisation MS (Toss *et al.* 2017). While direct determination of non-derivatised analytes is desirable in that it simplifies the analytical method, there has been mixed success in the development of such procedures. Ibanez *et al.* (2005) attempted to determine glyphosate, AMPA, and glufosinate without derivatisation but encountered difficulties including reduced sensitivity and lack of robustness of their proposed hydrophilic interaction liquid chromatography (HILIC) method. This ultimately resulted in their decision to employ derivatisation with FMOC-Cl. Similarly, Ehling and Reddy (2015) explored the direct analysis of glyphosate and AMPA using a variety of chromatographic stationary phases but also reported problems with lack of ruggedness, poor chromatographic peak shapes, and inadequate ESI-MS/MS sensitivity. Liao *et al.* (2018) stated that direct determination of glyphosate did not provide adequate sensitivity and selectivity to permit its analysis in baby food samples at concentrations as low as 10 µg/kg. For these reasons, derivatisation with FMOC-Cl has remained a popular procedure in numerous LC-MS-based methods (Arkan and Molnar-Perl 2015).

Based on initial investigations in our lab, it was observed that the sensitivity obtained for FMOC-Cl derivatives of the target analytes was significantly greater than for the non-derivatised compounds under negative electrospray ionisation conditions. A further complication of the direct determination of non-derivatised glyphosate, AMPA, and glufosinate in honey is the fact that the highly polar analytes of interest are difficult to separate from the polar carbohydrates which comprise the bulk of the honey matrix (approximately 80 % by weight simple sugars). While ion exchange solid-phase extraction (SPE) remains an option for isolating glyphosate, AMPA, and glufosinate from the sugars, the inclusion of an offline SPE clean-up step was undesirable due to the additional associated increases in labour, cost, and time. Derivatisation with FMOC-Cl increases the retention of glyphosate, AMPA, and glufosinate on reversed phase stationary phases making it possible to separate the derivatised analytes from highly polar carbohydrates which constitute the bulk of the honey matrix.

Numerous groups have employed online solid-phase extraction methods for the determination of one or more of glyphosate, AMPA, and glufosinate in water samples after offline derivatisation using FMOC-Cl (Vreeken *et al.* 1998; Meyer *et al.* 2009; Sanchis *et al.* 2012; Poiger *et al.* 2017). The advantages of online SPE versus offline SPE are three-fold: firstly to automate the clean-up procedure thereby reducing labour and preparation time; secondly to permit the direct transfer of the analytes of interest from the extraction column/cartridge to the analytical column; and thirdly to facilitate the refinement of the conditions under which the analytes are trapped and subsequently eluted for direct determination. The capability to monitor the chromatographic behaviour of the analytes during online SPE coupled to LC-MS/MS simplifies method development. It was therefore decided to investigate an analytical procedure employing offline derivatisation of glyphosate, AMPA, and glufosinate followed by online SPE separation of the derivatives from the bulk honey matrix with subsequent direct determination by LC-MS/MS.

Derivatisation using FMOC-Cl

Two challenges were encountered in establishing the derivatisation procedure. Firstly, derivatisation of the analytes using FMOC-Cl was discovered to not work efficiently when sodium tetraborate was used in the presence of the honey matrix. Honey is quite acidic in a relatively concentrated solution (2 g of honey plus 5 mL of water) and the borate solution did not have enough buffering capacity to permit the pH of the resulting mixture to be approximately 9 as commonly established in the derivatisation reaction employing FMOC-Cl (Arkan and Molnar-Perl 2015). Sodium carbonate has been used in the derivatisation reaction with FMOC-Cl with aminophosphonic acids (Huber and Calabrese 1985) while a carbonate buffer was used in conjunction with FMOC-Cl and tertiary amphetamines (Herraez-Hernandez and Campins-Falco 2000). Descombes *et al.* (1991) reported that borate and carbonate buffers both worked well in providing alkaline conditions (pH = 9.5) under which the derivatisation of catecholamines and

amphetamines could be achieved with FMOC-Cl. Upon switching to 0.1 M sodium carbonate for pH adjustment, it was observed that the derivatisation step proceeded smoothly.

The second challenge was realised when it became obvious that relatively dilute solutions of FMOC-Cl in ACN (e.g. 1 to 10 mg/mL) were not adequate to fully derivatise the analytes in the presence of the honey matrix. Nedelkoska and Low (2004) noted that excessive amounts of FMOC-Cl relative to the quantities of glyphosate present in the sample are required for complete derivatisation of the target analyte due to the reactivity of FMOC-Cl with matrix compounds containing primary and secondary amine functional groups. According to Ehling and Reddy (2015), concentrations of FMOC-Cl solutions used to derivatise glyphosate and AMPA have been previously reported to range from 1 to 28 mg/mL. Toss *et al.* (2017) used 0.14 mL of a 30 mg/mL solution of FMOC-Cl in acetonitrile to derivatise glyphosate and AMPA in surface water samples containing high levels of organic matter.

Honey is a complex matrix which may contain up to 1 % (w/w) of free amino acids and 0.2–1.6 % protein (Santos-Buelga and Gonzalez-Paramas 2017) which will potentially react with the FMOC-Cl. It was determined that increasing the FMOC-Cl concentration to 50 mg/mL in CAN and utilising 0.2 mL of this solution was necessary to provide the successful derivatisation of the analytes and their corresponding internal standards in the presence of the honey matrix.

Development of online SPE-LC-MS/MS method

The major sugars present in honey were poorly retained by the HLB extraction cartridge and could be flushed to waste without ever reaching the analytical LC column. The derivatised analytes were retained by the extraction cartridge and switching the position of the six-port valve allowed them to be subsequently eluted onto the analytical LC column for further chromatographic separation.

Figures 2 and **3** show the reconstructed MRM ion chromatograms obtained for the determination of a nominally blank honey (*i.e.* all analytes below the LOQ of 1 µg/kg) and the same honey fortified with 5 µg/kg each of glyphosate, AMPA, and glufosinate. During initial method development work it was discovered that it was virtually impossible to find a honey sample which was completely free of all three analytes. Each pair of ion chromatograms for the unspiked and spiked honey samples have been plotted on the same scale for each analyte. While there are additional peaks present in the chromatograms for both glyphosate and glufosinate in the blank honey, these peaks elute after the target analytes and therefore do not interfere in their analysis .

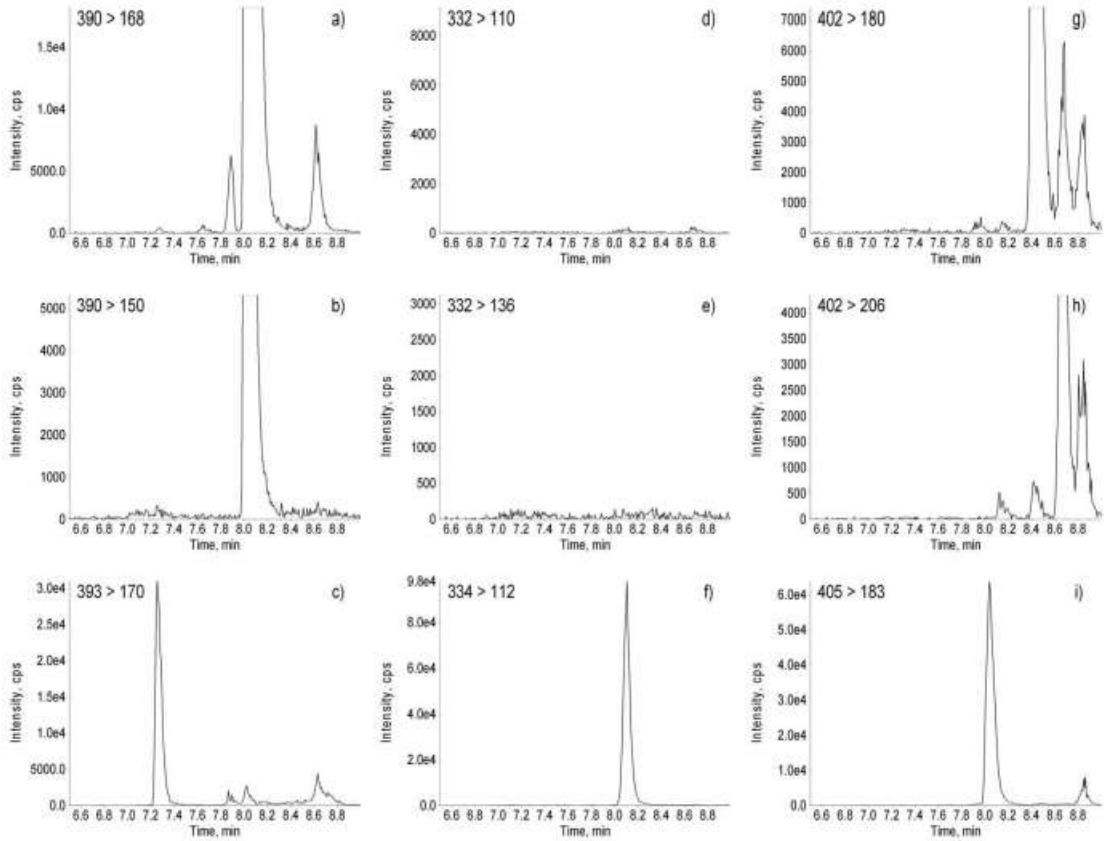


Figure 2: Reconstructed ion chromatograms for blank honey fortified with 25 µg/kg of each isotopically labelled internal standard. The quantitation and confirmatory MRM transitions (respectively) are: (a)+(b) glyphosate; (d)+(e) AMPA; and (g)+(h) glufosinate. The quantitation MRMs for the internal standards are: (c) 13C2,15N-glyphosate; (f) 13C,15N-AMPA; and (i) D3-glufosinate.

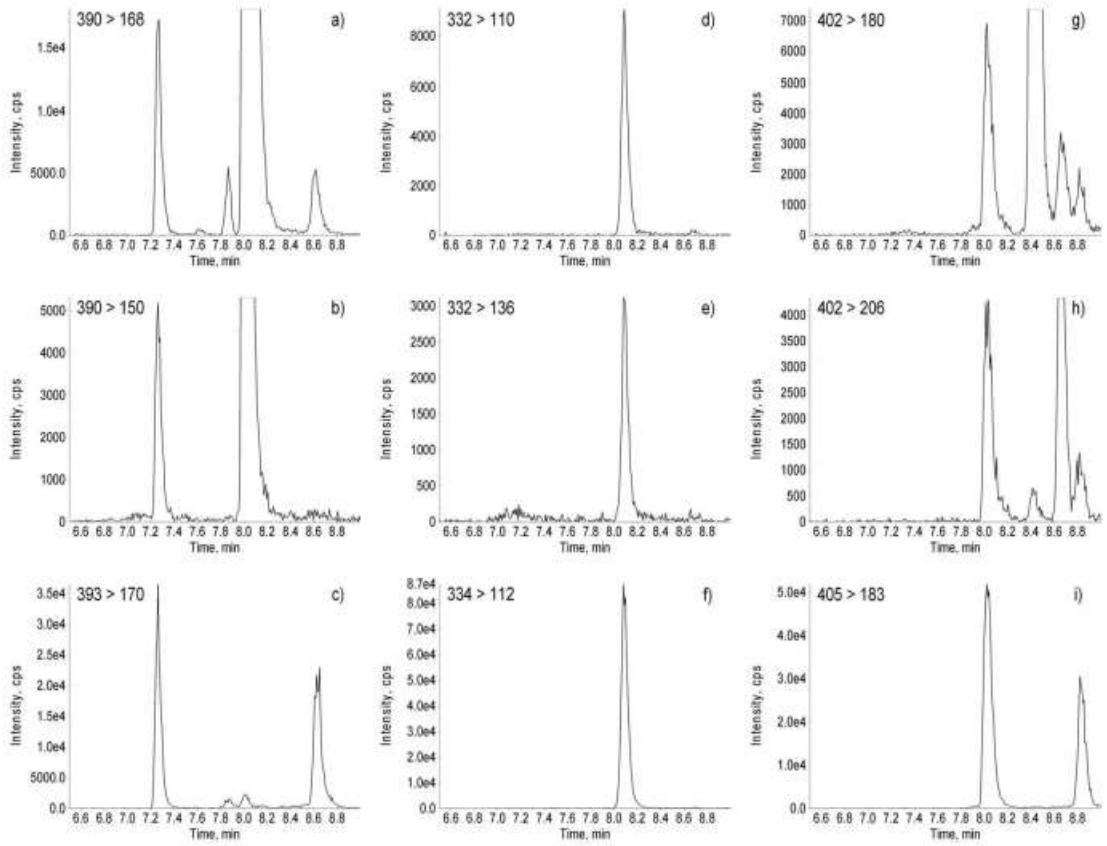


Figure 3: Reconstructed ion chromatograms for blank honey fortified with 5 µg/kg each of glyphosate, AMPA, and glufosinate as well as 25 µg/kg of each isotopically labelled internal standard. The quantitation and confirmatory MRM transitions (respectively) are: (a)+(b) glyphosate; (d)+(e) AMPA; and (g)+(h) glufosinate. The quantitation MRMs for the internal standards are: (c) 13C2,15N-glyphosate; (f) 13C,15N-AMPA; and (i) D3-glufosinate.

Criteria for confirmation of analyte identity

Two MRM transitions were monitored for each incurred analyte in order to permit confirmation of compound identity. A chromatographic peak must be present in both reconstructed ion traces within ± 0.05 min of the retention time of the associated isotopically labelled internal standard. The ratio of the peak areas for the quantitation and confirmation reconstructed MRM traces must be within ± 30 % relative to that obtained for authentic reference material analysed under the same set of operational parameters within the same analytical batch.

Evaluation of matrix effects

Matrix effects were evaluated by comparing calibration curves obtained for standards prepared in reagent water and honey solutions. Unfortunately it was extremely difficult to find a truly blank honey and it was decided that a set of calibration standards would be prepared using a nominally blank honey which did not contain any of the analytes above the LOQ of 1 µg/kg. The results of the calibration curves obtained for standards prepared in either water or honey are given in **Table 3**. The calibration curves were determined using two techniques: firstly by external standardisation and secondly by internal standardisation using each analyte's respective isotopically labelled analogue. The matrix effect (ME) was calculated based on the ratio of the slopes obtained for the calibration curves in matrix versus reagent water:

ME = 100 x (slope of calibration curve in honey) / (slope of calibration curve in reagent water)

where ME = 100 would indicate no matrix effect while ME < 100 or ME > 100 would indicate ionisation suppression

or enhancement, respectively. When the calibration is performed using external standardisation, there is minor ionisation enhancement (ME > 100) observed for glyphosate where ME = 109 %. The opposite ionisation effect (suppression) is observed for both AMPA and glufosinate which have ME values of 51 % and 54 %, respectively. However, when the calibration curves are established using internal standardisation by isotope dilution, the ME values are all within 100 ± 10 %. Based on these results it was concluded that the use of isotopically labelled internal standards for quantitation would adequately overcome the ionisation effects observed because of the honey matrix. Reagent-based calibration standards were subsequently used for all method validation experiments.

Table 3: Comparison of calibration standards prepared in reagent water and honey.

Compound	Standardisation	Equation of curve prepared in reagent water (r^2)	Equation of curve prepared in honey solution	Matrix effect (ME*)
glyphosate	external	$y = 6540.7x + 780.0$ ($r^2 = 0.99944$)	$y = 7134.5x + 13125.5$ ($r^2 = 0.99988$)	109%
AMPA	external	$y = 105014.4x - 121.9$ ($r^2 = 0.99972$)	$y = 5317.0x + 1117.5$ ($r^2 = 0.99912$)	51%
glufosinate	external	$y = 9029.6x + 386.2$ ($r^2 = 0.99976$)	$y = 4901.0x + 9180.4$ ($r^2 = 0.99954$)	54%
glyphosate	internal	$y = 0.07890x + 0.02175$ ($r^2 = 0.99826$)	$y = 0.08481x + 0.01717$ ($r^2 = 0.99924$)	107%
AMPA	internal	$y = 0.02129x + 0.00153$ ($r^2 = 0.99866$)	$y = 0.02059x + 0.00457$ ($r^2 = 0.99930$)	97%
glufosinate	internal	$y = 0.02574x + 0.00171$ ($r^2 = 0.99856$)	$y = 0.02480x + 0.03888$ ($r^2 = 0.99818$)	96%

*ME = 100 × (slope of curve in honey)/(slope of curve in water).

Method validation

The analytical method was validated by analysing a series of spiked replicate honey samples. A honey sample which had no analytes at a concentration above the LOQ of 1 µg/kg was found after a large number of honeys were screened using the proposed methodology. A set of spiked replicates fortified at three different concentrations were analysed in order to determine the accuracy and precision of the proposed method. The results of these analyses are summarised in **Table 4.** The inter-day reproducibility was also evaluated by carrying out the analysis of replicate samples over three separate days. The calculated accuracies obtained for the daily analysis of six spiked replicates at each of three concentration levels (5, 50, and 150 µg/kg) ranged from 95.2 % to 105.3 % for all three compounds. The daily precision (standard deviation) for all three analytes at all fortification levels ranged from 1.6 % to 7.2 %. The inter-day accuracy and precision for all three compounds at the three different levels studied over three separate days (a total of 18 replicates at each concentration level) were calculated to be between 97.7 % to 103.1 % and 2.1 % to 5.4 %, respectively. Based on these results, the method was deemed to be fit for purpose.

Table 4: Method validation data.

		Accuracy ± SD			
Compound	Fortification level (µg kg^{-1})	Day 1 (n = 6)	Day 2 (n = 6)	Day 3 (n = 6)	Inter-day (n = 18)
glyphosate	5	105.3 ± 5.4	102.9 ± 4.5	101.2 ± 6.3	103.1 ± 5.4
	50	100.4 ± 2.1	96.2 ± 4.4	104.5 ± 2.8	100.4 ± 4.6
	150	100.2 ± 4.3	96.1 ± 2.2	101.8 ± 3.1	99.3 ± 4.0
AMPA	5	98.9 ± 6.4	101.5 ± 4.4	96.0 ± 3.1	98.8 ± 5.1
	50	103.6 ± 4.4	103.0 ± 3.1	96.8 ± 3.5	101.1 ± 4.7
	150	100.1 ± 1.7	99.1 ± 3.5	96.6 ± 2.5	98.6 ± 2.9
glufosinate	5	101.8 ± 3.8	99.3 ± 3.4	97.3 ± 7.2	99.5 ± 5.1
	50	99.8 ± 3.4	99.2 ± 1.6	95.2 ± 2.5	98.0 ± 3.2
	150	97.7 ± 1.7	98.8 ± 2.4	96.6 ± 1.8	97.7 ± 2.1

The measurement uncertainty for each analyte was estimated using in-house method validation data according to the procedure described in the Codex guidelines on estimation of uncertainty of results (Codex Alimentarius Commission 2011). Method validation data obtained for the analysis of spiked replicates at the three different concentration levels covering a range from 5 to 150 µg/kg was used to calculate an expanded uncertainty (U′) with a coverage factor of 2 (95 % confidence interval) for each analyte. The expanded uncertainties were estimated as U′ = 14 % for glyphosate, 13 % for AMPA, and 11 % for glufosinate.

Application to honey samples

Two hundred randomly chosen honey samples, which were submitted to our laboratory for other testing, were analysed using the online SPE-LC-MS/MS method to obtain information regarding baseline levels of glyphosate, its main degradation product AMPA, and the other acidic herbicide, glufosinate. The results of these analyses are summarised in **Table 5**. Glyphosate was detected in almost all honey samples analysed with 197 out of 200 samples (98.5 %)

having residues equal to or above the LOQ of 1 µg/kg. The maximum concentration of glyphosate residue in the honey samples analysed was 49.8 µg/kg. AMPA was also frequently detected (198 or 99.0 % of 200 samples tested) up to a maximum concentration of 50.1 µg/kg. There were no samples where both glyphosate and AMPA were below the LOQ value.

Table 5: Concentrations of glyphosate, AMPA, and glufosinate in incurred honey samples.*

Compound	# of Detections	Median (µg kg^{-1})	90th Percentile (µg kg^{-1})	95th Percentile (µg kg^{-1})	Maximum (µg kg^{-1})
glyphosate	197	4.9	14.2	19.2	49.8
AMPA	198	10.3	20.8	28.7	50.1
glufosinate	125**	1.4	6.1	9.9	33.0

*n = 200 samples analysed.
**One sample did not meet the required ion ratio criterion for confirmation of compound identity and was not included in this value.

The third analyte, glufosinate, was detected much less frequently than either glyphosate or AMPA and also at lower levels in general. Glufosinate was found to be present in 125 of 200 samples analysed with the maximum concentration detected being 33.0 µg/kg. It must be noted that there was a single honey sample where the ratio of the two precursor > product ion MRM transitions for glufosinate was not within the acceptable relative ratio of ±30 % (average ion ratio for calibration standards = 59.3 % while the ion ratio for the sample was 7.0 %). Assuming that there was an interference in the quantitative MRM transition (thereby giving the unacceptably low relative ion ratio), if the confirmatory MRM transition was used for quantitation, the glufosinate concentration was estimated to be just above 1 µg/kg. All samples of honey containing either glyphosate or AMPA at concentrations above the LOQ of 1 µg/kg were successfully confirmed based on the criteria established for compound identification.

Interestingly, the ratio of glyphosate to AMPA was found to vary considerably in samples that contained both analytes. In some cases the two analytes were roughly equal in concentration while in others one of the pair was significantly higher than the other compound. This is illustrated by the scatter plot shown in **Figure 4** where the concentration of glyphosate is plotted versus the concentration of AMPA in the 200 honey samples which were analysed (note that only samples containing both glyphosate and AMPA at or above the LOQ were included in this plot). There are multiple factors which may influence the relative amounts of glyphosate and its degradation product AMPA. Differences in the chemical composition of the honeys tested as well as their age and handling/storage conditions prior to receipt by the laboratory may be important factors. The long-term stability of glyphosate and AMPA in honey has not been established. Other factors which may influence the relative ratios of the two compounds may include agricultural practices such as the timing of herbicide application relative to honey bee foraging, environmental decomposition of the targeted analytes, and differences in crops treated and subsequently pollinated by the bees. The contribution of glyphosate and AMPA residues present in the ambient environment to contamination of plant nectar and subsequently honey itself is further complicated by the variations in the levels of these compounds in environmental matrices such as soil and surface water. No conclusions can be drawn regarding any trend in the relative amounts of these glyphosate and AMPA in honey. The ratio of the concentration of glyphosate to that of AMPA present in samples containing both analytes at ≥1 µg/kg (195 samples) ranged from 0.05 to 9.16. It should also be noted that there were two samples containing glyphosate ≥1 µg/kg (7.7 and 8.8 µg/kg where the concentration of AMPA was below the LOQ. Conversely, there were three samples with AMPA concentrations ≥1 µg/kg (2.7, 9.0, and 10.6 µg/kg) where the glyphosate concentration was below 1 µg/kg. The concentration of glyphosate exceeded that of AMPA in 63 out of 200 honey samples tested.

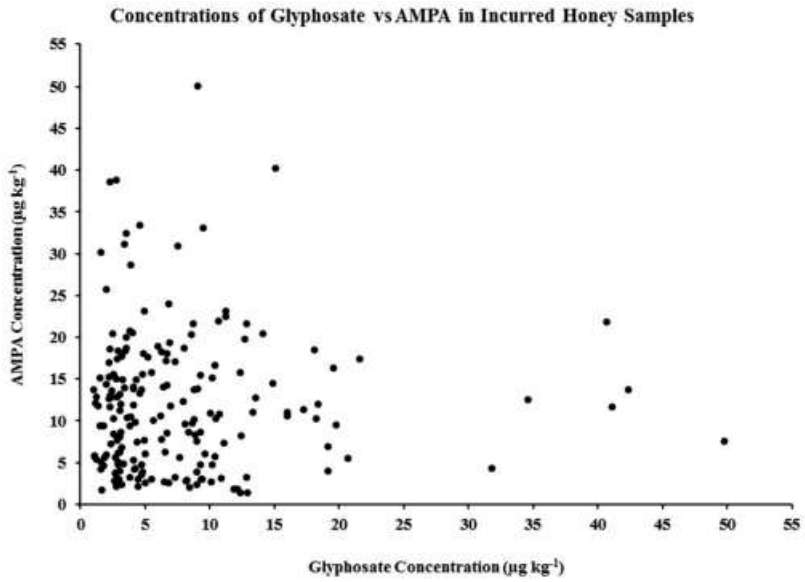


Figure 4: Scatter plot of glyphosate versus AMPA concentrations in samples containing both analytes at or above the limit of quantitation (LOQ = 1 µg/kg).

Comparison of residue levels in honey to other reported studies

Table 6 provides a comparison between the residues of glyphosate present in honey samples analysed in this study and those previously reported by other research groups. Bo *et al.* (2007) developed an analytical method for the determination of glyphosate and AMPA residues in a variety of foods including honey. Their reported LOQ was 50 µg/kg and while the method was employed for the analysis of several different food types it does not appear that it was actually applied to honey samples. In several subsequently reported studies, LOQ values were in the range of 10–50 µg/kg (Rubio *et al.* 2014; Chamkasem and Vargo 2017; Karise *et al.* 2017; Berg *et al.* 2018) which permitted frequent detection of glyphosate residues in honey. Zoller *et al.* (2018) and our work both achieved LOQ values of 1 µg/kg and also each had greater than 90 % of tested honey samples containing quantifiable residues of glyphosate. None of the honey samples in either our baseline study or in the survey of honey sold on the Swiss market (Zoller *et al.* 2018) had glyphosate residues above the EU MRL of 50 µg/kg. In a study of honey from numerous countries around the world (Rubio *et al.* 2014), 22 out of 69 samples tested contained glyphosate residues above the MRL of 50 µg/kg up to a maximum of 163 µg/kg. Glyphosate levels in honey samples mainly from the USA and a small number from other countries exceeded the MRL of 50 µg/kg in only 4 of 28 samples tested but with one sample containing 653 µg/kg (Chamkasem and Vargo 2017). Only 2 out of 33 honey samples from Estonia had glyphosate residues above the MRL of 50 µg/kg with a maximum of 62 µg/kg being detected (Karise *et al.* 2017). Berg *et al.* (2018) obtained 59 honey samples from Hawaiian beehives as well as 26 samples from commercially available products. A total of 8 of the 26 merchant samples had detectable residues, three of which were above the MRL of 50 µg/kg. A total of 16 of the 59 samples collected directly from beehives were determined to contain glyphosate residues above the LOQ of 15 µg/kg with 12 samples above the MRL of 50 µg/kg. The maximum concentrations of glyphosate detected in the merchant and hive samples were 87 and 342 µg/kg, respectively. John and Liu (2018) measured glyphosate residues in water, various food matrices, and human urine using an ELISA method. Only one honey was tested amongst the samples and was found to contain 22 µg/kg of glyphosate. In the 2016 EU report on pesticide residues in food (EFSA (European Food Safety Authority) 2018a), 18 of 220 honey samples were found to have detectable residues of glyphosate. The report does not include specific details regarding either the analytical methods used by the reporting laboratories or their LOQs for glyphosate in honey. Six honey samples contained glyphosate residues above the EU MRL of 50 µg/kg with levels ranging from 90 to 610 µg/kg.

Table 6: Glyphosate residues in honey from various studies.

	Country of study					
	USA (Rubio et al. 2014)	USA (Chamkasem and Vargo 2017)	Estonia (Karise et al. 2017)	Switzerland (Zoller et al. 2018)	USA (Berg et al. 2018)	Canada (this study)
Testing method	ELISA	LC-MS/MS	LC-MS/MS	LC-MS/MS	ELISA	LC-MS/MS
Source of honeys	Various countries of origin	Mainly from USA	Estonia	Not specified	Mainly from USA (Hawaii)	Mainly western Canada
# Samples tested	69	28	33	16	85	200
# Positives (%)	41 (59.4%)	17 (60.7%)	3 (9.1%)	15 (93.8%)	24 (28.2%)	197 (98.5%)
LOQ (μg kg^{-1})	15	10 to 16	50 (LOD = 10)	1	15	1
Maximum (μg kg^{-1})	163	653	62	15.9	342	49.8

Neither AMPA nor glufosinate were detected, with LOQs of 16 and 18 μg/kg respectively, in 19 honey samples analysed by direct determination of the underivatised analytes using LC-MS/MS (Chamkasem and Vargo 2017). None of the 16 honey samples analysed by Zoller *et al.* (2018) contained AMPA residues above the LOQ of 2.5 μg/kg. Considering the low levels of glyphosate found in these samples (median concentration of 3.0 μg/kg), it is entirely plausible that AMPA could be undetected since its LOQ was 2.5 times higher than for glyphosate.

It should be noted that the LC-MS/MS methods employed by Chamkasem and Vargo (2017) as well as by Karise *et al.* (2017) both involved the determination of glyphosate residues without derivatisation or subsequent extract clean-up. The combination of FMOC-Cl derivatisation and online SPE coupled directly to LC-MS/MS as performed in our method made it possible to achieve LOQ values which were at least one order of magnitude lower by comparison. The analytical method used by Zoller *et al.* (2018) did not employ a derivatisation step but did carry out an offline SPE clean-up step followed by extract dilution prior to LC-MS/MS analysis. Their LOQ values for glyphosate and AMPA were equal to and just slightly higher, respectively, than those obtained with our procedure.

Considerations for future studies

It should be noted that the current EU MRL for glyphosate in honey only includes the parent compound as the marker residue (EU 2016). A recent review by the European Food Safety Authority (EFSA) indicates that there is a proposal to include other related analytes in the residue definition for glyphosate in different foods (EFSA 2018b). While there is no specific mention of honey, it has been proposed that the residue definition for numerous other commodities be expanded to include the sum of glyphosate, AMPA, and the metabolite N-acetyl-glyphosate for enforcement purposes. It has also been recommended that residue analysis for risk assessment include glyphosate, AMPA, N-acetyl-glyphosate, and N-acetyl-AMPA. While several studies to date, including the work described herein, have reported residues of glyphosate and AMPA in honey, there is a need for the N-acetylated metabolites of these compounds to be considered for addition in future studies. The current EU MRL for glufosinate in honey includes the sum of the parent compound plus its metabolites 3-[hydroxyl(methyl)phosphinoyl]propionic acid (MPP) and N-acetyl-glufosinate (NAG) (European Union: Pesticides database 2016). While glufosinate was not detected in honey according to a single previously reported study (Chamkasem and Vargo 2017), its presence in honey samples analysed in our survey suggests the need to investigate MPP and NAG residues in future work.

Conclusion

A relatively simple method was developed for the determination of glyphosate, AMPA, and glufosinate residues in honey with an LOQ of 1 μg/kg for each analyte. A key component of the method was the utilisation of isotopically labelled internal standards to overcome matrix effects associated with the samples. Following a simple derivatisation step, it was possible to use online solid-phase extraction for the isolation of the derivatised analytes from the bulk of the honey matrix with subsequent direct determination of the residues by LC-MS/MS. A survey of honey samples from western Canada indicated the widespread contamination of these samples by glyphosate, AMPA, and glufosinate, albeit at low concentrations. While Health Canada has not currently established an MRL for either glyphosate or glufosinate in honey, in consideration of the EU MRLs of 50 μg/kg for each compound the risk to consumer health appears to be quite low based on the residues detected.

Chromatographic conditions

Chromatograph:	Shimadzu 30 LC System (SIL30AC autosampler, two LC30AD solvent delivery pumps, CBM20A module controller)
Column:	Agilent Zorbax Extend-C18 (50 mm x 2.1 mm, 1.8 μm)
Column oven temperature:	Not provided

Injection volume:	50 µL
Mobile phases:	(A) 10 mM ammonium carbonate in water (B) Acetonitrile

Gradient (linear transitions):

Time (Min)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)	Valve position
0.0	100	0	1.00	to waste
2.9	100	0	1.00	to waste
3.0	100	0	0.35	to waste
5.5	73.6	26.4	0.35	to column
12.0	5	95	0.35	to column
12.5	5	95	0.35	to column
13.0	5	95	0.60	to column
15.5	5	95	0.60	to column
16.0	5	95	0.35	to column
17.0	98	2	0.35	to column
19.0	98	2	0.35	to waste

Retention time:	Glyphosate: ~ 8.1 min 13C2,15N-glyphosate (IS): ~ 8.1 min AMPA: ~ 8.1 min 13C,15N-AMPA (IS): ~ 8.1 min Glufosinate: ~ 8.1 min D3-glufosinate (IS): ~ 8.1 min
Detector:	Sciex 4500 quadrupole tandem mass spectrometer
Scan type:	MRM
Ion source:	ESI negative

Source gas:	70 units	Source temperature:	700°C
CAD gas:	8 units	Source voltage:	−3500 V
Curtain gas:	20 units		

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Declustering potential (V)	Collision energy (eV)	Scan time (ms)
Primary transition (quantification)					
Glyphosate	390	168	−40	−16	50
Glyphosate (IS)	393	170	−40	−16	50
AMPA	332	110	−40	−16	50
AMPA (IS)	334	112	−40	−16	50
Glufosinate	402	180	−45	−14	50
Glufosinate (IS)	405	183	−45	−14	50
Secondary transition (confirmation)					
Glyphosate	390	150	−40	−34	50
AMPA	332	136	−40	−20	50

Glufosinate	402	206	−45	−20	50

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article describes the development and validation of a method for the analysis of glyphosate, AMPA, and glufosinate in honey. Aqueous honey solutions were derivatised offline prior to direct analysis of the target analytes using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Method validation fulfil EU requirements. The method showed good performance for all analytes with a LOQ of 1 µg/kg for each analyte.

The method can be considered valid for monitoring purposes and has been applied for the analysis of two hundred randomly chosen honey samples from Canada. Virtually all the samples were found to contain measurable residues of glyphosate and/or AMPA, which is at least in part due to the extremely LOQ (1 µg/kg). The ratio between parent glyphosate and AMPA was very variable, which is also in contrast to the findings of the EU monitoring (where no measurable residues of AMPA were found) but may also be accounted for by the very low LOQ. In spite of the large number of samples analysed, none showed residues of parent glyphosate exceeding the current EU MRL of 0.05 mg/kg.

According to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. As honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL. Therefore, the publication is considered relevant and reliable. It also includes a useful discussion of the residue levels of glyphosate in honey reported by other authors.

Assessment and conclusion by RMS:
The publication describes the development and validation of an analytical method for the determination of glyphosate, AMPA, and glufosinate in honey. The analytical method was subsequently used to analyse 200 randomly chosen honey samples. Residue levels of glyphosate, AMPA, and glyfosinate accounted for up to 0.0498 mg/kg, 0.0501 mg/kg, and 0.0330 mg/kg, respectively. The existing MRL for glyphosate in honey is therefore not exceeded based on this Canadian data set. The publication also includes monitoring data from other publications, but it is obvious that results between the different publications largely vary, not only in the residue levels determined in honey samples, but also in the percentage of positive findings.
Residue levels of glyphosate, AMPA, and glyfosinate accounted for up to 0.0498 mg/kg, 0.0501 mg/kg, and 0.0330 mg/kg, respectively. The existing MRL for glyphosate in honey is therefore not exceeded based on this Canadian data set. The publication also includes monitoring data from other publications, but it is obvious that results between the different publications largely vary, not only in the residue levels determined in honey samples, but also in the percentage of positive findings.

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered relevant for the MRL calculation for two reasons: (i) the data were not obtained by official monitoring laboratories; and (ii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

B.7.7.1.3.3. Reference 3

1. Information on the study

Data point	CA 6.10.1/005
Report author	Chiesa, L.M. *et al.*
Report year	2019
Report title	Detection of glyphosate and its metabolites in food of animal origin based on ion-chromatography-high resolution mass spectrometry (IC-HRMS)
Document No.	DOI 10.1080/19440049.2019.1583380

	E-ISSN 1944-0057
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

Glyphosate and glufosinate are broad spectrum herbicides, widely used in agriculture and in inhabited or industrialised areas, and aminomethylphosphonic acid is a degradation product of glyphosate. In 2015, the International Agency for Research on Cancer reported that glyphosate is a probable carcinogenic. In 2017, however, a scientific opinion of the European Chemicals Agency concluded that glyphosate is not proven to be carcinogenic, mutagenic or to have negative effects on reproduction. Nevertheless, aminomethylphosphonic acid was not considered. Due to their chemical-physical characteristics, these molecules present difficulties that have not yet allowed routine monitoring to be carried out. For these reasons, we developed and validated a simple and versatile liquid extraction, before IC-HRMS analysis, of three different complex matrices: honey, bass fish and bovine muscle. Among the satisfactory validation parameters, the LOQs in the range of 4.30 – 9.26 ng/g demonstrated high method sensitivity, compared to the few works present in literature. Finally, the method was applied to real commercial samples, which showed no traces of the selected pesticides.

Materials and Methods

Chemicals and reagents

Glyphosate, glufosinate ammonium, aminomethyl-phosphonic acid (AMPA) and the internal standard Glyphosate-2-^{13}C,^{15}N were purchased from Merck (Sigma–Aldrich, Merck KGaA, Darmstadt, Germany). All solvents used were of LC-MS or analytical grade. Formic acid (98–100 %) was obtained from Riedel-de Haën (Sigma–Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions

Stock standard solutions (1 mg/mL) for each standard were prepared in water and kept at −20°C. Working solutions containing each of the studied analytes at a concentration of 100 ng/mL were prepared daily in methanol containing 1 % of formic acid, as suggested by EU Reference Laboratory for pesticides (Anastassiades *et al.* 2016). Each working solution was maintained at 4°C during the method validation procedures. Plastic flasks and stoppers were used due to the fact that these pesticides tend to interact with glass surfaces.

Sample collection

Three different food matrices of animal origin were selected for the method validation: honey, fish (bass) and bovine muscle. Five commercial samples of each matrix were homogenised to create a pool to be used for the validation. After homogenisation, the samples were stored at −20° C until analyses.

Ten real Italian commercial samples each of organic honey, bass and bovine muscle were also collected from different supermarkets of Milan for the application of the method.

Sample extraction

The extraction procedure was very simple and identical for the three different matrices. Homogenised samples (1 ± 0.05 g) of honey, or minced fish or bovine muscle were weighed into 15 mL polypropylene centrifuge tubes. Samples were spiked with the internal standard: 0.2 µg/g for honey and 0.4 µg/g for fish and bovine muscle samples. Three mL of methanol was added followed by 7 mL of acidified deionised water (1 % formic acid). The samples were mixed for 1 min using a vortex and then sonicated for 15 min. After centrifugation (2500 × g, 4°C for 10 min), 1 mL of the supernatant was filtered through a mixed cellulose syringe filter (0.45 µm) directly into a plastic 2 mL vial, ready for determination by IC-MS/MS.

IC-HRMS orbitrap analyses

The analyses were performed by an Ionic Chromatography (IC) Dionex ICS-5000+ system (Sunnyvale, CA, USA) made up of Dual Pump (DP), a Conductivity Detector (EG), a Detector/Chromatography Module (DC) and an Autosampler (AS-AP). The ion chromatography separation column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm, 4 µm particle size) maintained at 30°C. The eluent flow rate was 0.30 mL/min with a gradient from 15 mM KOH (aq), held for 8 min, increased to 55 mM KOH (aq) at 20 min, held in these conditions for 4 min and back to 15 mM KOH (aq) at 24.1 min, with a cycle time of 30 min. The KOH eluent was neutralised using a Dionex AERS 500, 2 mm electrolytically regenerated suppressor (Thermo Scientific). The injection volume was 50 µL.

The detector was a Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionisation (HESI) source. Capillary temperature and vaporizer temperature were set at 330°C and 280°C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas were set at 35 and 15 arbitrary units, with S lens RF level of 60.

Instrument calibration was done every analytical session with a direct infusion of an LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan acquisition (FS) was combined with an Independent Data Acquisition mode (DIA), providing the MS2 spectra for confirmatory response, based on an inclusion list. The resolving power of FS was set at 70,000 Full Width at Half Maximum (FWHM). On the basis of our compound list, a scan range of m/z 50–250 was chosen; the automatic gain control (AGC) was set at 1×10^{-6} and the maximum injection time was 100 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5×10^{-4}, with an auto maximum injection time. The precursor ions are filtered by the quadrupole which operates at an isolation window of 1 m/z. Fragmentation of precursors was optimised as three- stepped normalised collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was based on the retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment. The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm glyphosate and its metabolites are reported in **Table 1**. Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HRMS system, determine the exact mass of the compounds, record and elaborate data.

Table 1: Main information (formula, retention time (tr), precursors, main products and polarity) about AMPA, glyphosate, glufosinate and the relative internal standard (IS) analysed by IC-HRMS.

Compound IC-HRMS	Formula	t_r (min)	Precursor (m/z)	Main products (m/z)			Polarity
AMPA	CH6NO3P	14.25	110.00125	62.96417	78.95904	80.97468	(-)
Glyphosate	C3H8NO5P	23.87	168.00673	62.96417	124.01687	149.99612	(-)
Glufosinate	C5H12NO4P	14.14	180.04312	85.02955	94.99042	136.05329	(-)
IS: Glyphosate-2-13C, 15N	[13]C2C[15]NH8O5P	23.85	171.01048	62.96415	80.97471	152.99988	(-)

Validation parameters

Validation was carried out following the European Commission (2017) SANTE/2017 Guidance document on method validation & quality control procedures for pesticide residues analysis in food & feed. The selectivity of the method was evaluated by injecting extracted blank honey, fish and bovine muscle samples. The absence of signal above a signal-to-noise ratio of 3 at the expected retention times of the target compounds was the parameter used to show the absence of interferences.

The matrix-matched calibration curves were obtained by spiking 1 g of the three different matrices with an appropriate volume of the standard working solution to cover the concentration range from 5 to 100 ng/g (five calibration points: 5, 10, 20, 50 and 100 ng/g). The limit of quantification (LOQ) of the methods was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤ 20 % (European Commission. 2017. SANTE/11813/2017). The repeatability, evaluated as a coefficient of variation, CV %, was calculated by analysing six replicates at two fortification level (10 and 50 ng/g). Recoveries were calculated by comparing the concentrations of the extracted compounds, spiked before extraction, with those spiked at the end of the extraction procedure at two fortification level (10 and 50 ng/g) for all compounds. The matrix effect was also evaluated using the Matuszewski *et al.* (2003) approach by comparing the corresponding peak areas for standards, spiked after extraction into the extracts, to the peak areas obtained in neat solution standards, expressed as percentage.

Results and Discussion

Extraction procedure

During the preliminary phase, the QuPPe extraction method proposed by EU Reference Laboratories for Residues of pesticides (Anastassiades *et al.*, 2016) was followed, with good results for glyphosate and AMPA but found not suitable for glufosinate in our matrices after the IC-HRMS analysis. In particular, we observed a different and opposite extraction and chromatographic behaviour of the molecules (in particular for AMPA and glufosinate) on the basis of the different solvents used and injected during the ion chromatography separation. Moreover, the final dilution 1/10 suggested by the Anastassiades *et al.* (2016) or by others (Adams *et al.*, 2017) did not improve chromatographic problems when AMPA or glufosinate was hardly detectable.

So we decided to modify the method starting from a smaller amount of matrix (1 g instead of 5 g) to decrease interferences, investigating also the influences of the different tested extraction solvents compatible with our instrumentation: water, methanol and the related acidified solutions with 1 % of formic acid. In particular, using only water (**Figure 1a**) or only methanol (**Figure 1b**) as extraction solvent we had poor results for AMPA, but very satisfactory chromatographic peaks for glyphosate and glufosinate. By using 1 % of formic acid in water (**Figure 1c**) we had an improvement of AMPA signal, but it was not yet satisfactory, while with 1 % of formic acid in methanol (**Figure 1d**) we observed the reverse situation, good chromatography for AMPA but not for glufosinate, which eluted with a too-jagged and wide peak. So after different trials, changing the percentage of formic acid and the composition of the methanol and acidified water mixture we reached the best compromise with 30 % of methanol and 70 % of acidified (1 % formic acid) water as extraction solution. **Figure 2** reports the extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes after method optimisation.

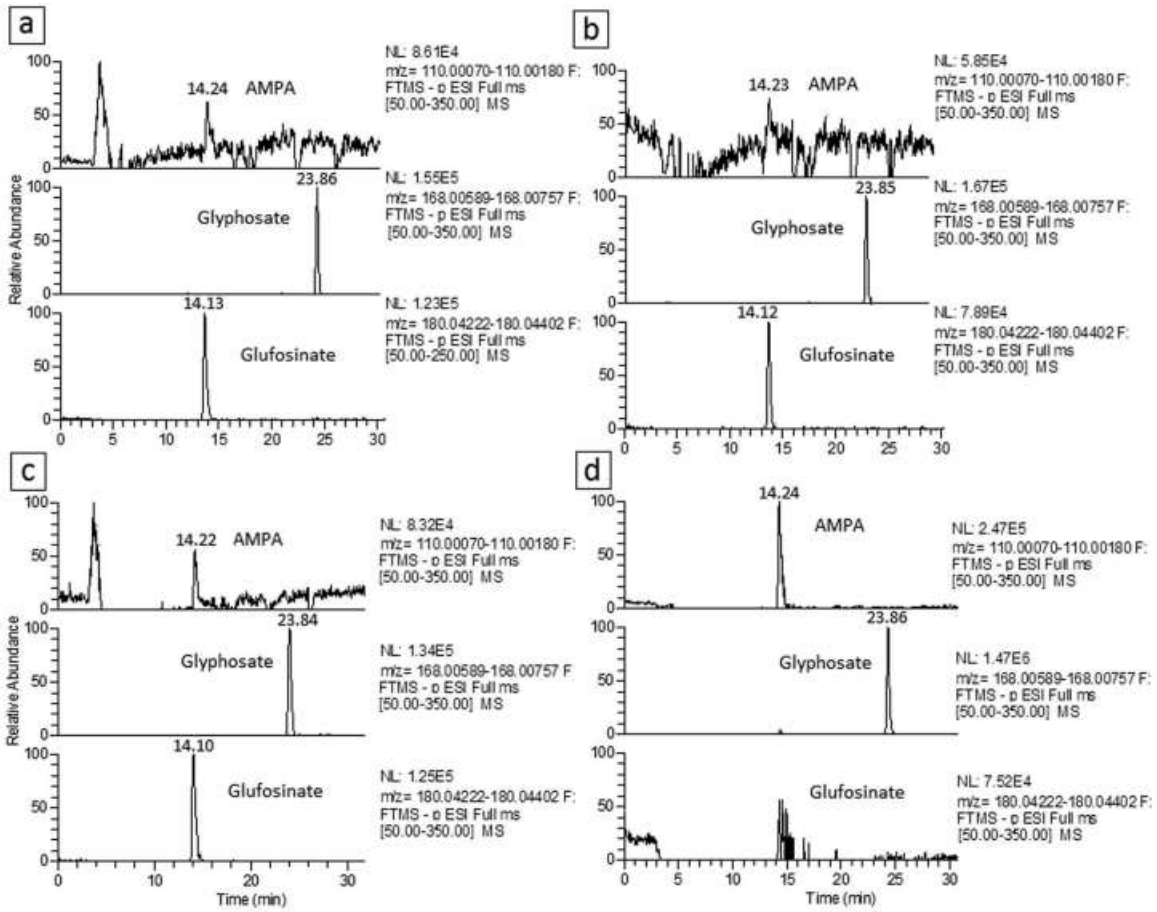


Figure 1: Extracted parent ion chromatograms from full-scan IC-HRMS analysis of AMPA, glyphosate and glufosinate and influences of the different tested extraction solvents: water (a), methanol (b), 1 % formic acid in water (c) and 1 % formic acid in methanol (d).

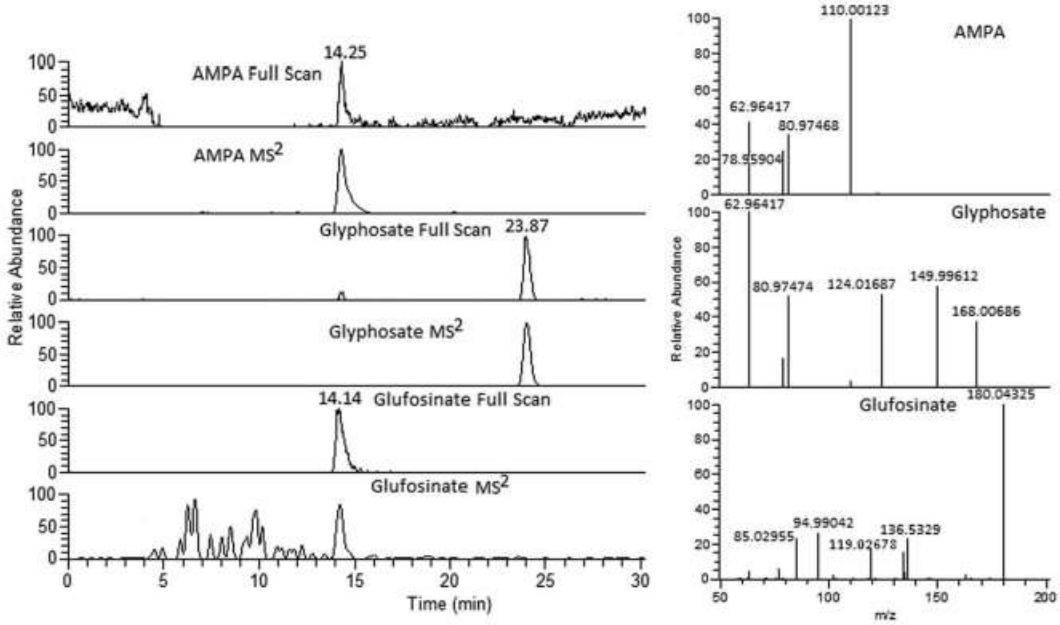


Figure 2: Extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes (concentration of 10 ng/g) after method optimisation.

IC-HRMS validation parameters

All instrument validation parameters are reported in **Table 2**. The method applied to the three different matrices (honey, bass fish, bovine muscle) showed high specificity, without any interference close to the retention time where the investigated compounds were expected to elute. The good selectivity of the method was demonstrated with a S/N ratio higher than 3 in presence of analytes at the lowest detectable concentrations. All identification criteria passed including retention time stability compared to the standard solution. The mean recoveries ranged between 75 and 112 %, indicating the efficiency of the extraction protocol. Matrix validation curves demonstrated a good linearity over the working range with a good fit ($R^2 > 0.99$) for all compounds. Repeatability was calculated using one-way analysis of variance (ANOVA); the CVs were substantially lower than 20 %, satisfying the criteria required by the European Commission (2017).

Table 2: Validation parameters about glyphosate, glufosinate and AMPA in the three different matrices analysed by IC-HRMS.

	LOQ (ng g^{-1})	Matrix effects %	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Matrix calibration curve	Linearity R^2
HONEY						
AMPA	9.26	84	11, 4	91, 95	y = 0.15747x-0.514838	0.9996
Glyphosate	4.30	94	7, 7	99, 100	y = 0.295172x+0.858303	0.9975
Glufosinate	5.05	84	13, 12	100,101	y = 0.442863x+0.00858678	0.9957
FISH MUSCLE						
AMPA	5.38	95	4, 2	96, 95	y = 0.0897754x-0.231747	0.9951
Glyphosate	5.08	93	8, 8	80, 93	y = 0.072573x+0.112081	0.9985
Glufosinate	4.36	96	7, 6	112, 96	y = 0.127407x-0.176602	0.9976
BOVINE MUSCLE						
AMPA	6.44	99	12, 9	75, 79	y = 0.091067x-0.113588	0.9925
Glyphosate	6.47	107	13, 10	75, 80	y = 0.063115x+0.0920263	0.9922
Glufosinate	6.25	106	5, 2	76, 80	y = 0.120407x-0.107902	0.9962

*The two concentration levels were 10 and 50 ng g^{-1}.

Regarding the LOQs in the range from 4.30 to 9.26 ng/g, our satisfactory results showed high method sensitivity for glyphosate and its metabolites, when compared to the few reports present in the literature. In fact, Picò *et al.* (2007)

reported LOQ of 0.05 mg/kg for glyphosate and AMPA in plant products, such as rice, wheat, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey, etc. using High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; Krüger *et al.* (2014) reported validation parameters spiking at 100 µg/g of glyphosate in animal and human residues through ELISA followed by GC-MS/MS analysis. The overview of approximate LOQs reported by Anastassiades *et al.* (2016) in the range of 0.01–0.02 mg/kg obtained by the QuPPE extraction followed by LC-MS/MS analysis or those of Chamkasem *et al.* (2016) in the range of 4–26 ng/g using LC-MS/MS system are a little higher than our results. In **Table 3** we report all the LOQs and other information on the different studies presented in the literature about glyphosate, AMPA and glufosinate in food of animal origin. Matrix effects were modest in the three different matrices with a percentage variation lower than the 20 % (from 84 % to 107 %) recommended by the European Commission (2017).

Based on our results the use of high-resolution mass spectrometry and hyphenation with ion chromatography has been demonstrated to be very effective for the analysis of these challenging analytes in very complex matrices of animal origin. Particularly, as stated by Rajski *et al.* (2018) in their analogous study on anionic pesticides in fruits and vegetables, the high ion-exchange capacity, the efficiency, the diameter reductions and the characteristic chemistry of bonded functional groups of IC columns are a major factor for the separation and identification of the highly polar pesticides, scarcely retained in reversed-phase LC, avoiding moreover any derivatisation step. The high-resolution mass spectrometry allowed obtaining low background matrix signals, improving the sensitivity in terms of LODs and efficient trapping and stability of low m/z ions, improving selectivity. The high MS resolving power and mass accuracy down to 1 ppm, combined with the rapid scan speed, also provide high specificity (Chiesa *et al.* 2018). The possibility to do retrospective analyses is an added value.

Table 3: Literature about glyphosate in products of animal origin.

Authors	Matrix	Analyte	LOQ	Analytical Instrumentation
Wang, Jaw, and Chen	Fish	Glyphosate	/	Beckman LS 1000C liquid scintillation counter.
Alferness and Iwata (1994)	Muscle, kidney, liver and fat beef, eggs and milk	Glyphosate, AMPA	0.01 µg g^{-1}	Capillary Gas Chromatography with Mass-Selective Detection
Picò et al. (2007)	Plant products, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey	Glyphosate, AMPA	0.05 µg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Bo et al. (2007)	Vegetables, fruits, cereals, pig and chicken muscles, aquatic products, honey	Glyphosate, AMPA	0.05 µg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Krüger et al. (2014)	Liver, kidney, lung, spleen, muscle and intestine of cow	Glyphosate	100 µg g^{-1}	ELISA- Gas Chromatography-Mass Spectroscopy
Chamkasem et al. (2016)	Milk	Glyphosate, Glufosinate, and AMPA	100 µg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Anastassiades et al. (2016)	Foods of plant origin, cereals and honey	Glyphosate, Glufosinate, AMPA and others	4–26 ng g^{-1}	Liquid chromatography/ tandem mass spectrometry
Liao et al. (2018)	Milk-based baby foods and other milk products, infant formulae, yogurt	Glyphosate, Glufosinate	/	Liquid chromatography/ tandem mass spectrometry
Chamkasem et al. (2018)	Honey	Glyphosate, Glufosinate, and AMPA	0.25 µg mL^{-1}	Liquid chromatography/ tandem mass spectrometer

Application to real commercial samples

Finally, we applied the proposed method for the analysis of 30 real samples: 10 organic honeys, 10 beef muscle pools and 10 sea bass muscle pools, each thoroughly homogenised. All the samples were of Italian origin, taken from different supermarkets of Milan. None of the selected samples showed any traces of glyphosate or metabolites, ensuring the good quality of the samples, especially when it comes to organic products such as honey, demonstrating the absence of pesticide contamination both of the sample and of the production area.

Conclusions

In this study, we developed and validated a new and versatile IC-HRMS method for the detection of glyphosate, AMPA and glufosinate in three complex different matrices, honey, bass fish and bovine muscle. These results are of great importance and topical in the field of food safety because of the scarce data regarding this topic, the extractive and analytical difficulties related to these analytes in relation to complex matrices, and the legislative situation not yet outlined on the use of glyphosate and residues in consumer products. The application of the method to real commercial samples did not show any traces of the pesticides. Further studies of the method's application and statistical evaluation are necessary to form a more complete view on this matter.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The purpose of the publication is to describe and discuss the performance of a residue analytical method for glyphosate, AMPA and glufosinate in, food of animal origin. As such, the publication is not relevant to risk assessment. However, since it also reports residue levels for the investigated compounds in 10 honey samples and since according to SANTE/11956/2016 rev. 9 it is possible to derive EU MRLs in honey based on monitoring data, the publication may be considered relevant to risk assessment and MRL setting. Based on the provided validation results, the method is considered reliable. The LOQ (defined as the lowest fortification level yielding acceptable recoveries) was 0.010 mg/kg for both glyphosate and AMPA (although different values, presumably estimated from the signal to noise ratio, are stated in Table 2). None of the 10 analysed honey samples showed residues of glyphosate or AMPA above the LOQ. However, it is important to note that all the samples were from organic production and this may need to be taken into account in the evaluation.

Assessment and conclusion by RMS:
The publication describes the development of an analytical method to determine residues of glyphosate, AMPA, and glyfosinate in three matrices, amongst others in honey. The analytical method was tested on commercial samples organic honey purchased in Italy. No residue levels of glyphosate, AMPA, and glyfosinate were determined in any of the samples above the LOQ of 0.01 mg/kg. The existing MRL for glyphosate in honey is therefore not exceeded based on this Italian data set.

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered relevant for the MRL calculation for three reasons: (i) the data were not obtained by official monitoring laboratories; (ii) all samples were from organic production and their suitability may therefore be questioned; and (iii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

B.7.7.1.3.4. Reference 4

1. Information on the study

Data point	CA 6.10.1/006
Report author	Berg, C.J. *et al.*
Report year	2018
Report title	Glyphosate residue concentrations in honey attributed through geospatial analysis to proximity of large-scale agriculture and transfer off-site by bees
Document No.	DOI 10.1371/journal.pone.0198876 E-ISSN 1932-6203
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Executive Summary

Honey taken directly from 59 bee hives on the Hawaiian island of Kauai was analyzed for glyphosate residue using ELISA techniques. Glyphosate residue was detected (> LOQ) in 27 % of honey samples, at concentrations up to 342 ppb, with a mean = 118 ppb, S.E.M. 24 ppb. Of 15 honey samples store-purchased on Kauai, glyphosate was detected in 33 %, with a mean concentration of 41 ppb, S.E.M. 14. Glyphosate residue was not detected in two samples from the island of Molokai but was in one of four samples from the island of Hawai‘i. Presence and concentration of glyphosate residues were geospatially mapped with respect to Hawaiian land divisions. Mapping showed higher occurrence of glyphosate that was over LOQ (48 %) and concentrations of glyphosate (mean = 125 ppb, S.E.M. 25 ppb; N = 15) in honey from the western, predominantly agricultural, half of Kauai versus the eastern half (4 %, mean

= 15 ppb; N = 1). Geographic Information System analysis of land use percentage was performed within a circular zone of 1 km radius around each hive. Various land use types within each circular zone were transcribed into polygons and percent land use calculated. Only agriculture land use showed a strong positive correlation with glyphosate concentration. High glyphosate concentrations were also detected when extensive golf courses and/or highways were nearby. This suggests herbicide migration from the site of use into other areas by bees. Best management practices in use for curtailing pesticide migration are not effective and must be carefully re-assessed.

Materials and Methods

Sample collection

Honey samples were collected directly from hives by beekeepers on the island of Kauai in three batches from 2013 through 2016 (**Table 1**). Samples were opportunistically obtained from all accessible parts of the island. Collections were constrained by lack of bee hives in the area or beekeepers' unwillingness to provide samples. A strict confidentiality agreement was needed to get participation in the study. For some sites, sample batches were collected over time, to increase sample size. The timing was irrespective of seasonality of honey production by the bees. Each sample came from a single unique hive and its location was precisely recorded. Two other batches of honey were obtained from merchants and comprised honey from many hives under control of the manufacturing company.

In the fall of 2013 (Batch 1) two honey samples were collected by beekeepers, by scraping the honey comb with the open mouth of a clean glass mason jars and sealing the jars. These samples were stored at room temperature in a closed box, in a cabinet, until shipment to Microbe Inotech Laboratories, Inc., St. Louis, MO, for analysis of glyphosate concentration.

During the spring of 2015 (Batch 2) 36 samples of honey were collected directly from their unique hives by beekeepers of Kauai, using only the certified pre-cleaned 40 ml amber borosilicate glass vials provided to collect and store the honey. Vials were immediately sealed under a signed and dated custody seal by the collector and delivered directly to one of the authors (CJB, RK), along with a signed confidentiality statement containing contact information, date of collection, and hive location. Samples were stored at room temperature in a closed box, in a cabinet until shipment for analysis.

In fall of 2016 (Batch #3) 21 samples were collected by beekeepers and delivered to one of the authors (CJB), under the same procedures and stored for shipment as Batch #2.

In the winter of 2016 (Batch #4) 23 samples of honey were purchased from local famers' markets, produce stands, and stores. Honey was decanted into glass vials, sealed, and stored as above. Commercially produced honey is a composite from many hives. Source location was broadly determined from the label or from discussion with merchants. Date of honey collection is unknown. Samples were sent to Abraxis Inc, laboratory for analysis.

Batch #5 comprises three honey samples. Two samples were from the island of Molokai. One was purchased at a store on Molokai and the other was obtained from the beekeeper's bottled supplies. Both samples were a composite from hives at each beekeepers' farm. The farms' hives, which were located on Google Earth Pro™, were widely separated and thus represented different bee foraging sites. The third sample was purchased at a Kauai store and the source locality identified as from the island of Hawaii by its label.

Table 1: Honey collection data and laboratory where glyphosate was analyzed by ELISA.

Batch Number	Sample ID	Date Collected	ELISA analysis location
Hive Samples			
Batch #1	37, 38	Fall 2013	Micro Inotech Lab.
Batch #2	1 to 36	Summer 2015	Surfrider Lab.
Batch #3	39 to 59	Fall 2016	Surfrider Lab.
Merchant Samples			
Batch #4	91 to 23	Winter 2016	Abraxis Lab.
Batch #5	60, 61, 62	Winter 2016	Surfrider Lab.

Sample analysis

ELISA analysis was performed at each laboratory using the Abraxis method [1]. Abraxis test kit (cat. #500086) and microtiter equipment were used. The sample preparation method for honey followed published procedures [1, 17] (S1 Appendix). Samples were processed and read with a microplate reader Model 4303 [18] from Abraxis Inc. and analyzed using Molecular Devices Soft max pro evaluation program (4-Parameter). Results from Surfrider laboratory

analysis were certified correct by Abraxis staff. Limit of quantitation (LOQ) was 15 ng/mL (15 ppb; 0.15 mg/kg). Samples are stated as having detectable levels of glyphosate only if they are > LOQ.

Abraxis' ELISA methods for analysis of glyphosate have been compared to standard liquid chromatography and tandem mass spectrometry methods but not for honey. Therefore, 14 samples from Batch #2 were analyzed by both methods for validation. The results closely correlated with $R^2 = 0.99$ (S2 Appendix). Only ELISA derived data were used in this study.

Geospatial analysis

Presence and concentration of glyphosate residues were geospatially mapped with respect to general geography of the island and land use. Ancient Hawaiian biogeographical and management land divisions (Moku) (**Figure 1**) [19] were identified using the Google Earth Pro™ (GEP).

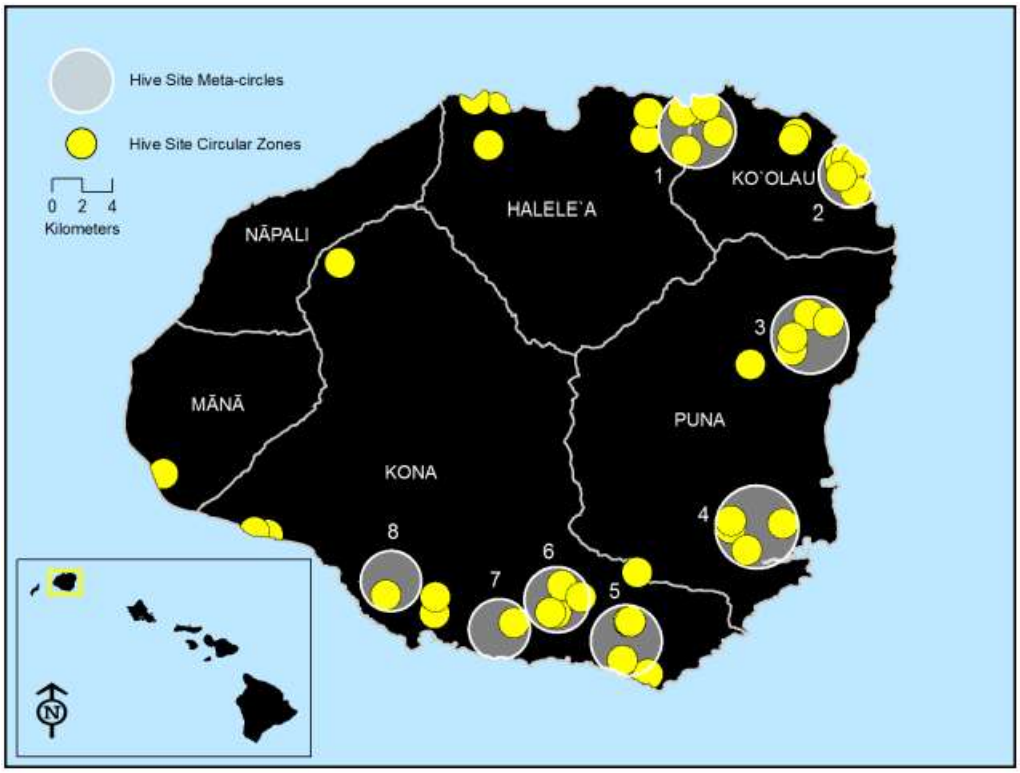


Figure 1: Distribution of 1 km radius circular zones (yellow) around hives on island of Kauai. Meta-circles of grouped circular zones are shaded in grey and numbered (N = 8). Moku divisions are indicated by white lines and each Moku is named.

Circular zones

Bees have been reported to forage as far as 9.5 km from the hive [20,21] with a mean distance closer to 1 km at times subject to patchiness of flowering resources [21]. Depending upon resource availability, the probability of plant visitation decreased non-linearly from the hive and > 85 % probability of visitation was at less than 1 km [22]. Beekeepers note that bees forage as close to the hive as possible [23], especially on Kauai where naturally occurring plants and crops bloom year-round. Foraging on Kauai may also be constrained by discrete watersheds, bounded by mountainous ridges.

Based on this information, and to avoid overlapping of foraging sites, a 1 km radius was used to define the bees' foraging zone around each hive. Geospatial information analysis was applied using the GEP program with Digital Globe™ (DG) images to delineate circular zones 1 km in radius around each hive (**Figure 1**).

The land area within each circular zone was further sub-divided into discrete polygons, based upon land cover designations derived from NOAA C-CAP twenty-one classifications [24] (**Table 2**). Habitat codes were reclassified to seven land use categories.

Individual polygons were delineated in GEP using an optical mouse and area covered was calculated. The land area of each habitat type was then summed to provide a measure of the total land area (m^2) in each land use polygon (**Figure 2**). Each circular zone comprised 314.16 hectares, unless ocean area was excluded. A total of 18,872 hectares

of land area were processed using the latest GEP images (years 2013–2014) and knowledge of current land use. Visual ground truthing was performed on sites known to differ from GEP images.

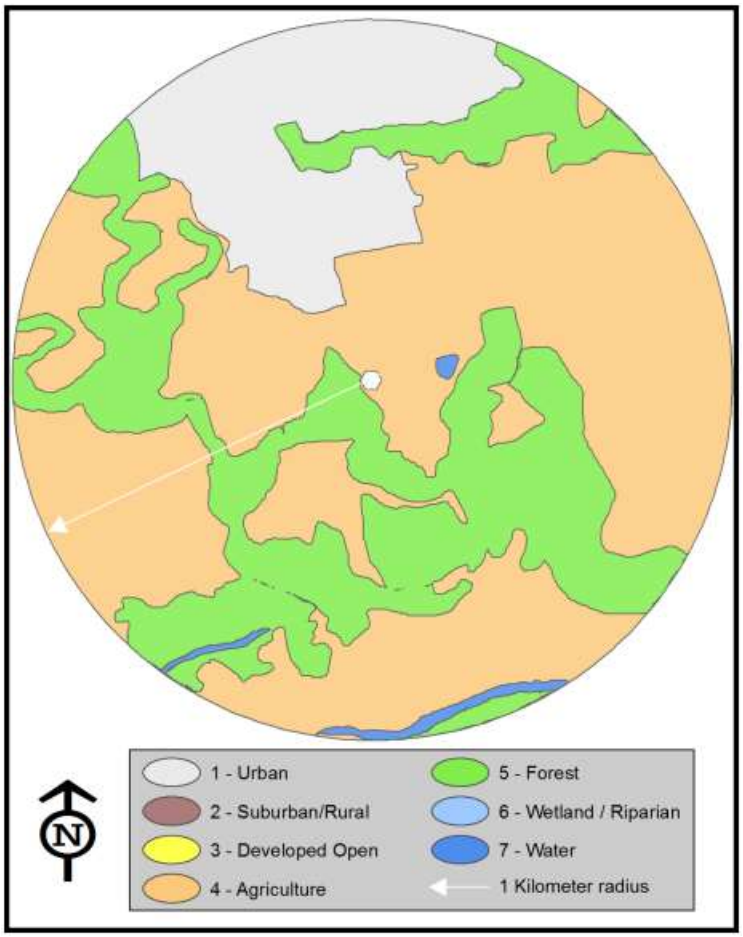


Figure 2: Circular zone around a central hive, drawn with 1 Km radius. Polygons represent different land uses categories. Site #16 provided as an illustration.

The percent of the current land use was calculated for each habitat type represented by the polygons within the hive sites' circular zones. These percentages were then correlated with the concentration of glyphosate residue from the hive in the circular zone. One hive (#48, Mānā Moku) was excluded from polygon land use calculations, as it had been moved among sites within the Moku.

A second independent geospatial analytical method for land use categorisation used the NOAA Coastal–Change Analysis Program (C-CAP) [24] and ArcGIS Version 10.5 [25] (S3 Appendix). It derived area (m^2) within the 1 km radius circular zones using a program that automatically identified different types of ground cover (**Table 2**). A comparison of the two methods for accuracy in determining current land use patterns showed GEP preferable, so it was used in this study (S3 Appendix).

Table 2: Land use NOAA C-CAP classification descriptions.

This Study	Land use category	C-CAP	Land use classifications	Description of ground cover
		1	Unclassified	
1	Urban	2	High Developed	heavily built-up urban centers as well as large constructed surfaces in suburban and rural areas. Large buildings
		3	Medium Developed	constructed surface mixed with substantial amounts of vegetated surface. Small buildings
2	Suburban/Rural	4	Low Developed	class 3, with the addition of streets and roads with associated trees and grasses
3	Developed Open	5	Developed Open	parks, lawns, athletic fields, golf courses, and natural grasses occurring around airports and industrial sites
4	Agriculture	6	Orchard	herbaceous (cropland) and woody (e.g., orchards, nurseries, and vineyards) cultivated lands
		7	Pasture land	grasses, legumes or grass-legume mixtures planted for livestock grazing or the production of seed or hay crops
		8	Grassland	Grassland: grasses and non-grasses (forbs) that are not fertilized, cut, tilled, or planted regularly
		20	Bare land	bare soil, rock, sand, silt, gravel, or other earthen material with little or no vegetation
5	Forest	9	Deciduous forest	Deciduous Forest areas dominated by single stemmed, woody vegetation
		10	Evergreen forest	67 percent of the trees remain green throughout the year. Both coniferous and broad-leaved
		11	Mixed Forest	areas in which both evergreen and deciduous trees are growing and neither predominate
		12	Scrub/shrubs	woody vegetation: true shrubs,young trees, and trees or shrubs that are small
6	Wetland/ Riparian	13	Palustrine Forested Wetland	non-tidal wetlands dominated by woody vegetation >5m
		14	Palustrine Scrub/Shrub Wetland	non-tidal wetlands dominated by woody vegetation less than or equal to 5 meters
		15	Palustrine Emergent Wetland	non-tidal wetlands dominated by persistent emergents, emergent mosses, or lichens
		16	Estuarine Forest Wetland	tidal wetlands dominated by woody vegetation >5m, salinity >0.5ppt
		17	Estuarine Scrub/Shrub Wetland	tidal wetlands dominated by woody vegetation <5m, salinity >0.5ppt
		18	Estuarine Emergent Wetland	erect, rooted, herbaceous hydrophytes. Perennial plants usually dominate these wetlands
7	Water	19	Unconsolidated Shore	substrates lacking vegetation: beaches, bars, and flats
		21	Open water	open water with less than 25 percent cover of vegetation or soil.

Meta-circles

Analysis was done to determine if non-glyphosate using areas (e.g. containing forest, water, organic farms and residential) could be differentiated from areas of higher glyphosate use, as determined by conversations with the beekeepers. Eight meta-circles were made, comprising multiple 1-km circular zones that were grouped as having the same general land use description (**Table 2**, **Figure 1**) and situated in grouped watersheds. These meta-circles were encircled within a computer-generated circumference (mean 1707 hectares) that fully contained 3 to 9 circular zones of the same land-use practices (ranging from 1256 to 2365 hectares). In total, 41 samples were included within these eight meta-circles.

Large-scale divisions (East-West side of island, Moku)

The island of Kauai is divided by mountainous ranges and orographic rainfall in to two different biogeographical zones [16]. The drier leeward west-side of Kauai comprises the Moku of Kona, Nāpali, and Mānā for approximately 73,710 hectares, 51.3 % of the island's area, while the wetter windward east-side comprises the Moku of Puna, Koʻolau, and Haleleʻa for approximately 70,049 hectares, 48.7 % of the island's area. Moku are identified by geological and biogeographic features [19] (**Figure 1**).

Statistical analysis

Data was analyzed with Microsoft Excel and Access (means, medians, S.D., S.E.M, t-tests, linear and exponential line fits). Analyse-it, a plug-in for Excel, was used for correlations and AICc line fits. TIBCO Spotfire Analyst® was used to produce the Trellis plots and non-parametric Kruskal-Wallis analysis.

Results

Island-wide

ELISA measured glyphosate concentrations in honey taken directly from the hive ranged from < LOQ to 342 ppb (**Table 3**). Sixteen (27.1 %) of 59 samples had glyphosate concentrations detected over the ELISA limit of quantitation (LOQ = 15 ppb).

Calculations of mean concentrations were done in two manners: using all sample ELISA data (N = 59, mean = 33.5

ppb, standard error of the mean, S.E.M. = 9.3) or for only those samples with ELISA values greater than the LOQ (N = 16, mean = 118.3, S.E.M. = 24.0).

Spatial and temporal variations at hive sites

Six separate sites had samples taken from multiple unique hives on those sites. At two of these six sites (Samples # 52, 53; 54, 56, 58), all hives had no glyphosate detected. At three of these six sites (Samples # 18, 59; 8, 14, 20, 21; 34, 35, 36), all hives had glyphosate > LOQ. At one site (Samples # 55, 57), only one hive had detectable glyphosate (Sample # 57) (27 ppb), while the other hive had none detected.

An extremely large feral beehive sampled in 2013 had 92 ppb glyphosate (**Table 3**, Sample # 37). In 2015, this site had four samples taken from widely spaced parts of the hive (Samples 8, 14, 20 & 21). Analysis yielded values ranging from 33 ppb to 342 ppb (mean = 147.7 ppb, S.E.M. = 69.7 ppb).

Two different sites were sampled in 2015 and again in 2016. Each of these two sites had multiple hives. Both sites showed an increase in concentration levels over time (0 ppb to 27 ppb for samples 55 & 57; 25 ppb to 95 ppb for Samples 18 & 59).

Of the store-bought samples (**Table 4** and Table A in S4 Appendix), 33.3 % of those from Kauai had glyphosate residue > LOQ (mean = 41 ppb, S.E.M. = 14.2)

East-West side of island

Presence and concentration of glyphosate residues were mapped with respect to ancient Hawaiian biogeographical and management land divisions (Moku) [19]. When all 59 samples were analyzed, there was a higher glyphosate concentration (mean = 61.6 ppb, N = 31, S.E.M. = 16.2) (**Table 5** and Tables B and C in S4 Appendix) in honey from the leeward western half of Kauai versus the windward eastern half (mean = 2.4 ppb, N = 28, S.E.M. = 0.9). Mean values between the western and eastern sides are different (t-test, $p = 0.001$, df = 57) (Table D in S4 Appendix).

If only glyphosate values > LOQ are used (N = 16), the western Moku had 15 samples, 48.4 % of which had glyphosate > LOQ (mean = 125.1 ppb). The eastern Moku had only 1 sample over the LOQ (3.6 %). This sample value (15.2 ppb) is just greater than the LOQ.

A Trellis plot was made showing the glyphosate concentration across samples, grouped by side of island and by Moku. When all 59 samples are plotted, there is a clear pattern of the higher glyphosate concentrations in the western Moku vs the eastern Moku (Figure 3). No samples were collected from the remote western Moku of Napali.

Moku

Moku differed greatly in concentration of glyphosate in honey (Table B in S4 Appendix). Puna and Ko'olau Moku had no samples > LOQ and Halele'a had only one > LOQ. No samples were collected from remote Napali and only one sample from Mana. Concentrations from the west side Kona Moku were different from the three east-side Moku ($p < 0.003$) (Table E in S4 Appendix).

Since it is not known if these samples are from a normally distributed population, a non-parametric Kruskal-Wallis test was performed. This test confirmed the above parametric tests that glyphosate distributions were different depending upon the side of the island and the Moku ($p = 0.0008$ and 0.004, respectively) (Table F in S4 Appendix).

Source location of honey purchased from merchants on Kauai was obtained from the label and discussions with vendors. Percentage of samples with glyphosate residue > LOQ and mean concentrations of glyphosate differed among Moku sampled (**Table 6** and Table A in S4 Appendix). Area with the greatest percentage of samples with glyphosate was in the agricultural district of Kona on the west side of the island. This is the same trend seen as with the hive samples (**Figure 3**).

Table 3: Glyphosate concentration and percent of land use (by category) within the circular zones surrounding the hives.

Google Earth Polygon Land Use Classification								[Glyphosate]
Sample #	% Urban	% Suburbs	% Open	% Ag	% Forest	% Wetland	% Water	ppb
1	71.4%	1.1%	6.6%	3.1%	0.0%	17.6%	0.2%	< LOQ
2	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
3	0.0%	13.5%	0.0%	70.9%	15.5%	0.0%	0.0%	
4	31.1%	0.0%	9.0%	30.0%	29.7%	0.0%	0.3%	
5	22.6%	0.0%	13.3%	21.0%	42.8%	0.0%	0.3%	< LOQ
6	19.8%	0.0%	3.3%	76.9%	0.0%	0.0%	0.0%	80
7	0.0%	10.4%	66.5%	3.2%	19.8%	0.0%	0.0%	
8	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	61
9	0.0%	46.6%	23.1%	1.1%	29.2%	0.1%	0.0%	
10	0.0%	6.5%	87.5%	4.4%	0.0%	0.0%	1.6%	< LOQ
11	0.0%	0.0%	4.2%	69.7%	26.1%	0.0%	0.0%	
12	0.0%	0.0%	48.2%	19.8%	32.0%	0.0%	0.0%	15
13	0.0%	30.9%	26.6%	8.6%	34.0%	0.0%	0.0%	
14	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	342
15	15.58%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	
16	15.3%	0.0%	0.0%	53.8%	30.1%	0.0%	0.9%	
17	0.0%	4.0%	1.8%	0.0%	94.2%	0.0%	0.0%	
18	25.4%	0.0%	74.1%	0.1%	0.0%	0.0%	0.3%	25
19	52.9%	0.0%	44.6%	2.4%	0.0%	0.0%	0.0%	< LOQ
20	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	155
21	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	33
22	0.0%	45.8%	3.0%	33.9%	13.3%	0.0%	4.1%	
23	0.0%	50.2%	14.8%	0.4%	34.6%	0.0%	0.0%	
24	0.0%	1.5%	11.2%	64.3%	23.0%	0.0%	0.0%	
25	6.8%	10.1%	57.1%	2.3%	23.5%	0.0%	0.3%	
26	0.0%	1.0%	6.7%	68.6%	23.7%	0.0%	0.0%	
27	18.9%	0.0%	50.5%	0.0%	29.4%	0.0%	1.2%	
28	0.0%	0.0%	47.5%	11.5%	41.0%	0.0%	0.0%	< LOQ
29	0.0%	14.4%	0.5%	75.0%	10.1%	0.0%	0.0%	
30	0.0%	7.0%	30.2%	8.7%	54.1%	0.0%	0.0%	
31	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
32	0.0%	30.2%	8.3%	1.8%	57.7%	0.0%	2.0%	
33	22.2%	5.4%	61.4%	0.0%	7.8%	3.2%	0.0%	
34	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	187
35	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	178
36	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	172
37	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	92
38	36.2%	0.0%	2.8%	61.0%	0.0%	0.0%	0.0%	78
39	20.7%	4.2%	44.3%	3.5%	27.4%	0.0%	0.0%	
40	0.0%	49.0%	0.0%	12.9%	38.1%	0.0%	0.0%	< LOQ
41	17.1%	0.3%	0.0%	58.9%	23.8%	0.0%	0.0%	60
42	0.0%	0.0%	1.4%	67.9%	30.7%	0.0%	0.0%	
43	0.9%	1.2%	81.8%	4.7%	11.4%	0.0%	0.0%	
44	0.0%	28.2%	0.0%	25.3%	44.2%	0.0%	2.3%	< LOQ
45	0.0%	3.2%	0.0%	0.0%	95.5%	0.0%	1.3%	
46	6.8%	56.2%	0.0%	0.0%	37.0%	0.0%	0.0%	< LOQ
47	0.0%	0.0%	0.0%	19.5%	80.5%	0.0%	0.0%	
48	19.7%	0.0%	10.2%	16.3%	50.2%	0.0%	3.6%	292
49	20.0%	49.6%	0.0%	0.0%	29.0%	0.0%	1.3%	
50	0.0%	1.5%	0.0%	50.4%	48.1%	0.0%	0.0%	
51	0.0%	16.4%	0.0%	0.0%	83.6%	0.0%	0.0%	
52	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
53	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
54	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
55	18.0%	4.2%	2.2%	42.6%	31.2%	0.0%	1.8%	
56	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
57	15.6%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	27
58	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
59	25.4%	0.0%	74.1%	0.2%	0.0%	0.0%	0.3%	95

Table 4: Concentration and percentage of glyphosate detected in store-bought honey. Samples originated from three Hawaiian islands and international blends. Samples categorised as Organic or Non-Organic.

Category			Samples N	> LOQ %	> LOQ Mean ppb
Location					
	Hawaii	Island:			
		Kaua'i	15	33.3	41.0
		Hawai'i	4	25.0	16.4
		Molokai	2	0	NA
	International		5	40.0	51.5
Type					
	Organic		5	20.0	30.6
	Non-Organic		21	33.3	42.0

Table 5: Glyphosate concentration by side of island and the six Moku. All 59 sample values used. Napali Moku had no samples ("ns").

Moku	Glyphosate Mean ppb	Median	S.D.	S.E.M.	Count
Windward:					
Koolau	0	0	0	0	10
Puna	2.5	0	5.1	1.7	9
Halelea	4.1	0	6.3	2.1	9
Totals	2.41	0	4.9	0.92	28
Leeward:					
Kona	53.9	11.7	80.9	14.8	30
Mana	292.2	292.2	na	na	1
Napali	ns	na	na	na	ns
Totals	61.61	13	90.3	16.2	31

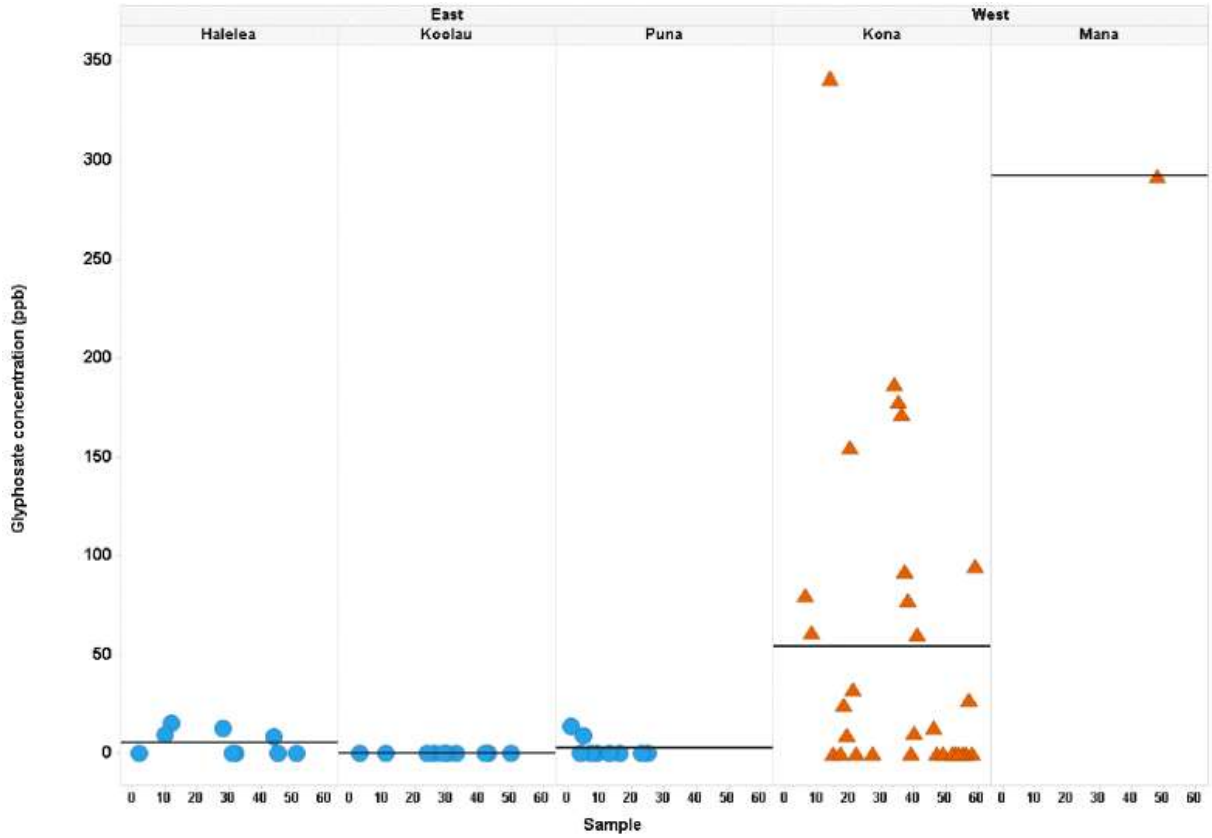


Figure 3: Glyphosate concentrations across samples by side of island and within each Moku. Mean glyphosate (ppb) is shown by the horizontal line for each Moku. Side of the island and Moku names are listed at the top of the plot. Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles.

Table 6: Prevalence and concentration of glyphosate in Kauai honey from store-bought samples.

Moku	All samples N	> LOQ N	> LOQ % total	> LOQ mean	> LOQ SEM
Puna	6	1	16.7	15.0	na
Koolau	5	1	20.0	61.8	na
Kona	4	3	75.0	43.1	22.2

Circular zones and land use polygons

Land use within an area of 1 Km radius around each of the hives was determined using Google Earth Pro™ (GEP) (N = 59 hives from Kauai). These circular zones were divided into single land use polygons and the total meter^2 coverage for each of the seven land types was calculated. The percent of the total allocated to each of the seven land use types of each site was summarised with the glyphosate concentration found in the samples from that site (**Table 3**).

AICc analysis was performed to determine correlations between presence of glyphosate in honey and various land uses. Non-zero glyphosate data (N = 23) was used for these analyses. The exponential model for land use and glyphosate was chosen, as it has the highest correlation and strongest AICc values, compared with other line fits (Table G in S4 Appendix). Agriculture land use in the immediate 1 km radius vicinity of the hive showed the highest positive correlation with glyphosate concentration (**Table 7**, $R^2 = 0.594$) and the strongest AICc compared with the other land use categories. Open, Suburbs, Urban, and Forest land use all showed weak negative correlations (negative Parameter Estimates) between land use and glyphosate concentration. Wetland and Water land use showed very weak positive correlations. The negative correlations (e.g. Forest) is due to these land use types not being independent variables; rather, they are multicollinear (Figure A in S4 Appendix).

Concentration of glyphosate in honey was plotted versus the percent land use in agriculture. Samples with non-zero glyphosate were used (N = 23). **Figure 4** shows that the higher glyphosate concentrations are correlated with sites that have high percent agriculture land use (> 60 % agriculture).

The hives in the western Moku (orange triangles) have a strong correlation with higher glyphosate when there is higher percent land use as agriculture. Hives in the eastern Moku (blue circles) had very low glyphosate, even with 60 % to 80 % of the area in agriculture (**Table 3**).

Table 7: Correlation of glyphosate concentration (ppb) in honey samples and the percent land use.

Land Use	R^2	AICc	SE of fit (RMSE)	Parameter estimate	95% CI	95% CI	SE	p-value	Exponential Equation
Agriculture	0.594	-8.664	0.784	2.552	1.594	3.511	0.461	0.000	$Y = 12.58 * 12.84^X$
Forest	0.326	2.967	1.010	-3.977	-6.572	-1.383	1.247	0.004	$Y = 65.24 * 0.01874^X$
Open	0.123	9.030	1.152	-1.465	-3.242	0.311	0.854	0.101	$Y = 50.03 * 0.231^X$
Suburbs	0.086	9.973	1.176	-2.276	-5.638	1.087	1.617	0.174	$Y = 46.98 * 0.1027^X$
Urban	0.049	10.897	1.200	-1.422	-4.274	1.430	1.371	0.311	$Y = 47.01 * 0.2412^X$
Wetlands	0.017	11.660	1.220	3.659	-9.110	16.427	6.140	0.558	$Y = 36.23 * 38.8^X$
Water	0.011	11.796	1.224	13.180	-44.017	70.377	27.504	0.637	$Y = 34.83 * 5.296e+05^X$

Meta-circles

In order to expand land use to watersheds or larger areas, meta-circle analysis was done on eight clusters of circular zones situated all around the island (**Figure 1**). They comprise similar environments. Discussions with beekeepers were used to develop a general description of each meta-circle (**Table 8**) as to predominant land use and glyphosate use.

The percent of each of the seven types of land use was calculated for each circular zone in each meta-circle (**Table 3** and Table H in S4 Appendix). Then the mean percent of each type of land use was calculated for each meta-circle. The highest percent land use was used to describe the meta-circle, if that land use type was at least 70 %. If it was less than 70 %, then a composite was used; the second highest type of land use was added to the highest land use type. This process was repeated until the composite land use designation comprised at least 70 % of the meta-circle. This composite description is shown in **Table 8**, in the column "Composite land use type".

The mean concentration of glyphosate in honey was calculated using all samples within each meta-circle (N = 48 samples total). The percentage of samples which had glyphosate > LOQ was also calculated (N = 16 total). Only three meta-circles had significant glyphosate residues and all were in areas on the western side of Kauai. The two meta-circles with the most glyphosate, Ag. 1 and Ag. 2, were in areas of large scale agriculture use. The Koloa meta-circle had some agricultural use and contained the circular zones with large amounts of golf courses and or highway present, as discussed below.

A Trellis plot was made to show glyphosate concentration across samples, grouped by meta-circle (**Figure 5**). Within each meta-circle, samples are plotted versus the percentage of agriculture for that sample. There is a clear pattern of the higher glyphosate concentrations for samples in the western meta-circles (orange) vs samples in the eastern meta-circles (blue). The samples with glyphosate > LOQ (triangles) are also all in the western meta-circles, while the eastern meta-circles all have glyphosate < LOQ (circles) (**Figure 5**).

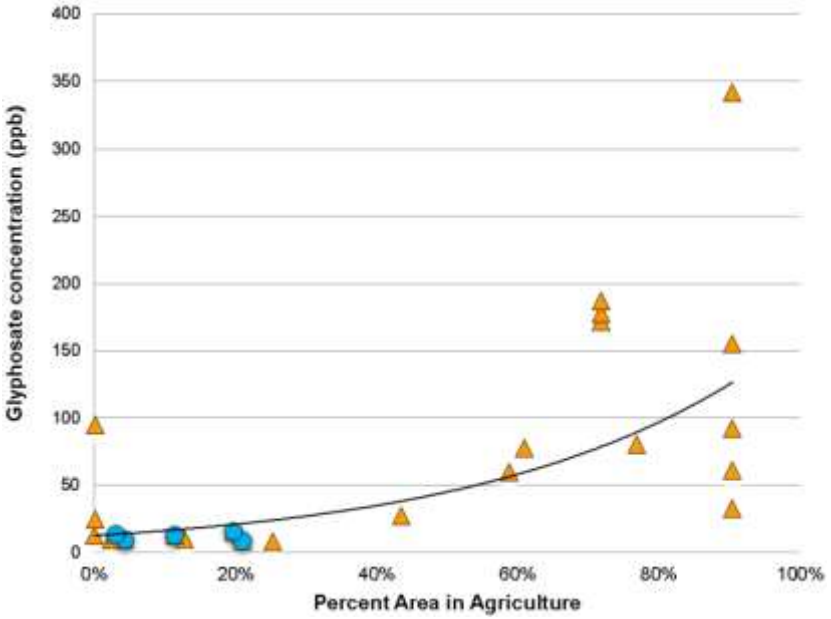


Figure 4: Glyphosate concentration versus the percent land use in agriculture (N = 23). Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles. Exponential fit is $Y = 12.6\ e^{12.8X}$, $R^2 = 0.594$.

Table 8: Meta-circle composition, mean glyphosate concentration, and percent prevalence. Meta-circle # corresponds to Figure 1.

Meta-circle #	Meta-circle	Number of Circular Zones	General Description	Composite % land use	Composite land use type	Mean ppb	% > LOQ
1	Kīlauea	5	Rural, Suburbs	72.0%	Open, Forest	< LOQ	0%
2	Moloaʻa	6	Organic farming	89.2%	Agriculture, Forest	< LOQ	0%
3	Kapaʻa	4	Suburbs	82.0%	Open, Forest, Suburbs	< LOQ	0%
4	Līhuʻe	4	Urban, open, agriculture	87.7%	Urban, Agriculture, Forest	< LOQ	0%
5	Kōloa	9	Suburbs, golf, resort	74.7%	Agriculture, urban, Forest	16.3	33%
6	Lāwaʻi	5	Suburbs	82.9%	Forest, Suburbs, Open	< LOQ	0%
7	Ag. 1	3	Large scale agriculture	71.9%	Agriculture	179.0	100%
8	Ag. 2	5	Large scale agriculture	90.5%	Agriculture	136.6	100%

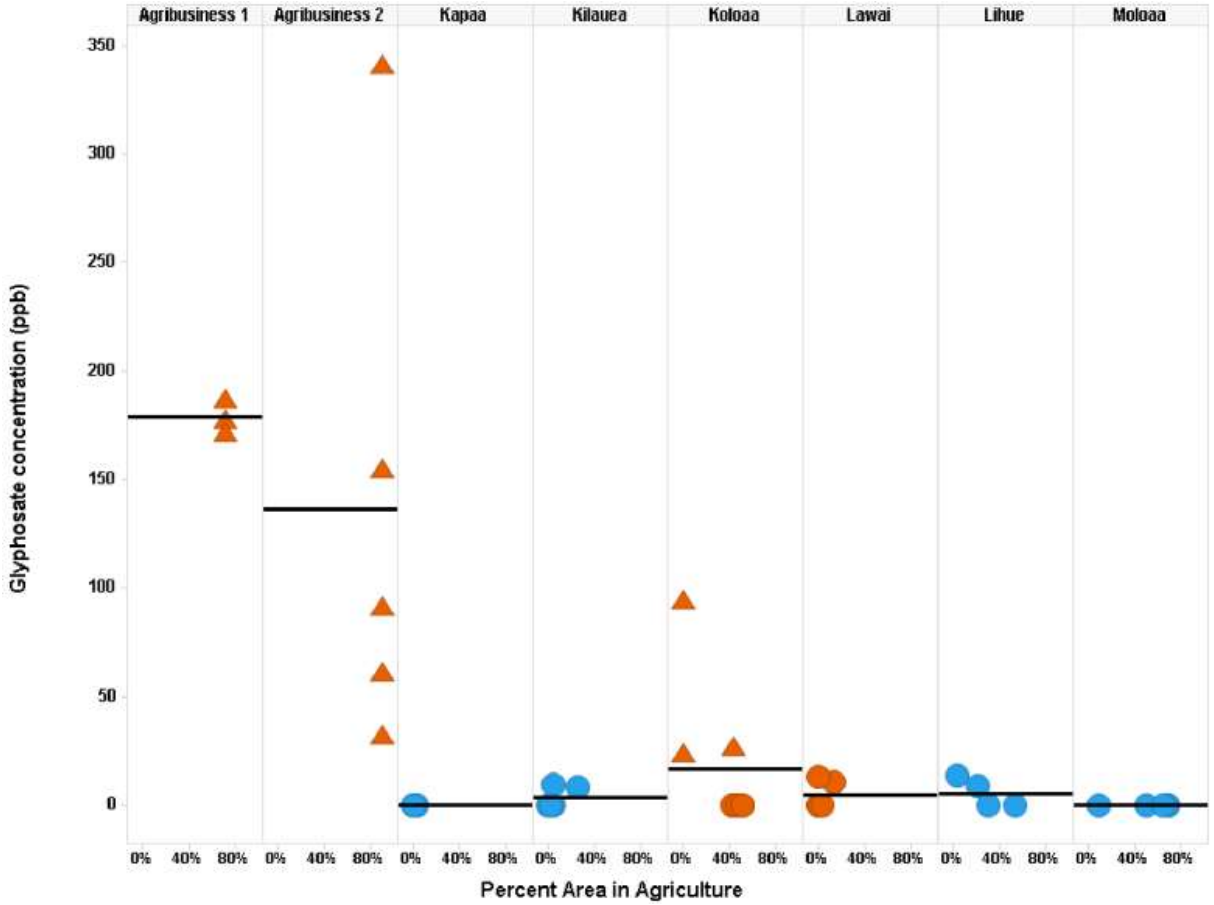


Figure 5: Glyphosate concentrations across samples within each meta-circle. Mean glyphosate (ppb) is shown by the horizontal line for each meta-circle. Meta-circle names are listed at the top of the plot. Samples from the western Moku are shown as orange and eastern Moku as blue. Samples with glyphosate > LOQ are shown as triangles, while those < LOQ are as circles.

Golf courses and highways

A smaller specific land use, golf course, was identified from GEP images, but was subsumed in the "Developed Open" C-CAP category (**Table 2**). There were only eight circular zones which encompassed golf course(s) and all had glyphosate residues in honey (**Table 9A**). Percent area in golf course varied from 1.2 % to 16.2 %. Three of those samples (samples #34, 35, 36) were from different hives on the same farm and were also associated with high percent (> 70 %) agricultural land use. Two hives with the highest percent land use as golf course (samples # 18 and #59) were from the same residence with very low agricultural land use.

Major highways were identified as another small specific land use. These were subsumed under the Urban and Suburban/Rural categories (**Table 2**). Portions of highways were contained within 76 % of the circular zones (Table I in S4 Appendix). Those in the top 10 % of cumulative length of highway (> 4.6 km) had three samples with glyphosate > LOQ (25 to 95 ppb) (**Table 9B**). Frequent spraying of golf courses and highways may explain the presence of glyphosate (> LOQ) in samples # 18, 57, and 59.

Table 9: (A) 8 samples with highest % area Golf; (B) 6 samples with highest km highway present.

A				
Sample #	**Glyphosate ppb**	**% Ag**	**% Golf**	**Km Highway**
34	187	71.9%	1.2%	1.1
35	178	71.9%	1.2%	1.1
36	172	71.9%	1.2%	1.1
19	10	2.4%	1.6%	3.4
1	14	3.1%	4.8%	2.0
28	13	11.5%	13.7%	2.0
18	25	0.1%	16.2%	4.7
59	95	0.2%	16.2%	4.7
B				
55	0	42.6%	0.0%	4.6
57	27	43.6%	0.0%	4.6
59	95	0.2%	16.2%	4.7
18	25	0.1%	16.2%	4.7
52	0	51.6%	0.0%	4.7
53	0	51.6%	0.0%	4.7

Discussion

The presence of glyphosate residue in honey samples taken directly from the hive has been shown to correlate with areas that geospatial analysis has identified as comprised mainly of large-scale mono-crop agriculture. This suggests both a source and a pathway whereby pesticides migrate from site of use into other areas. Glyphosate residue > LOQ was found in 27.1 % of the hives and 33.3 % of store bought honey from Kauai, lower than the 59 % in store bought honey from around the world [1]. With hive-collected honey, geospatial analysis was able to further identify: which side of the island (west), which Moku (Kona and Mana), which areas (agriculture meta-circles), and most specifically which land use (agriculture) had the greatest prevalence and greatest concentration of glyphosate in honey.

Purchased samples from the other Hawaiian islands had lower mean concentrations and a smaller percentage contaminated than those from Kauai. The mean concentration of glyphosate from international samples purchased on Kauai was 51.5 ppb, similar to the 64 ppb in Rubio [1]. Samples from Brazil and a sample from a blend of USA and Argentina approximated values reported earlier, while the blend from Brazil, Mexico and Uruguay did not [1].

One of five Kauai purchased samples (20 %) labeled organic had glyphosate residues > LOQ (mean 30.6 ppb) compared to 45 % (mean 50 ppb) reported elsewhere [1]. This supports supposition of some migration of pesticides from areas of application to organic farms. The twenty-one Kauai samples not labeled as organic had both a higher occurrence (33.3 %) and higher mean concentration (42.0 ppb) of glyphosate than the organic labeled samples, suggesting application of glyphosate near the hives. Honey from traditional agriculture sites around the world had 62 % with glyphosate > LOQ and mean 66 ppb [1], expressing widespread use of glyphosate in agriculture.

The actual process of how Kauai bees obtained, carried and processed glyphosate is not known and was not addressed in this study, but is discussed elsewhere [13,14]. As honey was obtained directly from the hive using clean vials, this eliminated the possibility of contamination occurring during processing. Each sample was unique to a single hive, not blended from various sites. A survey of beekeepers confirmed that their hives did not get sprayed with glyphosate. Uptake could have occurred if the bees themselves got sprayed while foraging, if flowers frequented by the bees contained glyphosate from either direct spraying or aerial drift, or if water that the bees drank on plants or on the ground was contaminated in some way. In all cases, contamination could have occurred at a distance from the hive. Geospatial analysis mallowed the determination that within a 1 km radius of the hive, glyphosate contamination was most closely associated with large scale agriculture. The proximity of golf courses and highways were also associated, but to a lesser degree. General land use changes and landscape composition may have indirect detrimental effect on bee fitness, although the association between pesticide and landscape composition was not investigated.

The presence of both restricted use pesticides and glyphosate in bee pollen and honey, even at very low levels, identifies an important pathway whereby pesticides migrate from site of application to the hive and into the human food supply [12–14, 26]. Geospatial analysis can help honey producers estimate spatial pesticide exposure risks inherent in intensive agriculture. When bees are used for commercial large-scale crop pollination, hive placement can be optimised so that the bee colonies are not seriously impacted by pesticides that the bees must endure while foraging [26–27]. Linking spatial and temporal dynamics of flowering crops, agri-environmental schemes, and pesticide applications would lead to better understanding of environmental risk assessment, management of pollination services, and protecting biodiversity [26–28].

Supporting information
Supporting information with is available online:

S1 Appendix. Abraxis technical bulletin.
https://doi.org/10.1371/journal.pone.0198876.s001

S2 Appendix. ELISA verification with mass spectrometry.
https://doi.org/10.1371/journal.pone.0198876.s002

S3 Appendix. Geospatial analytical method comparison.
https://doi.org/10.1371/journal.pone.0198876.s003

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey.
https://doi.org/10.1371/journal.pone.0198876.s004

This information is summarised at the end of this document.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The publication provides residue levels for glyphosate in honey produced in Hawaii (majority of samples) but also Argentina, Brazil, Canada, Mexico, Uruguay and USA (mainland). It is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. As honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated indirectly by comparison with an LC-MS/MS method. A total of 14 honey samples were analysed with the two methods and the results were shown to be similar. The publication, however, does not provide validation data for the LC-MS/MS method (recovery rates from fortified samples).

The study showed a higher detection rate of glyphosate than in the EU-monitoring for 2016-2017. Besides the different origin of the samples, this may also be due to the use of different analytical methods with different LOQs. In line with the EU-monitoring the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.342 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

Assessment and conclusion by RMS:
The publication describes the monitoring results of residues of glyphosate in honey from different Hawaiian apiaries. Residue levels of glyphosate in honey samples directly from the Hawaiian hives (n = 59) ranged from below the LOQ (0.015 mg/kg) to 0.342 mg/kg, with 27% of these samples (n = 16) having residues above the LOQ. Residue levels of glyphosate from store-bought honey originating from Hawaii, but also Argentina, Brazil, Canada, Mexico, Uruguay and USA (mainland), were in the range of <0.015-0.087 mg/kg, with up to 40% of these samples having residues above the LOQ, depending on the country of origin. Based on these data sets, the existing MRL for glyphosate in honey (0.05* mg/kg) would therefore be exceeded.

The obtained data are more or less in line with the monitoring data obtained from the official monitoring labs in the EU (see Volume 3, B.7.7.1.2) where residue levels of glyphosate ranged between <LOQ to 0.61 mg/kg, although it is noted that the percentage of samples in which residues were above the LOQ was lower compared to the percentage determined in this publication (10% in the EU monitoring data set compared to up to 40% positive findings in this publication).

The publication also investigated the geospatial variation of glyphosate levels in different honey samples. As one would expect, residue levels of glyphosate were higher in samples of hives that were located close to agricultural sites, as well as highways or golf courses.

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered fully reliable and/or relevant for the MRL calculation for two reasons: (i) the data were not obtained by official monitoring laboratories; and (ii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

S1 Appendix. Abraxis Technical Bulletin

Glyphosate in Honey and Corn Syrup Sample Preparation

1. Intended Use

For the detection of Glyphosate in honey and corn syrup.

2. Sensitivity

0.015 ppm in matrix

3. Materials and Reagents Required

Analytical balance
Microcentrifuge tubes
4 mL glass vials with Teflon-lined caps
Disposable pipettes
Micropipettes with disposable plastic tips
Vortex mixer
Microcentrifuge
Timer
Plate shaker or Micro-well plate holder with insert retainer for vortex mixer
1 N Hydrochloric Acid (HCl)
Glyphosate sample diluent
Abraxis Glyphosate Plate ELISA Kit

4. Notes and Precautions

This procedure is intended for use with honey and corn syrup (light and dark). Other matrices should be thoroughly validated before use with this procedure.

Hydrochloric Acid must be handled with care. Wear appropriate protective clothing (gloves, glasses, etc.). Avoid contact with skin and mucous membranes. If contact occurs, wash with copious amounts of water and seek appropriate medical attention.

Due to the viscous nature of the prepared samples, the microtiter plate should be placed on a plate shaker or vortex mixer fitted with a micro-well plate holder adapter for the incubations with the antibody and conjugate solutions. This will allow for the appropriate mixing of all reagents in the microtiter wells.

5. Sample Preparation Procedure

5.1 Weigh 0.5 g of sample into an appropriately labeled microcentrifuge tube.

5.2 Add 0.5 mL of 1 N HCl. Vortex for 2 minutes.

5.3 Add 3.96 mL of Glyphosate Diluent to a clean, appropriately labeled 4 mL glass vial. Add 40 µL of the acid-treated sample (from step 5.2) to the Glyphosate Diluent in the vial (1:100 sample dilution). Vortex. This will then be analyzed as sample, see Derivatisation of Standards, Control, and Samples in the Reagent Preparation section of the Glyphosate Plate ELISA Kit user's guide.

6. Evaluation of Results

The Glyphosate concentration in the samples is determined by multiplying the ELISA results by a factor of 200.

Samples showing a concentration lower than standard 1 (0.075 ppb) should be reported as containing < 15 ppb of Glyphosate. Samples showing a higher concentration than standard 5 (4.0 ppb) can be reported as containing > 800 ppb of Glyphosate or diluted further and re-analyzed to obtain an accurate quantitative result.

7. Performance Data Recovery

Honey samples were spiked with various amounts of Glyphosate, prepared as described above, and then derivatised and assayed using the Glyphosate Plate Assay. Average recovery was 113 %.

Corn syrup samples (light and dark) were spiked with various amounts of Glyphosate, prepared as described

above, and then derivatised and assayed using the Glyphosate Plate Assay. Average recovery was 104 %.

S2 Appendix. ELISA verification with mass spectrometry

To verify ELISA techniques for measuring glyphosate in a honey matrix (LOQ of 15 ng/g, 15 ppb) honey remaining in 14 vials from the Batch 2 samples analysed with ELISA were sent to Quality Services International GmbH (QSI), (Bremen, Germany) for analysis of herbicide residue by gas chromatography/mass spectrometry (GC-MS/MS) and/or liquid chromatography mass spectrometry (LC-MS/MS) methods (QSI method # 88505) with a LOQ of 0.01 mg/kg (10 ppb) (Table A). All concentrations derived from ELISA were used in analysis, however QSI did not report readings for levels < 10 ppb, so a value of zero was assigned for data analysis.

Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	Glyphosate ppb	Glyphosate ppb
Sample #	ELISA	QSI
1	13.6	17
5	8.8	10
6	**80.2**	**63**
7	0	0
10	9.2	0
12	15.2	0
14	**341.6**	**330**
16	0	0
18	**24.6**	**21**
19	9.6	12
25	0	0
27	0	0
28	12.6	0
35	**178.0**	**190**
LOQ=	15	10

Results for all 14 samples analysed by both methods correlated well (Figure A). Standard error of y for each x-value is 8.6 ppb. Only 4 samples had both the ELISA and LC/GC/MS/MS values over their respective LOQ, but the correlation coefficient remained high (Figure A).

Although sample size was small, a correlation coefficient $R^2 = 0.99$ supports the ELISA tests for accuracy, in addition to the use of blank and standards within each test run [1]. Comparison of ELISA techniques for monitoring glyphosate with chromatography-mass spectrometry have consistently found high correlations between the techniques in tests of various matrices, e.g. water [2,3], animal urine and animal tissues [4]. The use of Abraxis methods of ELISA determination of glyphosate in honey is well substantiated.

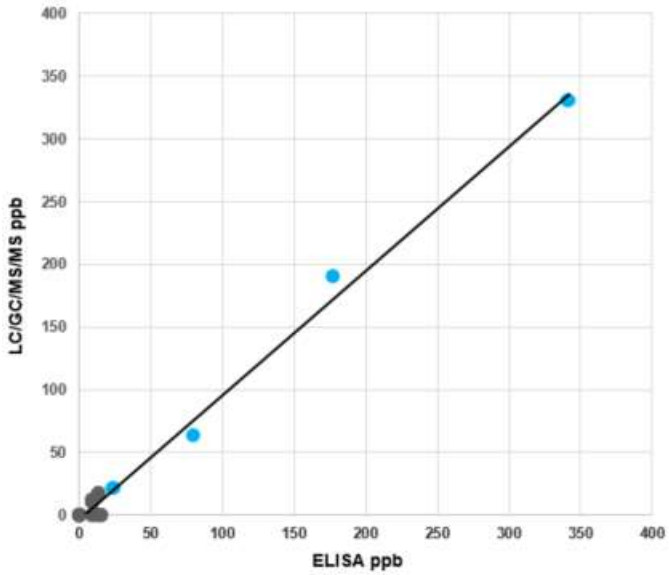


Figure A: Correlation of glyphosate concentration in honey split-samples using ELISA and LC-GC-MS/MS techniques. Linear fits: Y = 0.99 x – 3.1, R^2 = 0.993 (N = 14; black and blue circles); Y = 0.99 x + 6.1, R^2 = 0.994 (N = 4; blue circles).

S3 Appendix. Geospatial analytical method comparison

Development, application and comparison of two means for quantifying current land use practices within 1 km radius of a bee hive.

Geospatial analysis was performed in ArcGIS 10.5 on two separate Habitat datasets

1. Coastal Change Analysis Program (C-CAP) High Resolution Land Cover (1-4 meter resolution). Derived from high resolution imagery and analyzed according to the Coastal Change Analysis Program (C-CAP) protocol to determine land cover.

2. Vector polygons digitised in Google Earth Pro™ (GEP) using Digital Globe™ (DG) images (2013-2014) of 30-50 cm resolution as a base layer.

Coastal Change Analysis Program (C-CAP)

C-CAP analysis was conducted in January of 2016. Data downloaded was produced at a 1-4 meter resolution and utilised 35 full or partial WorldView2 multispectral scenes and the 2005 high-resolution Kauai C-CAP data set. The imagery and base classification were included in a multi-step semi-automated change detection process to extract land cover features in the 2010 imagery. Habitat within this dataset is classified into one of 21 different habitat classifications using a 2.5 meter cell size.

In order to extract out raster cells within the 2 kilometer boundary (1 Km radius) per hive site, the data set was masked using a vector dataset. This dataset was created by plotting each of the 38 hive sites in ArcGIS using their UTM location. Locations were converted into a point shapefile and then buffered by 1 km to create the 2 kilometer boundary polygon. Individual polygons were dissolved into one record to create the Mask to extract out pixels of the CCAP raster. Masking a raster using a vector is similar to the "Clip" geoprocessing routine done between two vector datasets. A vector representing an outline of the island was used to further mask the raster, removing pixels that were beyond the coastline, seemingly representing ocean (Figure A, B).

In order to quantify the percentage of habitat within each hive site boundary area (buffer a k.a. circular zone), the raster pixels were converted into a polygon feature class (vector) for vector geoprocessing. This polygon conversion resulted in 26,176 records/polygons, representing 26,176 cells within the original Raster dataset residing in the hive site boundary area. The "Intersect" geoprocessing tool was used next to assign to each record the corresponding hive site number it fell within. Habitat codes were reclassified, reducing the number of habitats considered by the analysis to seven land use categories. These were used in identifying the candidate habitats bees are believed to be foraging. Using the "Dissolve" geoprocessing tool, the habitat polygons were dissolved by Hive Site and reclassified Habitat Code, and the results stored in a geodatabase so that the area for each habitat could be reported using the Shape Area field. Totals for the amount of habitat polygon cells residing within each hive site boundary were then summed and

the percentage for each habitat within the boundary calculated.

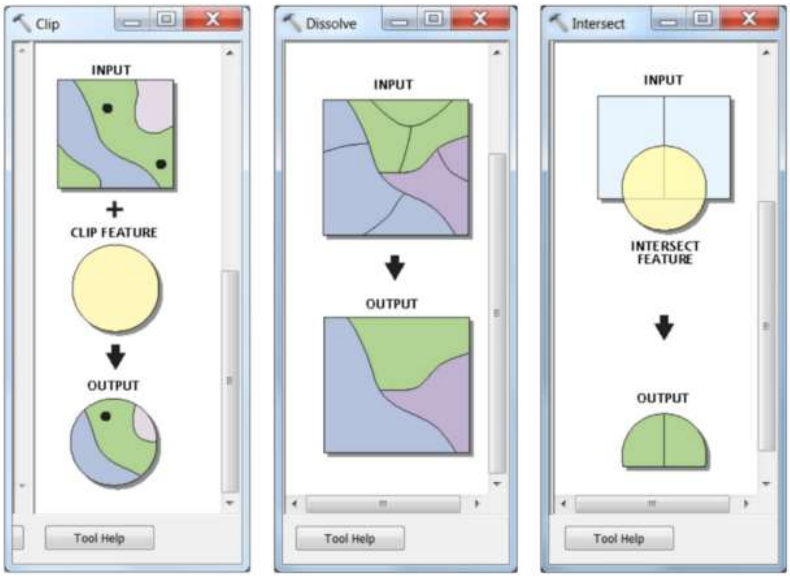


Figure A: ArcGIS 10.5 geoprocessing tools: Clip, Dissolve and Intersect.

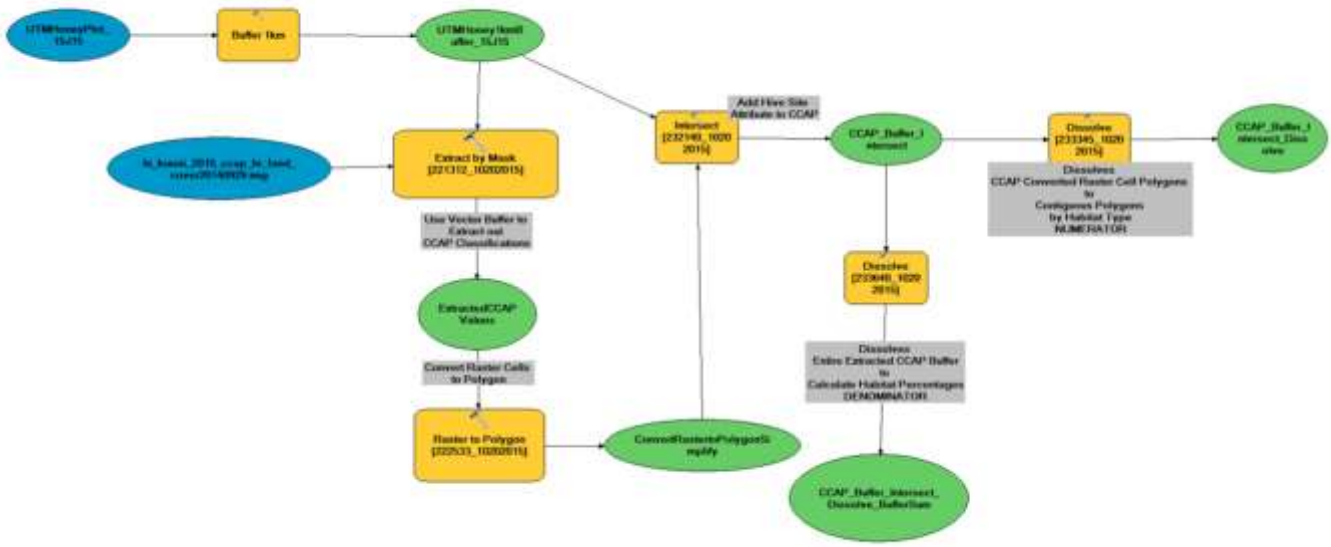


Figure B: Schematic of geoprocessing tools used to improve calculations of polygon areas for the C-CAP dataset.

Google Earth Pro™ (GEP)

Digitizing in a Geographic Information System is the process of converting geographic data from a hard-copy or scanned image into a vector dataset by tracing features; features are captured in coordinates and stored as either a point, line or polygon vector dataset. For this analysis, "heads up digitizing" in GEP was used to create discrete habitat polygons based on the reclassified habitats in the C-CAP analysis. Polygons created in GEP were stored as a KML/KMZ file, imported into ArcGIS 10.5 and converted into a feature class residing in a geodatabase so that areas of each habitat polygon could be calculated in square meters.

Upon importing the polygons from Google Earth, numerous topological errors were discovered in the polygons themselves, the most pervasive being knots, loops and slivers. These occur when "…the digitizer has an unsteady hand and moves the cursor or puck in such a way that the line being digitised ends up with extra vertices and/or nodes". Knots and loops result when a line forming a boundary of a polygon folds back on itself, creating small polygon like geometry known as "weird polygons".

Polygon features are enclosed areas created from a series of vertices that are connected with a continuous line traveling

in one direction whereby the starting and ending point are coincident (Fig C). Because the depiction of the polygon begins with a start point and travels in one direction, the resulting geometry of the polygon means the GIS can interpret what area is 'right' as opposed to 'left' of the boundary, as well as what area is enclosed by the boundary of the entire polygon; when a knot or loop occurs, the topology of the polygon actually becomes confounded due to the extra node between them. As a result, right and left sides of the boundary violates the topological relationship of the polygon itself, preventing performance of common geoprocessing tasks (clip, intersect and dissolve).

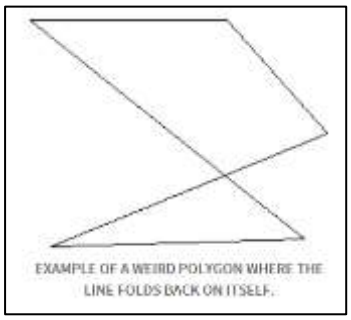


Figure C from https://www.gislounge.com/digitizing-errors-in-gis/

Another confounding topological error involves slivers. "Slivers are gaps in a digitised polygon layer where the adjoining polygons have gaps between them or where the two adjacent polygons overlap in error". This can inadvertently lead to areas among the polygons to have conflicting attributes as to what habitat the slivers represent (Fig D).

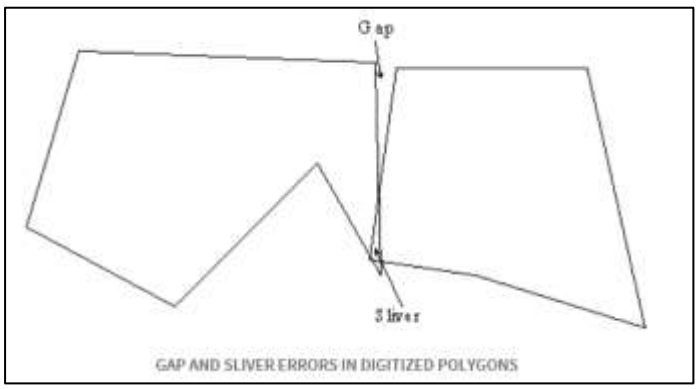


Figure D from https://www.gislounge.com/digitizing-errors-in-gis/

Manual digitizing habitat polygons is time consuming and tedious. For this analysis, and to reduce anticipated issues related to slivers, it was decided early on in the digitizing process that the largest habitat within a circular zone could be left un-transcribed and the void filled utilizing geoprocessing tools in ArcGIS. Unanticipated topological inconsistencies related to knots and loops however prevented these geoprocessing tools to be run and thus required that topology of all individual polygons to be inspected and corrected.

"Topology in GIS is generally defined as the spatial relationships between adjacent or neighboring features" Planar topology requires that intersections for lines and polygons in a digital data layer is enforced and that no two lines or polygons cross. This process involves removing twisted or self-intersecting polygons (i.e. knots and loops) so as to ensure that the "inside" of the polygon is on the correct side of the boundary. It also includes removing overlaps (i.e. slivers) found by intersecting each polygon with all other polygons.

Tools from ET Geowizards 11.3 were used to correct planar topology, rigorously testing and correcting for topological correctness and verifying the spatial relationships between neighboring polygons. Eight circular zone sites were chosen to validate the hand-drawn polygon designations and to determine if the process would improve calculations of polygon areas. Overestimation of the initial polygons varied by only 2.5 % (n=51, t-test no significant difference in paired data p=.875)

Once the topology of the GEP dataset was reconciled, the "Intersect" geoprocessing tool was used to fill voids and assign a habitat code. The dataset was then "clipped" using an "island" polygon to remove those portions of the circular zone that extended past the coastline. Since there were multiple polygons representing a given habitat within a circular zone, the "dissolve" tool was used to consolidate records so that percent habitat calculations could be

completed for each circular zone.

Total area of each habitat type for each 1 km hive site circular zone was summed and the percentage calculated (Table 2 in text). Each circular zone comprised approximately 314.16 hectares, unless ocean surface area was removed. A total of 18,872 hectares of land area was classified for the vector polygon dataset. Visual ground truthing was performed to ensure images in the GEP imagery matched images on the ground.

Comparing results between the C-CAP and GEP Datasets

C-CAP high-resolution land cover for 2010, produced at 2.4 m resolution, was applied to the 38 sites from the 2013 and 2015 sampling and compared to the same data grouped and processed using GEP polygons. For Agriculture and Urban land-cover categories, the two methods produced similar mean values, were not significantly different (t-test), and were well correlated (Table A). For Forest, Open and Water land-cover, the mean values were significantly different.

Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	C-CAP Mean	GEP Mean	t-test	correlation
% Ag	39.4%	36.9%	0.423	0.880
% Forest	36.4%	22.4%	0.000	0.836
% Open	12.4%	18.7%	0.078	0.584
% Urban	9.9%	9.6%	0.898	0.832
% Wetland	1.2%	1.2%	0.990	-0.034
% Water	1.0%	0.6%	0.041	0.532

The percentage coverage for Agriculture calculated with the C-CAP method was plotted versus the percentage coverage for Agriculture calculated with the GEP.

The plot illustrates the difference between GIS analyses of the two datasets and the general under-representation by C-CAP (Figure E).

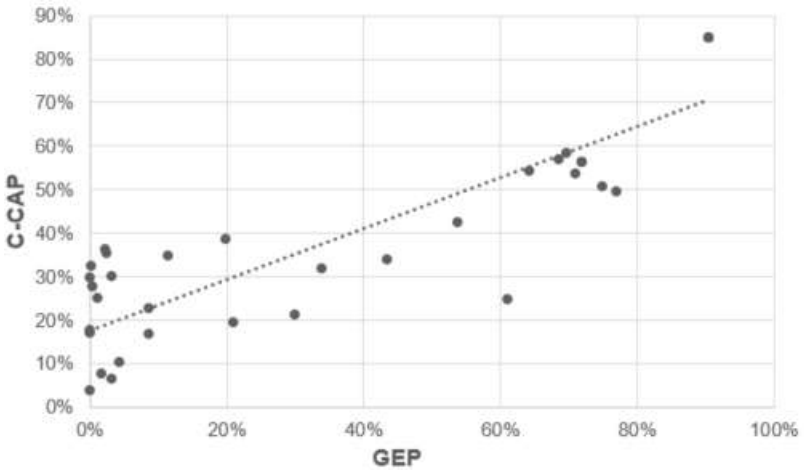


Figure E: Correlation of % Agriculture in areas surrounding hive sites using C-CAP versus GEP analysis. Linear fit: $Y = 0.586 x + 0.177$, $R^2 = 0.775$.

When glyphosate concentrations are plotted against percent acreage in agriculture using the two methods (Fig F), the general trends as expressed by exponential curves are very similar, but the GEP polygon method produces a stronger correlation ($R^2 = 0.71$, AICc = -9.794).

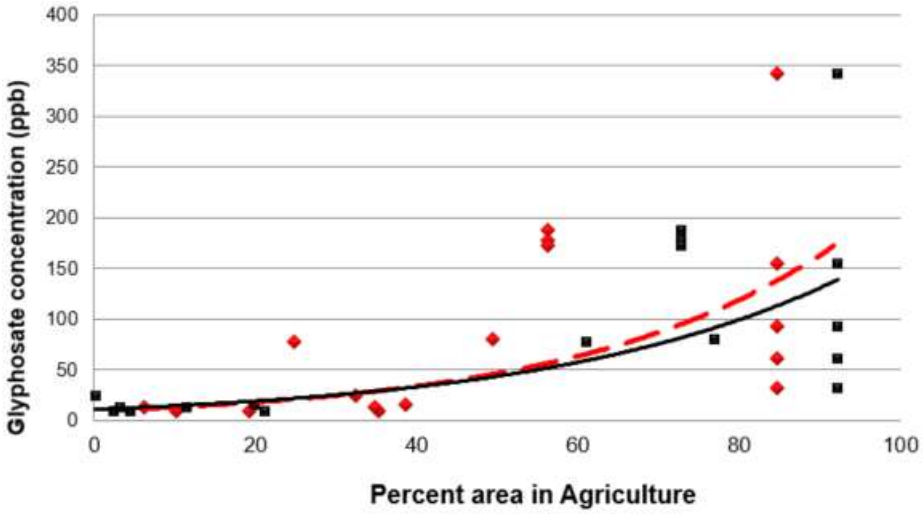


Figure F: Correlation of % Agriculture and Glyphosate concentration surrounding hive sites using C-CAP and GEP analysis. Excel Analyse It software exponential fits produced Y = 9.648 $e^{0.23121x}$, R^2 = 0.48, AICc = 0.173 (C-CAP, red diamonds; dash line) and Y = 11.02 $e^{0.1628x}$, R^2 = 0.71, AICc = -9.794 (GEP Polygons, black squares; solid line).

There are many factors that would explain the differences in the land use designation and the choice of GEP polygons as the most accurate method for determining land use contemporary with honey production. These include:

- Cell size is 2.4 m for C-CAP vs Digital Globe has a 30-50 cm range. A smaller cell size allows for finer delineation and identification of objects.
- Date the image was accessed: 2010 for C-CAP but 2013-2014 for GEP with ground-truthing in areas in question.
- C-CAP would designate a ground cover as forest, but GEP showed it to be an orchard.
- C-CAP would identify open fields as "Open", but GEP showed that cattle are on it, so it is “Agriculture”.
- C-CAP does not recognize little streams or ponds but GEP resolution does.
- C-CAP sees "Forest" but Google Earth shows "Riparian"
- C-CAP see “Urban” but finer detail allows designation as “Rural/Suburban”

Conclusion

Although manually digitizing GEP polygon delineations is more tedious and time consuming, for the above stated reasons and the stronger correlation of the GEP derived curve, only the GEP polygon delineation method was used for final analysis of the relationship between land use and glyphosate concentration

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey

Table A: Store-bought honey; sources and glyphosate concentration.

Sample Origin				**Sample #**	**Glyphosate ppb**
Hawaii	**Island:**	**Moku**	**Area**		
	Kauai	Kona	Waimea Valley	5	15.2
		Kona	Koloa	9	0
		Kona	Kalaheo	11	87
		Kona	Poipu	19	27.2
		Koolau	Waipake	3	5
		Koolau	North/Northeast Kauai	4	6.4
		Koolau	North Shore Kauai	6	60.8
		Koolau	Kilauea	8	0
		Koolau	Kilauea	12	11.2
		Puna	Puhi	1	15
		Puna	Hanamaulu	2	6.2
		Puna	Kapa'a	7	0

		Puna	Kapa'a	10	7
		Puna	Puhi	20	10.4
		Puna	Hanamaulu	21	6.4
	Hawaii Island		Hawaii Island	15	12
			Kealakekua, Big Island	16	7.4
			Kealakekua, Hawaii Island	60	16.4
			Big Island and Oahu	18	8
	Molokai		Molokai	61	0
			Molokai	62	0
Country		Product of Brazil and Canada		17	0
		Product of Brazil and Canada		22	30.6
		Product of Brazil and Canada		14	8.2
		Product of Mexico, Brazil and Uruguay		13	0
		Product of USA and Argentina		23	72.4

Table B: Kauai hive samples categorised by side of island and Moku with glyphosate concentration.

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
WINDWARD							
	Halele'a	2	0				
		10	9.2				
		12	15.2				
		28	12.6				
		31	0				
		32	0				
		44	8.2				
		45	0				
		51	0	9	0	5.0	6.3
	Ko'olau	3	0				
		11	0				
		24	0				
		26	0				
		29	0				
		30	0				
		33	0				
		42	0				
		43	0				
		50	0	10	0	0	0
	Puna	1	13.6				
		4	0				
		5	8.8				
		7	0				
		9	0				
		13	0				
		16	0				
		23	0				
		25	0	9	0	2.5	5.1
LEEWARD							
	Kona	6	80.2				
		8	61.4				
		14	341.6				
		15	0				
		17	0				
		18	24.6				
		19	9.6				

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
		20	155.2				
		21	32.6				
		22	0				
		27	0				
		34	187.2				
		35	178				
		36	171.8				
		37	92.2				
		38	77.6				
		39	0				
		40	10.4				
		41	60				
		46	13				
		47	0				
		49	0				
		52	0				
		53	0				
		54	0				
		55	0				
		56	0				
		57	27.4				
		58	0				
		59	95	30	11.7	53.9	80.9
	Mana	48	292.2	1	292.2	292.2	na
	Napali	None	None	None			

Table C: Summary statistics of glyphosate with Kauai hive samples categorised by side of island.

Windward	Count	28
	Median	0
	Mean	2.41
	SD	4.87
Leeward	Count	31
	Median	13
	Mean	61.61
	SD	90.34

Table D: t-test comparing glyphosate from Windward (Eastern) and Leeward (Western) sides of Kauai. Data from Table B1.

Windward-Leeward:	
t-test probability	0.001
degrees of freedom	57

Table E: t-test comparing glyphosate between Moku pairs. Mana Moku had only one sample, thus could not be compared.

Moku differences	**t-test p**
Kona -Koolau	0.001
Kona - Puna	0.002
Kona - Halelea	0.003
Koolau -Halelea	0.043
Puna -Koolau	0.180
Puna - Halelea	0.361

Table F: Kruskal-Wallis analysis of impact of side of island and Moku on glyphosate concentration.

Y (numerical)	**X (categories)**	**H-stat**	**DF**	**N**	**p-value**
Glyphosate	Side	11.3	1	58	0.00077
Glyphosate	Moku	13.3	3	58	0.0041

Table G: AICc analysis of fits for glyphosate concentration vs. % Agriculture.

	Exp.	**Power**	**Linear**	**Log**	**Polynomial**
R2	0.594	0.174	0.417	0.155	0.429
AICc	-8.664	7.662	194.88	195.232	197.055

Table H: Sample #'s included within Meta-circles and their glyphosate concentrations.

Meta-circle #	**Meta-circle Name**	**Sample #**	**Glyphosate ppb**	**Glyphosate ppb Mean**
1	Kilauea	10	9	
		32	0	
		33	0	
		43	0	
		44	8	3.5
2	Moloaa	11	0	
		24	0	
		26	0	
		30	0	
		42	0	
		50	0	0.0
3	Kapaa	7	0	
		9	0	
		23	0	

Meta-circle #	Meta-circle Name	Sample #	Glyphosate ppb	Glyphosate ppb Mean
		25	0	0.0
4	Lihue	1	14	
		4	0	
		5	9	
		16	0	5.6
5	Koloa	15	0	
		18	25	
		52	0	
		53	0	
		54	0	
		55	0	
		56	0	
		57	27	
		59	95	16.3
6	Lawai	27	0	
		39	0	
		40	10	
		46	13	
		49	0	4.7
7	Agribusiness 1	34	187	
		35	178	
		36	172	179.0
8	Agribusiness 2	8	61	
		14	342	
		20	155	
		21	33	
		37	92	136.6

Table I: Samples by Side and Moku with % Agriculture, % Golf, Hiway Km, and Glyphosate concentrations.

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
1	East	Puna	13.6	3.1 %	4.8 %	2.00
2	East	Halelea	0	0.0 %	0.0 %	2.39
3	East	Koolau	0	70.9 %	0.0 %	1.59
4	East	Puna	0	30.0 %	0.0 %	2.02
5	East	Puna	8.8	21.0 %	0.0 %	1.74
6	West	Kona	80.2	76.9 %	0.0 %	2.03
7	East	Puna	0	3.2 %	0.0 %	0.00
8	West	Kona	61.4	90.5 %	0.0 %	1.65
9	East	Puna	0	1.1 %	0.0 %	0.00
10	East	Halelea	9.2	4.4 %	0.0 %	2.04
11	East	Koolau	0	69.7 %	0.0 %	0.00
12	East	Halelea	15.2	19.8 %	0.0 %	0.00
13	East	Puna	0	8.6 %	0.0 %	0.00
14	West	Kona	341.6	90.5 %	0.0 %	1.65
15	West	Kona	0	43.6 %	0.0 %	4.53
16	East	Puna	0	53.8 %	0.0 %	0.00
17	West	Kona	0	0.0 %	0.0 %	0.00
18	West	Kona	24.6	0.1 %	16.2 %	4.66
19	West	Kona	9.6	2.4 %	1.6 %	3.36
20	West	Kona	155.2	90.5 %	0.0 %	1.65
21	West	Kona	32.6	90.5 %	0.0 %	1.65
22	West	Kona	0	33.9 %	0.0 %	1.44
23	East	Puna	0	0.4 %	0.0 %	0.00
24	East	Koolau	0	64.3 %	0.0 %	1.66
25	East	Puna	0	2.3 %	0.0 %	0.00
26	East	Koolau	0	68.6 %	0.0 %	1.10
27	West	Kona	0	0.0 %	0.0 %	2.29
28	East	Halelea	12.6	11.5 %	13.7 %	2.04
29	East	Koolau	0	75.0 %	0.0 %	2.03
30	East	Koolau	0	8.7 %	0.0 %	2.06
31	East	Halelea	0	0.0 %	0.0 %	2.36
32	East	Halelea	0	1.8 %	0.0 %	2.63
33	East	Koolau	0	0.0 %	0.0 %	1.98
34	West	Kona	187.2	71.9 %	1.2 %	1.08
35	West	Kona	178	71.9 %	1.2 %	1.08
36	West	Kona	171.8	71.9 %	1.2 %	1.08
37	West	Kona	92.2	90.5 %	0.0 %	1.65
38	West	Kona	77.6	61.0 %	0.0 %	2.18
39	West	Kona	0	3.5 %	0.0 %	0.32
40	West	Kona	10.4	12.9 %	0.0 %	0.52
41	West	Kona	60	58.9 %	0.0 %	0.00
42	East	Koolau	0	67.9 %	0.0 %	0.00
43	East	Koolau	0	4.7 %	0.0 %	1.51
44	East	Halelea	8.2	25.3 %	0.0 %	0.00
45	East	Halelea	0	0.0 %	0.0 %	0.00
46	West	Kona	13	0.0 %	0.0 %	1.20
47	West	Kona	0	19.5 %	0.0 %	0.00
48	West	Mana	292.2	16.3 %	0.0 %	0.00
49	West	Kona	0	0.0 %	0.0 %	2.10
50	East	Koolau	0	50.4 %	0.0 %	2.26
51	East	Halelea	0	0.0 %	0.0 %	2.25
52	West	Kona	0	51.6 %	0.0 %	4.73
53	West	Kona	0	51.6 %	0.0 %	4.73
54	West	Kona	0	47.3 %	0.0 %	4.35
55	West	Kona	0	42.6 %	0.0 %	4.58

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
56	West	Kona	0	47.3 %	0.0 %	4.35
57	West	Kona	27.4	43.6 %	0.0 %	4.60
58	West	Kona	0	47.3 %	0.0 %	4.35
59	West	Kona	95	0.2 %	16.2 %	4.66

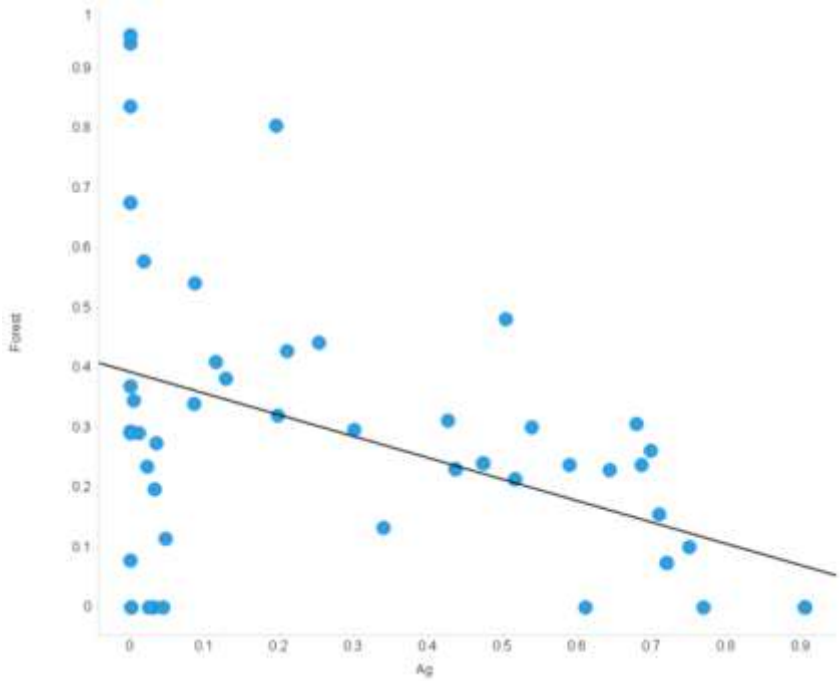


Fig A: Multicollinearity amongst land use types. Samples are plotted with their % Forest vs % Agriculture. $Y = 0.39 – 0.36*X$, $R^2 = 0.23$

B.7.7.1.3.5. Reference 5

1. Information on the study

Data point	CA 6.10.1/007
Report author	Karise, R. *et al.*
Report year	2017
Report title	Are pesticide residues in honey related to oilseed rape treatments?
Document No.	DOI 10.1016/j.chemosphere.2017.09.013 E-ISSN 1879-1298
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicble
Acceptability/Reliability	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Executive summary

Pesticide treatments before and during the flowering of honey bee forage crops may lead to residues in honey. In northern regions oilseed rape belongs to the main forage crops that is mostly cultivated by means of intensive agriculture, including several pesticide treatments. However, in addition to the focal forage crops, pesticides from non-forage crops can spread to wild flowers around fields, and thus the residues in honey would reflect the whole range of pesticides used in the agricultural landscape. The aim of our study was to clarify which currently used pesticides are present in honey gathered from heterogeneous agricultural landscapes after the end of flowering of oilseed crops.

Honey samples (N = 33) were collected from beehives of Estonia during 2013 and 2014, and analysed for residues of 47 currently used agricultural pesticides using the multiresidue method with HPLC-MS/MS and GC-MS and a single residue method for glyphosate, aminopyralid and clopyralid. Residues of eight different active ingredients with representatives from all three basic pesticide classes were determined. Although no correlation was detected between the cumulative amount of pesticide residues and percent of oilseed crops in the foraging territory, most of the residues are those allowed for oilseed rape treatments. Among all pesticides, herbicide residues prevailed in 2013 but not in 2014. Despite the relatively small agricultural impact of Estonia, the detected levels of pesticide residues sometimes exceeded maximum residue level; however, these concentrations do not pose a health risk to consumers, also acute toxicity to honey bees would be very unlikely.

Materials and methods

Study location
Honey samples were gathered from Eastern and Southern Estonia (Ida-Viru, Tartu, Polva and Valga Counties) in 2013 (N = 14) and 2014 (N = 19). This area is representative of typical agricultural landscapes in Estonia with mostly intensively managed fields, forested areas and human settlements. Among other field crops, both winter and spring oilseed rape are often grown in Estonia, and both belong to the common forage crops of honey bees. Within a 2 km radius of each hive there is on average 34.6 ± 20.7 % cultivated land (min. 0.81 %, max. 70.2 %), 48.1 ± 20.6 % forest, 5.3 ± 7.6 % waste and vacant land, 7.6 ± 5.0 % grassland and 2.1 ± 3.6 % garden. The average oilseed crop coverage within the foraging territory remained between 0 and 12.9 %.

Pesticide selection
The 47 active ingredients analysed were selected for the survey as being the most commonly used in Estonian fields according to the pesticide ordering lists of the Tartu County Farmers Association for the year 2013-2014. These include the most commonly used contemporary herbicides (21), fungicides (15) and insecticides (10), and plant growth regulator and retardant (1). The active ingredients searched for were: 2,4D, alpha-cypermethrin, amido-sulphuron, aminopyralid, azoxystrobin, clopyralid, cypermethrin, cyproconazole, deltamethrin, dicamba, dimethachlor, dimethoate, ethyl trinexapac, fenoxaprop-p-ethyl, fenpropidin, florasulam, fludioxonil, fluoxastrobin, flutriafol, fuberidazole, glyphosate, imazalil, imidacloprid, indoxacarb, iodosulfuron-methyl-sodium, lambda-cyhalotrin, MCPA, mefenpyr-diethyl, pencycuron, picloram, pinoxaden, prochloraz, propaquizafop, propiconazole, propoxycarbazone-sodium, prothioconazole, pymetrozine, pyroxsulam, quizalofop-p-ethyl, spiroxamine, sulfosulfuron, tau-fluvalinate, tebuconazole, thiacloprid, triadimenol, triasulfuron and tribenuron-methyl.

Sample collection and handling
A total of 33 honey samples were collected from beehives in the eastern and southern part of Estonia (Tartu County and its near vicinity) during 2013 and 2014 for analysis of pesticide residues. Each honey sample originated from a different apiary, each of which consisted of 10-20 honey bee hives. The sampled hive was selected randomly for testing. The distance between sampled apiaries was at least 4 km in 2013 and at least 8 km in 2014 to preclude overlapping of the main forage area. The samples were gathered from honeycombs within beehives during the honey harvest in mid-July after the end of oilseed rape flowering. Due to the funding allocated for this study, it was decided to concentrate only on honey samples, and in order to cover more apiaries from the largest possible territory, we sampled only one hive per apiary. The honey was extracted from the comb wax and thereafter kept at 5 °C until analysis.

Chemicals and materials
The reference standards of pesticides were purchased from AccuStandard (New Haven, USA) and Dr. Ehrenstorfer (Germany). HPLC grade acetonitrile and methanol were purchased from Merck-Millipore (Darmstadt, Germany). ACS grade formic acid (≥ 96.0 %), acetic acid (glacial, ≥ 99.85 %), and ammonium formate (99 %) were purchased from SigmaeAldrich (St. Louis, MO, USA). Ultrapure deionised water was generated by a Millipore Milli-Q™ system (Billerica, MA, USA). A buffer-salt mixture (1 g trisodium citrate dihydrate, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate and 4 g of anhydrous magnesium sulphate) and a mixture of dSPE (900 mg anhydrous magnesium sulphate, 150 mg PSA and 150 mg C18E) were obtained from Phenomenex (Torrance, CA, USA).
Stock solutions of approximately 1000 mg/L concentration were prepared by weighing 10 mg of standard in a 10 mL graduated flask and dissolving it in acetonitrile. The purity of the standard was taken into account in the preparation of standard solutions of final concentration. The mix of working standard solution with a concentration of 0.01 mg/L was prepared by diluting the appropriate volume of stock solution in acetonitrile. The stock and working standard solution were stored at – 20°C.

Sample preparation
Different sample extraction and detection procedures were used for analysis of the selected pesticides. Most compounds were analysed using QuEChERS extraction methodology followed by detection using GC-MS and UHPLC-MS/MS. Analysis of glyphosate, aminopyralid and clopyralid was performed as single analyses using extraction with methanol.
For analysis of glyphosate, aminopyralid and clopyralid, 5.0 ± 0.1 g of samples were weighed into a 50 mL polypropylene centrifuge tube, then 10 mL of water and 10 mL of methanol were added for extraction. The samples were shaken for 20 min and centrifuged for 10 min at 4500 rpm. An aliquot of extract was transferred to an autosampler vial for analysis by UHPLC-MS/MS.

UHPLC-MS/MS analysis
An Acquity UHPLC system (Waters, USA) coupled to QTrap 5500 (AB SCIEX, USA) equipped with an electrospray ionisation source was used for the analysis of pesticides in honey. The chromatographic conditions for analysis of glyphosate residues in honey are summarised in Table 1 below.

Table 1: Chromatographic conditions for analysis of glyphosate in honey

UHPLC system and conditions		
Column:	Thermo Scientific, Hypercarb, 100 x 2.1 mm, 5 µm	
Column temperature:	40°C	
Injection volume:	10 µL	
Mobile phase:	1 % acetic acid in water	
Column flow:	0.3 mL/min	
MS system and conditions		
	Quantification	*Confirmation*
Scan type:	MRM	MRM
Ionisation mode:	ESI negative	ESI negative
Ion source temperature:	500°C	500°C
Ion spray voltage [V]:	-4500	-4500

Curtain gas nebulizer [psi]:	45	45
Ion source gas 1 [psi]:	40	40
Ion source gas 2 [psi]:	60	60
Declustering potential [V]:	-50	-50
Collision energy [V]:	-20	-16
Mass transition for evaluation [*m/z*]:	168 → 63	168 → 150

Results

Performance of the method
The performance of the method was evaluated according to the EC guidance document SANCO/12571/2013. The method showed good linearity with the determination coefficients, higher than 0.990 for all compounds included in the study. The mean variation of coefficients for repeatability of the method ranged from 3.0 % to 16 %, and the recovery ranged from 78 % to 115 %.
The limit of quantification (LOQ) for which the S/N ratio exceeds 10 was assumed at a concentration level of 0.01 mg/kg for all pesticides with the exception of aminopyralid, clopyralid, glyphosate, dicamba and picloram for which the LOQ was 0.05 mg/kg.

Analysis of the honey samples
The amounts and composition of pesticide residues found in the honey samples differed between years. The residues of glyphosate in honey samples are summarised in Table 2. The agricultural practices generally do not vary so much, but the need for different kinds of pesticides can vary widely from year to year.

Table 2: Concentrations of glyphosate residues found in honey samples in Estonia 2013-2014

Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [1] [µg/kg]**
1	2013	3.4	n.d.
2	2013	5.7	14
3	2013	6.2	**56**
4	2013	12.1	n.d.
5	2013	10	n.d.
6	2013	9.2	n.d.
7	2013	12.9	**62**
8	2013	9.2	n.d.
9	2013	9.1	n.d.
10	2013	14	n.d.
11	2013	8.6	n.d.
12	2013	5.1	n.d.
13	2013	9.2	n.d.
14	2013	9.3	n.d.
Average	*2013*	*8.86*	*44*
% of samples	*2013*		*21*
15	2014	0	n.d.
16	2014	8.6	n.d.
17	2014	1	n.d.

Table 2: Concentrations of glyphosate residues found in honey samples in Estonia 2013-2014			
Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [1] [µg/kg]**
18	2014	3.8	n.d.
19	2014	0	n.d.
20	2014	2.8	n.d.
21	2014	8.8	n.d.
22	2014	2.7	n.d.
23	2014	11.6	(9)
24	2014	12.3	n.d.
25	2014	8.9	n.d.
26	2014	13.3	n.d.
27	2014	11.6	n.d.
28	2014	1.8	n.d.
29	2014	5.9	n.d.
30	2014	5.4	n.d.
31	2014	8.7	n.d.
32	2014	3.5	n.d.
33	2014	4.9	n.d.
Average	*2014*	*6.08*	*9*
% of samples	*2014*		*16*

[1] The numbers in parenthesis represent values under the limits of detection (LOD). The numbers in bold represent values above the maximum residue limits (MRL).

Honey as a product contains surprisingly few pesticide residues compared to bee bread or pollen (Thompson *et al.*, 2014). Pesticide residues in different matrixes differ in their chemical composition and physical characteristics. Fat or lipid soluble compounds tend to contaminate wax, whereas water-soluble compounds are more readily found in nectar or honey. Besides contaminated nectar, honey contamination may also occur via translocation of the compounds from comb wax to honey (Kochansky *et al.*, 2001; Tremolada *et al.*, 2004).

The relatively large areas with natural vegetation, and the low amounts of pesticides used in Estonian agriculture (Eurostat, 2015) has shaped the notion that the bee forage environment should be unpolluted in Estonia and probably also in other Nordic countries. Our results, however, suggest the situation may be of concern.

Despite the general low input of pesticides compared to the average usage over the European countries (Eurostat, 2015), some compounds found in honey samples exceeded the MRL. On the background of landscape characteristics, this might arise from relatively homogeneous land cover type – in Estonia, as in Ireland and the United Kingdom, the landscape in 2015 is dominated by larger areas composed of the same land cover type, also the number of structural green elements in the landscape is small (Eurostat, 2015). Larger forest areas may serve as barriers for bees, for instance. Forests have been shown to negatively affect bumble bees with larger foraging territories (Diaz-Forero *et al.*, 2011). Such barriers may concentrate bees on other land, thus increasing the risk of forage on polluted plants. Honey bees prefer to forage in larger open areas rich in flowers, and flowering crops make up an important part of the forage. Since it is one of the most profitable crops, oilseed rape crops are common in crop rotations: covering 15 % and 11 % of total cultivated land in 2010 and 2015 accordingly (Statistics Estonia, 2012).

In northern regions, the most common group of pesticides sold are herbicides: these comprise more than 70 % of pesticides sold in Estonia (Eurostat, 2015). The higher amounts of herbicide active ingredients needed for effective treatments compared to insecticides, for instance, may also be one reason why herbicide residues in particular were higher in our samples. The amounts of herbicides used on fields may differ from year to year depending on the weather conditions throughout the spring and summer. The amounts of herbicides sold in Estonia were higher in 2013 compared to 2014 (Eurostat, 2015) and this appears to have been reflected in our honey samples. Although pesticide

residues may be retained in soils from the previous year or even from treatments made decades ago (Hilber *et al.*, 2008; Lozowicka *et al.*, 2016), the authors believe this probably did not affect our results because the samples with higher concentrations in 2013 did not show higher residue level in 2014. Most of the locations sampled in 2013 were also sampled in 2014. We suppose that in those cases where we found herbicide residues higher than the MRL, the bees must have foraged on recently treated fields. For instance, glyphosate residues may remain very high in nectar for up to seven days after treatment, as demonstrated by Thompson *et al.* (2014). Glyphosate-based herbicides are the most common herbicides worldwide. Moreover, its usage nowadays has gone beyond pest control purposes – being more of an agricultural instead of a pest management tool (Steinmann *et al.*, 2012). We believe that this is something to consider for reducing the levels of pesticide residue found in food: by excluding the routine spray applications and retaining the weed management purpose of glyphosate, one could facilitate a less polluted environment.
The concentrations of all residues found from honey samples in this study remained below the lethal dose to honey bees. LD50 is measured for 2,4D was 0.0115 mg/bee (Extension Toxicology Network, 1996), clopyralid > 100 mg/bee (Dow AgroSciences, 2007) and glyphosate 100 mg/bee (Thompson *et al.*, 2014), tebuconazole 83 mg/bee, azoxystrobin 200 mg/bee, dimethoate 0.11 mg/bee, thiacloprid 27.89 mg/bee, and tau-fluvalinlate 45 mg/bee (Sanchez-Bayo and Goka, 2014). This means that the concentrations found are definitely below acute lethal dosages, although sub-lethal effects cannot be excluded when considering that at least nurse bees consume the contaminated food until they produce the royal jelly, and also larger larvae are fed with nectar and pollen collected by foragers.

Conclusion

Our results demonstrate that intensively treated oilseed rape fields can be a source for pesticide residue contamination in honey, however no direct correlation was found. We believe that pesticides escape from fields over larger neighboring areas with wild vegetation and contaminate the nectar of wild plants. Our study indicates that most of the agrochemical residues in Estonian honey can originate from oilseed treatments, however the same active ingredients are used for different crops, which is why no direct references can be made. The compounds that were represented in the highest amounts belonged to herbicides, the most frequently used pesticide group in Northern European climatic conditions. In the context of honey as human food, the concentrations of pesticide residues do not pose any health risk to consumers, although in some cases the levels detected exceeded the MRLs. Concerning the health of bees, the residues remained below acute lethality, however some sub-lethal effects cannot be excluded.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The publication is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Although only limited information is given about the validation of the method for the determination of glyphosate residues, the analytical results are most likely reliable. The residue levels found for glyphosate are consistent with the EU-monitoring data published by EFSA for 2016-2017 in that: 1. Most of the samples do not show quantifiable residues of glyphosate. 2. Some samples show residues > 0.05 mg/kg, which indicates that it is appropriate to increase the existing MRL. 3. The measured residue levels are far below the levels found in the tunnel residue study.

Assessment and conclusion by RMS:
The publication describes the monitoring results of residues of, amongst others, glyphosate in honey sampled in Estonia. Residue levels of glyphosate in the investigated specimens accounted from <0.05 mg/kg to 0.062 mg/kg, i.e. the existing MRL for glyphosate in honey (0.05* mg/kg) is therefore exceeded based on this Estonian data set. As already stated by the applicant, the data are well in line with the monitoring data obtained from official EU monitoring labs (see Volume 3, B.7.7.1.2).

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered relevant for the MRL calculation for two reasons: (i) the data were not obtained by official monitoring laboratories; and (ii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

B.7.7.1.3.6. Reference 6

1. Information on the study

Data point	CA 6.10.1/008
Report author	Rubio, R. *et al.*
Report year	2014
Report title	Survey of glyphosate residues in honey, corn and soy products
Document No.	DOI 10.4172/2161-0525.1000249
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

Samples of honey (sixty nine), pancake and corn syrup (twenty six), soy sauce (twenty eight), soy milk (eleven), and tofu (twenty) purchased in the Philadelphia, US metropolitan area were analyzed for glyphosate residue using ELISA. The limit of quantification (LOQ) and range of the method were determined for honey, pancake syrup, and corn syrup to be 15 to 800 ppb; soy sauce, soy milk, and tofu 75 to 4000 ppb. Glyphosate residues above the limit of quantification were not found in pancake and corn syrup, soy milk, and tofu. Of the sixty-nine honey samples analyzed, forty-one samples, or fifty-nine percent (59 %), had glyphosate concentrations above the method LOQ (15 ppb), with a concentration range between 17 and 163 ppb and a mean of 64 ppb. Eleven of the tested honey samples were organic; five of the organic honey samples, or forty-five percent (45 %), contained glyphosate concentrations above the method LOQ, with a range of 26 to 93 ppb and a mean of 50 ppb. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62 %), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb. In addition to comparison of production method (organic *vs.* conventional), the honey results were evaluated according to pollen source and by country of origin, grouped by GMO usage (prohibited, limited, or permitted). Glyphosate concentrations above the method LOQ (75 ppb) were also found in ten of the twenty-eight soy sauce samples evaluated (36 %), with a concentration range between 88 and 564 ppb and a mean of 242 ppb; all organic soy sauce samples tested were below the method LOQ.

Materials and Methods

Chemicals and reagents
Chemicals were of reagent grade and were purchased from Sigma Chemical Company, St. Louis MO, USA, except as indicated. Glyphosate (> 98 % purity), Chem Service, West Chester, PA, USA. Glyphosate micro titer plate ELISA, Abraxis PN 500086; Glyphosate sample diluent, PN 500082, Abraxis LLC, Warminster, PA, USA. Glyphosate stock solution was prepared in deionised water to 1.0 mg/mL; spiking solutions were prepared from the working solution using deionised water.

Samples and sample preparation/extraction
In total, 153 representative samples were purchased from markets in the Philadelphia metropolitan area (69 honey, 26 corn and pancake syrup, 28 soy sauce, 11 soy milk, and 20 tofu products).
Honey, corn and pancake syrup samples: A 0.50 g aliquot of sample was weighed into a micro centrifuge tube and 0.50 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/ extraction described above produced a 1:200 sample dilution.
Soy sauce: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.
Soy milk: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then centrifuged at 6000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Tofu: A 1.0 g aliquot of sample was weighed into a 20 mL vial and 10.0 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then allowed to separate for 2 minutes. Approximately 1 mL of the mixture was transferred into a micro centrifuge tube and centrifuged at 6,000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Determination of glyphosate in samples
The instructions provided in the ELISA kit user's guide were followed, in brief, glyphosate calibrators provided in the kit and the samples to be tested are derivatised for ten minutes and then added, along with an antibody specific for glyphosate to micro titer wells coated with goat anti-rabbit antibody and incubated for thirty minutes with shaking. A glyphosate horseradish peroxidase (HRP) enzyme conjugate is then added. At this point a competitive reaction occurs between the glyphosate, in the calibrators or samples, and the enzyme labeled glyphosate for the antibody binding sites on the micro titer well. The reaction is allowed to continue for sixty minutes. After a washing step an enzyme substrate (hydrogen peroxide) and the chromogen (3,3',5,5'-tetramethylbenzidine) are added. The enzyme-labeled glyphosate bound to the glyphosate antibody catalyzes the conversion of the substrate /chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilised by the addition of diluted acid and read in a Molecular Devices micro titer plate reader (450 nm). Since the labeled glyphosate (conjugate) was in competition with the unlabeled glyphosate (sample) for the antibody sites, the color developed is inversely proportional to the concentration of glyphosate in the sample.

Data analysis
The evaluation of the assay was performed using Molecular Devices Soft max pro evaluation program (4-Parameter). The program calculates the mean absorbance value for each of the standards (Bi) and calculates the %Bi /B0 for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance (B0). The program then constructs a non-linear regression model of a standard curve by plotting the % Bi/B0 for each standard on the vertical linear (y) axis versus the corresponding glyphosate concentration on the horizontal logarithmic (x) axis. The % Bi/B0 for samples is interpolated using the standard curve yielding sample concentration levels of glyphosate from the standard curve. Correlation coefficients of the assays were >0.995 and standard deviation between standard replicate analysis were < 10 %.

Validation, performance and quality control
Specificity had been previously determined (ELISA user's guide), (**Table 1**). Recovery, limit of quantitation, range and limit of quantification were determined to test the validity of the dilution/ extraction procedures of each of the matrices used in combination with the glyphosate ELISA.

Table 1: Cross-reactivity table. The reactivity of glyphosate to various related compounds expressed as LOD and as the dose required for 50 % absorbance inhibition (50 % B/Bo).

COMPOUND (B/Bo)	LOD(ng/mL)	50% B/Bo(ng/mL)
Glyphosate	0.05	0.5
Glyphosine	50	3,000
Glufosinate	2,000	70,000
AMPA	35,000	>1,000,000
Glycine	>10,000	>1,000,000

Results and Discussion

The method performance for glyphosate analysis was determined by conducting recovery tests on each of the matrices. To determine the accuracy of the glyphosate analysis for the sample matrices analyzed in this study, matrix samples that were glyphosate negative and positive (positive samples were not encountered with tofu, soy milk, pancake and corn syrup) were spiked as follows: 15, 40, 100, 200 and 400 ng/mL (honey, pancake and corn syrup); 75, 200, 500, 1000 and 4000 ng/mL [soy sauce, soy milk and tofu (ng/g)]. Analysis was performed in duplicate for all unspiked and spiked samples at all levels. Average recovery obtained for glyphosate negative honey samples fortified with glyphosate was 119 %, (SD = 10). Average recovery for glyphosate positive honey (unspiked contained 44 ng/g glyphosate) after fortification was 116 % (SD = 10). Average recovery for negative soy sauce was 94 % (SD = 5), and for positive fortified soy sauce (unspiked contained 417 ng/mL) was 86 % (SD = 5). The limit of quantification and range of the method were determined for honey, pancake and corn syrup to be 15 to 800 ng/g; soy sauce, soy milk, and tofu 75 to 4000 ng/ mL or ng/g, respectively.

In this study, the first sample matrix analyzed for the presence of glyphosate was honey; 69 samples were analyzed and classified into 18 groups depending on the country of origin listed on the bottles: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. The glyphosate concentrations obtained are shown in (**Figure 2**). Forty-one out of the sixty-nine honey samples analyzed, or fifty nine percent (59 %), had glyphosate concentrations above the method LOQ (15 ng/g) with a concentration range between 17 and 163 ng/g and a mean of 64 ng/g.

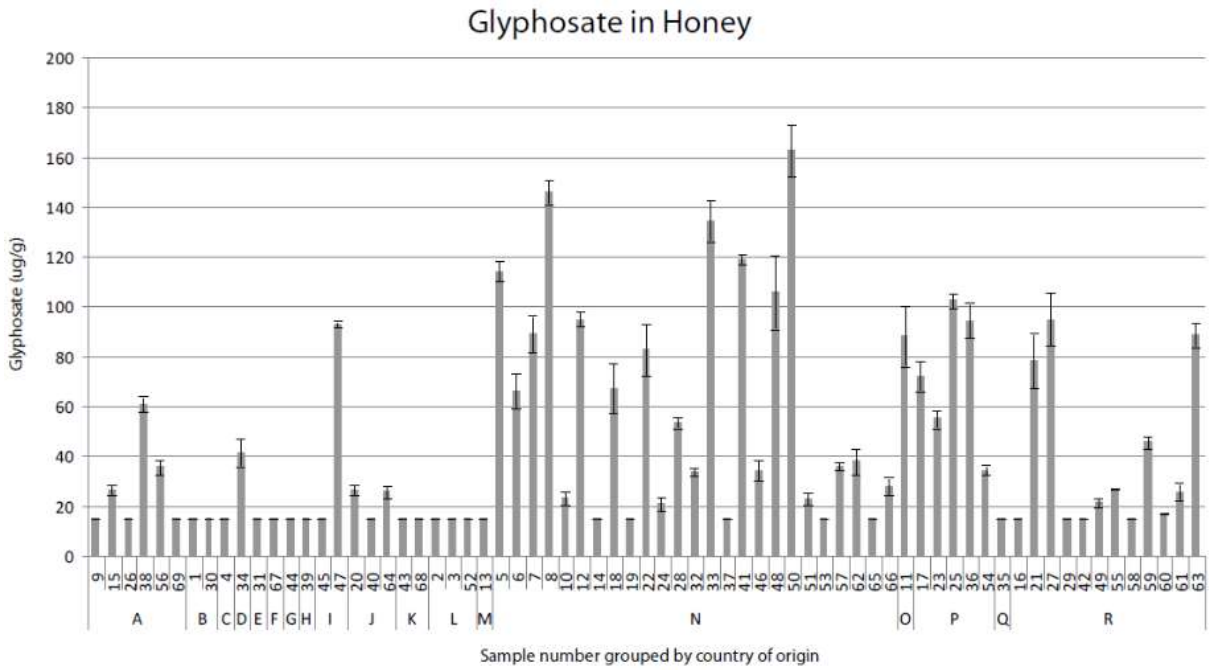


Figure 2: Concentration of glyphosate (ng/g) in honey samples listed by honey origin: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis. [It is noted that the unit on the y-axis is wrong – it should be ng/g instead of µg/g]

The glyphosate concentration in honey grouped by flower (pollen) source is shown in (**Figure 3**). The pollen types listed on the bottles were: clover (12 samples), exotic (11 samples), wildflower (11 samples), unknown (35 samples). (**Figure 4**) depicts the concentration of glyphosate in honey samples grouped by growing method of source pollen: organic (11 samples) and traditional (58 samples); 5 of the 11 organic samples had glyphosate concentrations above the method LOQ with a range of 26 to 93 ng/g and a mean of 50 ng/g. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62 %), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb.

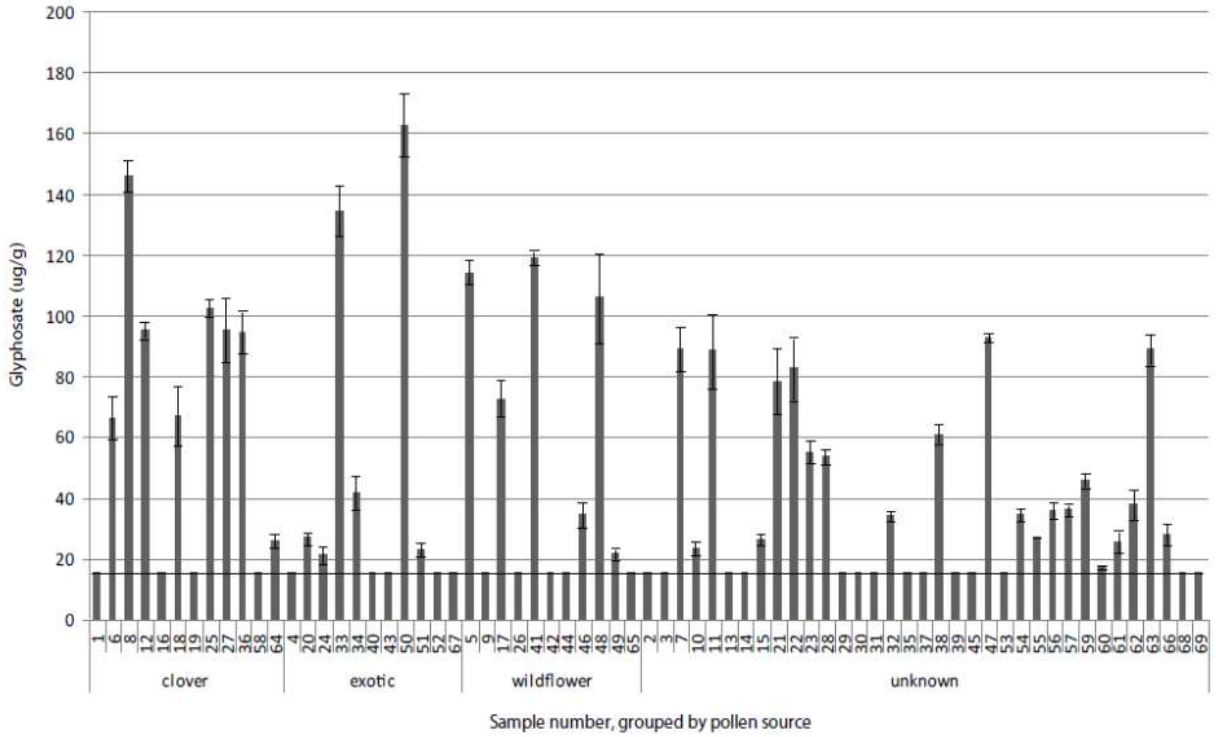


Figure 3: Concentration of glyphosate (ng/g) in honey samples by flower (pollen) source. Dashed line represents LOQ of method (15 ng/g). Exotic flowers were sophora, manuka, orange, cactus, summer flower, lychee, alfalfa, acacia). Error bars represent concentrations obtained during duplicate analysis.

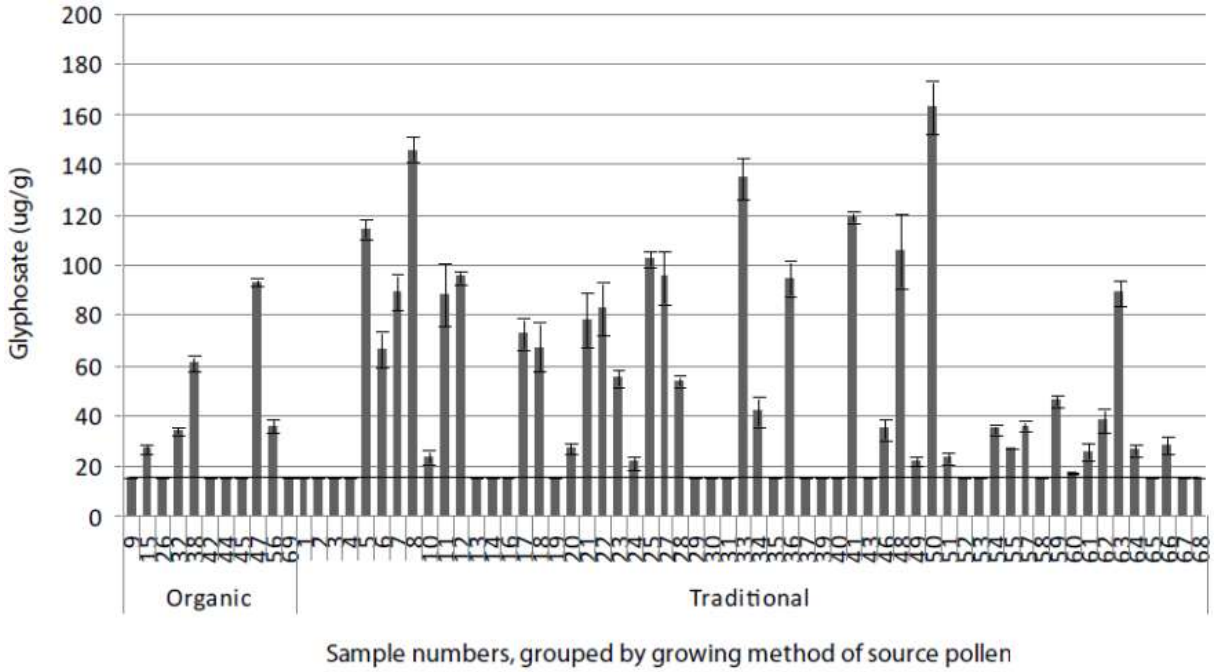


Figure 4: Concentration of glyphosate (ng/g) in honey samples by growing method of source pollen (Organic *vs.* Traditional). Dashed line represents LOQ of method (15 ng/g).) Error bars represent concentrations obtained during duplicate analysis.

Figure 5 depicts the concentration of glyphosate in honey by country and whether the use of genetically modified organisms (GMO) seeds is prohibited or permitted. The graph also shows where some minimum uses of GMO traits are allowed (Spain, and blend of Vietnam/Ukraine). The glyphosate concentration in honey originating in countries that do not allow or allow limited GMO traits (3 out of 14 samples above the LOQ) ranged from 26 to 41 ng/g with a mean of 31 ng/g. The glyphosate range for those countries that allow GMO (30 out of 43 samples above LOQ) was 21 to 163 ng/g with a mean of 71 ng/g. Samples of unknown origin (8 out of 12 samples above LOQ) ranged from 17 to 95 ng/g with a mean of 50 ng/g.

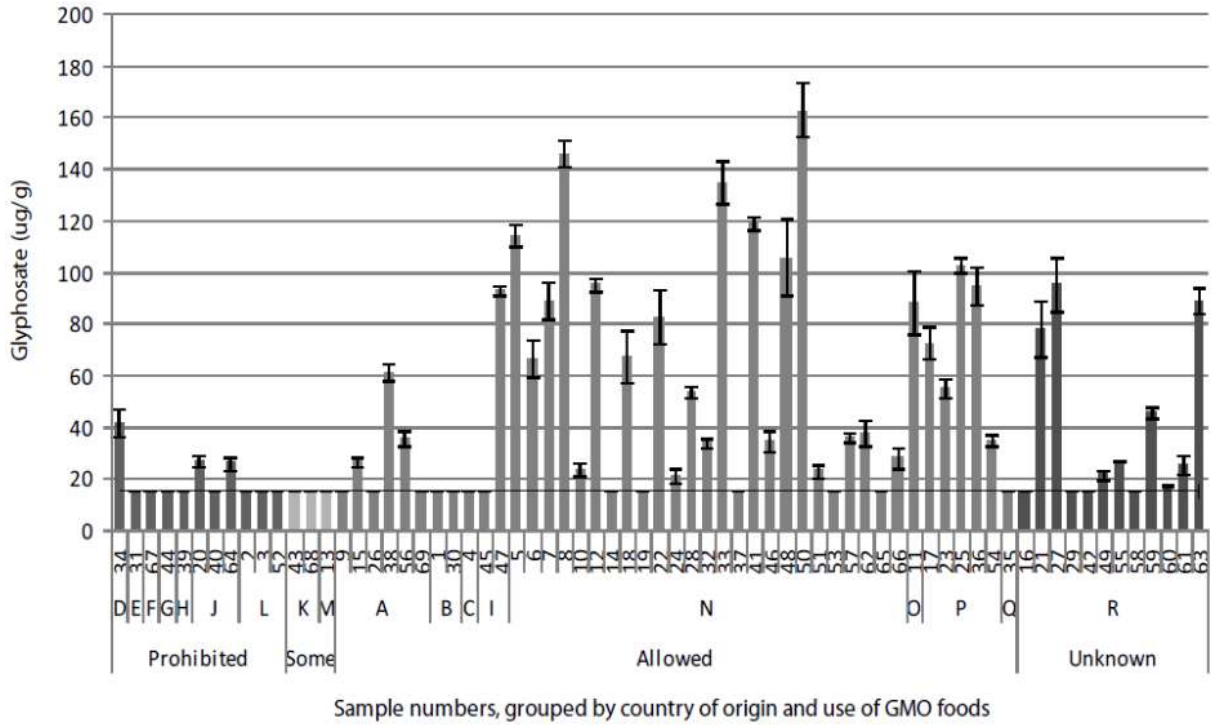


Figure 5: Concentration of glyphosate (ng/g) in honey samples listed by honey origin and the allowance of GMO use: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis.

The second matrix group analyzed for glyphosate was soy sauce. The analysis consisted of 28 samples, (**Figure 6**). Ten out of 28 samples (36 %) had glyphosate concentrations above the method LOQ (75 ng/mL) with a concentration range between 88 and 564 ng/mL and a mean of 242 ng/mL. (**Figure 7**) shows the concentration of glyphosate in soy sauce by method of soy bean growing (organic vs. traditional). The recent report from the Chinese Academy of Medical Science and the Beijing Union Hospital [20] reported an average glyphosate concentration in soy sauce of 133 ng/mL in samples that did not specify on the bottle whether or not the raw material was GM soybean. In our study, the small subset of organic labeled samples (three) was all below the limit of quantitation of the test.

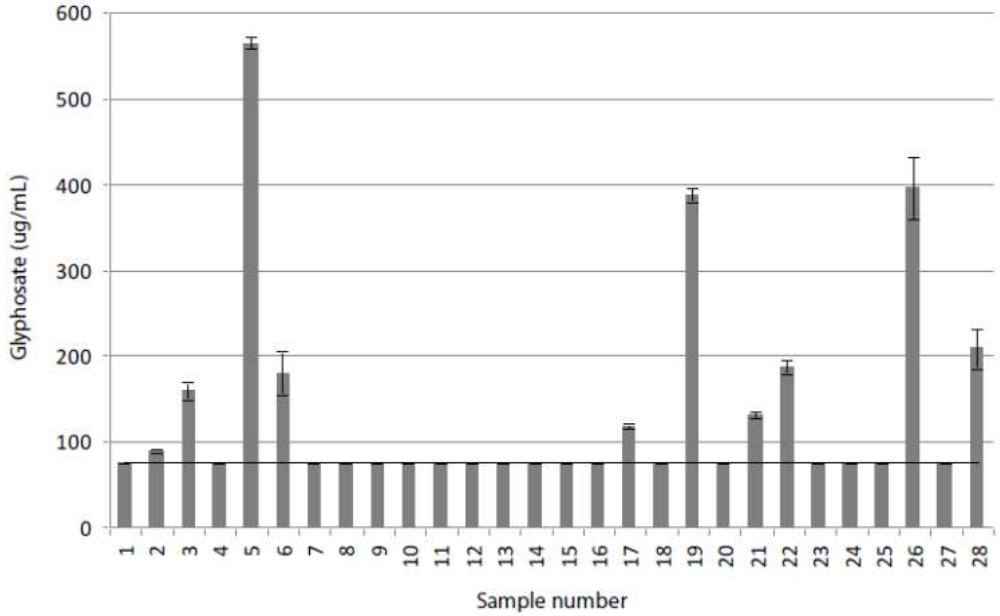


Figure 6: Concentration of glyphosate (ng/mL) in soy sauce samples. Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.

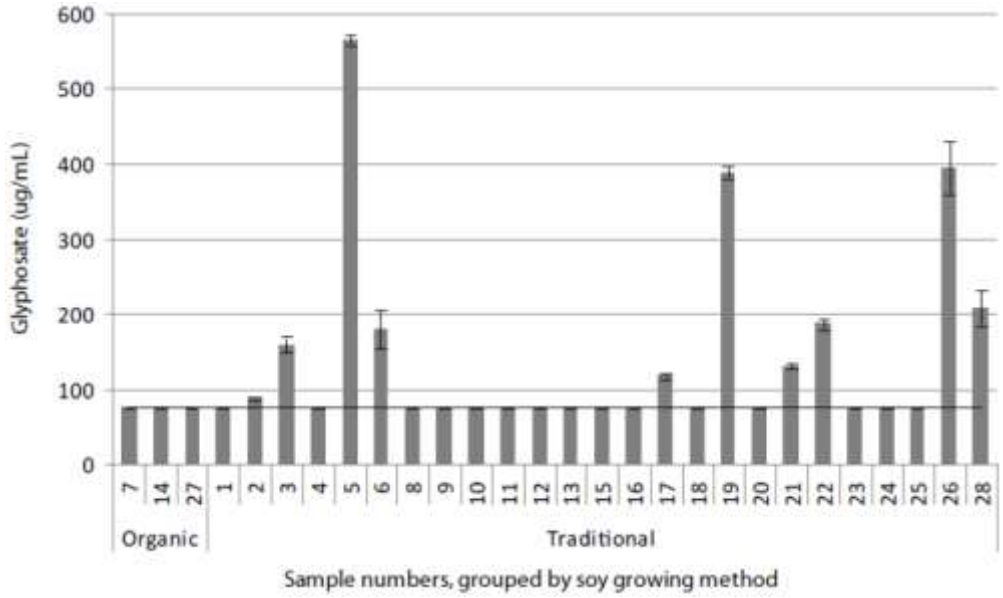


Figure 7: Concentration of glyphosate (ng/mL) in soy sauce samples by growing method of soy beans (Organic *vs.* Traditional). Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.

Corn and pancake syrup (26 samples), soy milk (11 samples), and tofu (20 samples) tested were negative for glyphosate at the LOQ of the method (15 ng/g for pancake and corn syrup, and 75 ng/mL or ng/g for soy milk and tofu, respectively).

Studies on glyphosate residues in food are scarce. Among the few studies found was a recent report published on the incidence of glyphosate in soy sauce, conducted by the Chinese government [20]. Searches were conducted by the authors using various scientific databases on the concentration and incidence of glyphosate in honey, but these failed to provide any information. The honey samples analyzed in the present study show that 59 % of all samples contained glyphosate residues (ranging from 17 to 163 ng/g, mean 64 ng/g); the residue concentration does not seem to depend on pollen source or growing method, even organic honey contained glyphosate residues (5 out 11 samples, or 45 %, mean glyphosate concentration 50 ng/g). Comparing the concentration of glyphosate in honey by countries that use GMO extensively with countries that allow the use of some GMO traits and those that do not allow GMO, shows that,

in general, glyphosate levels are lower in samples from countries that do not allow or allow limited use of some GMO traits, such as Spain and Vietnam/ Ukraine blend (mean 31 ng/g), compared to those countries that allow planting of GMO traits (71 ng/g). It should be noted, however, that some residues of glyphosate (although < 50 ng/g) were found in honeys originating from Germany and New Zealand, countries where no GMO planting is allowed.

The European Union has specific guidelines for the labeling of organic honey [25,26]. According to those guidelines, the location of apiaries is strictly controlled and states that "Nectar and pollen sources available over a three-kilometer radius around the apiary sites must consist essentially of organically produced crops or crops treated with low-environmental-impact methods. Apiaries must also be far enough away from any non-agricultural production source that could lead to contamination (*e.g.* urban centers, waste dumps, waste incinerators, etc.). Member States have the option of prohibiting the production of organic honey in certain regions or areas that do not meet these conditions. Organic honey must not contain chemicals residues (synthetic pesticides, etc.)." The United States has no such guidelines for the organic production of honey, but uses organic farming certification for honey labeling purposes; one reason is that it is practically impossible to regulate without testing all honey for residues since bees can fly up to 3 miles in search of nectar and it is difficult to be certain that they do not feed on nectar contaminated by crop spraying or industrial sources. In the EU, glyphosate residues in non-organic honey regulatory limits are 50 ng/g [27], the United States does not have a limit in honey. The limit in drinking water in the United States is 700 ng/mL; the reference dose is 1.75 mg/kg/day; the One-Day Health Advisory level is 20 mg/L [28]. Also, it is widely known that like milk and olive oil, honey is one of the foods that is most commonly mislabeled and adulterated [29] providing yet another source of glyphosate contamination in honeys that, according to the bottle label, originated in non-GMO countries.
Bee colony collapse disorder (CCD) is a growing threat to the efficient production of food around the world. Honey bees pollinate nearly 130 species of plant life [30], such as fruits, vegetables, nuts, and seed crops. Honeybees are therefore indirectly responsible for an estimated one-third of the world food supply [31]. Although several factors are involved in CCD, including numerous pathogens and parasites, the extensive use of pesticides [32,33] such as neonicotinoids have provided evidence that these products are harmful to honey bees and have lead to a recent ban or restriction in the use of three neonicotoids by the European Union [34]. Although glyphosate is not acutely toxic to bees, it is chronically toxic to animals and is reported to disrupt the endocrine system [35,36] and a recent study indicates that honey bees exposed to increasing sub-lethal concentrations of glyphosate exhibit a decrease in acetyl cholinesterase (AChE) activity [37]. The high rate of glyphosate use creates the potential for wide-spread contamination of our food chain. Glyphosate is used throughout the bee foraging period in high amounts and is found in the air, water, and in plant parts frequented by bees, such as flowers and buds, potentially contaminating the nectar collected by bees from contaminated plants [38]. Based on its prevalence in the environment, as well as our findings in honey samples, we propose that future studies should be conducted to determine if glyphosate is in fact a contributing factor in CCD.

Conclusions

This study indicates the presence of glyphosate residues in honey and soy sauce, but not in pancake and corn syrups or soy based products such as soy milk and tofu. Forty one out of sixty nine (59 %) honey samples analyzed contained glyphosate at a concentration above the method LOQ (15 ng/g) with a range between 17-163 ng/g and a mean of 64 ng/g. Ten out of twenty eight (36 %) soy sauce samples contained glyphosate at a concentration above the method LOQ (75 ng/mL) with a range between 88-564 ng /mL and a mean of 242 ng /mL. Future studies should be conducted on many other food products to determine the extent of glyphosate residue contamination.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a survey of glyphosate residues in honey (n = 69), pancake and corn syrup (n = 26), soy sauce (n = 28), soy milk (n = 11) and tofu (n = 20) purchased in USA, but originating from various countries around the globe. In the context of the dossier for the renewal of the EU approval of glyphosate and with regard to the supported representative uses, the residue data for pancake and corn syrup, soy sauce, soy milk and tofu are not considered relevant. However, the residue data for glyphosate in honey are potentially relevant since according to the guideline SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Only few of the analysed honey samples originated from Europe but, as honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated by determining the recovery rates from fortified samples. The validation results are not provided in detail, but the average recoveries and relative standard deviations were satisfactory, although the validation was not conducted exactly in accordance with EU or OECD guidelines (i.e. with at least 5 replicates at the LOQ and 5 replicates at a higher level). The limit of quantification was estimated at 0.015 mg/kg. The specificity of the method was investigated by assessing the response of the ELISA test to a series of substances chemically related to glyphosate and it was shown that the response of these substances was at least 1000 times less than that of glyphosate. While this experiment allows to exclude some possible sources of false-positive results, it does not allow to completely rule out that other (not tested compounds) may yield false positive results. Despite these limitations, the obtained analytical results are considered fairly reliable.

59 % percent of the 69 honey samples contained glyphosate residues above the method LOQ (0.015 mg/kg) with a concentration range between 0.017 and 0.163 mg/kg and a mean of 0.064 mg/kg. While the individual results are not provided, it seems that about 31 % of the samples (22 from 69) showed residues of glyphosate above the EU MRL of 0.05 mg/kg. The samples originating from the EU all showed residues < 0.05 mg/kg. Overall, the findings reported in the publication are in line with the results of the EU-monitoring since the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.163 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

Assessment and conclusion by RMS:

The publication describes the monitoring of residues of glyphosate in commercial honey sampled in the Philadelphia metropolitan area (United States). Residue levels of glyphosate in the investigated specimens (n = 69) accounted from <0.015 mg/kg to 0.163 mg/kg, i.e. the existing MRL for glyphosate in honey (0.05* mg/kg) is exceeded based on this mixed data set. As already stated by the applicant, the data are well in line with the monitoring data obtained from official EU monitoring labs (see Volume 3, B.7.7.1.2). The study provided additional interesting information regarding the distribution of glyphosate residues between countries and different types of agricultural practise (organic vs. conventional production; the allowance of use of GMO crops), but these are considered less relevant for evaluation.

The Guidance Document SANTE/11956/2016 rev. 9 indeed gives the possibility to set temporary MRLs based on monitoring data, however, the obtained data are not considered relevant for the MRL calculation for two reasons: (i) the data were not obtained by official monitoring laboratories; and (ii) the RMS calculates the MRL based on the available tunnel residue trial. For further details, it is referred to Volume 1, Section 2.7.10.

B.7.8. REFERENCES RELIED ON

B.7.8.1. Literature search strategy

A literature search for glyphosate and its metabolites AMPA, *N*-acetyl-AMPA, *N*-acetyl-glyphosate, *N*-methyl-AMPA, *N*-glyceryl-AMPA, *N*-malonyl-AMPA, HMPA, methylphosphonic acid and *N*-methylglyphosate was conducted according to the requirements stated in the EFSA Guidance document (EFSA Journal 2011;9(2):2092). The basic input parameters were the substance name, UPAC, chemical name or CAS number.
The search filter for the residues section was:
uptake OR translocation OR rumen OR storage stability OR storage OR stability OR metabolic OR metabolism OR breakdown OR nature of residues OR residue? OR magnitude of residues OR process? OR effects of processing OR dessicant OR preharvest OR preemerg? OR ?resistant? OR ?toleran? OR transgenic OR hydroly? OR rotation? OR succeed? OR plant? OR crop? OR feed? OR animal? OR livestock? OR hen OR cattle OR ruminant? OR goat? OR cow? OR pig? OR dietary OR assessment OR risk assessment OR consum? OR exposure

The literature search was performed by accessing 11 bibliographic databases via the service provider STN (AGRICOLA, BIOSIS, CABA, CAPLUS, EMBASE, ESBIOBASE, MEDLINE, TOXCENTER, FSTA, PQSCITECH, and SCISEARCH). A justification for the choice of the databases has been provided.
Due to a large amount of public literature available for the active substance glyphosate, the search has been divided into six parts, by splitting publication periods and metabolites into different searches (see table B.7.8.1-1).

Table B.7.8.1-1: Overview of the searches conducted for glyphosate and its metabolites

Search	Performed for	Covering publication period	Conducted on
Part 0	glyphosate, AMPA, N-acetyl-AMPA and N-acetyl-glyphosate	Jan 2010 – Dec 2011	28th Oct 2019
Part 1	glyphosate, AMPA, N-acetyl-AMPA and N-acetyl-glyphosate	Jan 2012 – Dec 2017	08th Jun 2018
Part 2a	glyphosate, AMPA, N-acetyl-AMPA and N-acetyl-glyphosate	Jan 2018 – Dec 2018	04th Jul 2019
Part 2b		Jan 2019 – Jun 2019	10th Jul 2019
Part 3	glyphosate, AMPA, N-acetyl-AMPA and N-acetyl-glyphosate	Jul 2019 – Dec 2019	7th Jan 2020
Part 4	HMPA	Jan 2010 – Feb 2020	24th Feb 2020
Part 5a	N-methyl-AMPA, N-glyceryl-AMPA, N-malonyl-AMPA	Jan 2010 – Feb 2020	27th Feb 2020
Part 5b	methylphosphonic acid	Jan 2010 – Feb 2020	27th Feb 2020
Part 6	N-methylglyphosate	Jan 2010 – April 2020	04th May 2020

AMPA = (aminomethyl)phosphonic acid
HMPA = (hydroxymethyl)phosphonic acid

As the number of records from these searches in some cases was still very large, some searches were further split into 'focussed searches for grouped data requirements'.

Subsequently, all six parts of the literature search were combined, and upon removal of duplicates 11,326 articles in total were identified. All 11,326 articles were subsequently assessed for their relevance at title/abstract level (via 'rapid assessment' according to the procedure and requirements in the EFSA Guidance document (EFSA Journal 2011;9(2):2092)).
Articles identified as 'non-relevant' in the rapid assessment belong to one of the following categories. These articles were excluded from further evaluation:

- Publications related to efficacy (resistance related articles, new uses of control of pest/crops) or to agricultural / biological research (crop science, breeding, fertilization, tillage, fundamental plant physiology / micro / molecular biology).
- Publications dealing with analytical methods / development.
- Publications describing new methods of synthesis (discovery / developments) or other aspects of basic (organic / inorganic) chemistry.
- Patents.
- Wastewater treatment.
- Abstracts referring to a conference contribution that does not contain sufficient data / information for risk assessment.

- Publications focusing on genetically modified organisms / transgenic crops; no data directly relevant to glyphosate evaluation (e.g. crop compositional analysis, gene flow, protein characterization).
- Publications where glyphosate or a relevant metabolite were not the focus of the paper.
- Secondary information including scientific and regulatory reviews.
- Articles dealing with political / socio / economic analysis.
- Observations caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity).
- Study design, test system, species tested, exposure routes etc. are not relevant for the European regulatory purposes.
- Findings related to ecotoxicology, toxicology, metabolism, environmental fate.
- Publications not dealing with EU representative uses / conditions (e.g. field locations, soil properties, non-EU monitoring etc.).

A total of 9,784 of the 11,326 articles were identified as 'non-relevant' in the rapid assessment and excluded from further evaluation. For the remaining 1,542 articles, identified as potentially 'relevant' or of 'unclear relevance' in the rapid assessment, the full-text documents have been reviewed in detail ('detailed assessment').
Articles that have been identified as 'non-relevant' in the detailed assessment belong to one of the following categories:

- Publications dealing with a Roundup formulation that is not the representative formulation for the AIR5 dossier in Europe.
- Publications dealing with general pesticide exposures (not glyphosate specific).
- The presented endpoints are not relatable to the EU level risk assessment.
- Opinion articles where no new data is provided that can be used for risk assessment.
- Findings based on cellular and molecular level that cannot be related to the risk assessment.
- Criteria outlined for the 'rapid assessment', that needed the full text document to determine.

A total of 852 articles of the remaining 1,542 articles were identified as 'non-relevant' in the detailed assessment and were excluded from further evaluation. The remaining 690 articles identified as 'relevant' in the detailed assessment were classified according to the EFSA Guidance Document (Category A, B and C).

Category A: Studies that provide data for establishing or refining risk assessment parameters. These studies have been summarised and are presented in the respective dossier sections.
Category B: Studies that are relevant to the data requirement, but in the opinion of the applicant provide only supplementary information that does not alter existing risk assessment parameters (a justification for such a decision should be provided).
Category C: Studies for which relevance cannot be clearly determined. For each of these studies the applicants should provide an explanation of why the relevance of such studies could not be definitively determined.

For articles, which have been identified as category A, a reliability assessment has been performed by the applicant. The applied reliability criteria relevant for the residues section were the following:

- For guideline-compliant studies (GLP studies): OECD, OPPTS, ISO, and others. The validity/quality criteria listed in the corresponding guidelines are met.
- (No) previous exposure to other chemicals is documented (where relevant).
- The test substance is dissolved in water or non-toxic solvent.
- Test item is sufficiently documented, and reported (i.e. purity, source, content, storage conditions).
- Only glyphosate or its metabolites is the test substance (excluding mixture), and information on application of the test substance is described.
- The endpoint measured can be considered a consequence of glyphosate (or a glyphosate metabolite).
- Study design / test system is well described, including when relevant: concentration in exposure media (dose rates, volume applied, etc.), dilution/mixture of test item (solvent, vehicle) where relevant.
- Analytical verifications performed in test media (concentration) / collected samples, stability of the test substance in test medium should be documented.
- Assessment of the statistical power of the assay is possible with reported data.
- Statistical methodology is reported (e.g., checking the plots and confidence intervals).
- Field locations relevant / comparable to European conditions.
- Characterization of soil: texture (sandy loam, silty loam, loam, loamy sand), pH (5.5-8.0), cation exchange capacity, organic carbon (0.5-2-5%), bulk density, water retention, microbial biomass (~1% of organic carbon).

- For tests including agricultural soils, they should not have been treated with test substance or similar substances for a minimum of 1 year.
- Data on precipitation is recorded.
- The residue data can be linked to a clearly described GAP table, appropriate in the context of the renewal of approval of glyphosate (crop, application method, doses, intervals, PHI).
- Analytical results present residues measurements which can be correlated with the existing residues definition of glyphosate, and where relevant its metabolites.
- Analytical methods are clearly described; and adequate statement of specificity and sensitivity of the analytical methods is included.
- Monitoring data: description of matrix analysed, and analytical methods to be fully described.

Articles of category A which have been identified as non-reliable were downgraded to articles of category B (relevant but supplementary).

Specifically for the residues section, the results of the literature search are shown in the following table (table B.7.8.1-2).

Table B.7.8.1-2: Results of the article selection process for residues

	Number
Total number of records after merge of all searches a) and removal of duplicates.	475
Number of articles excluded after rapid assessment (title / abstract).	405
Total number of full-text documents assessed in detail.	70
Number of articles excluded after detailed assessment (*i.e.* not relevant).	30
Number of articles not excluded after detailed assessment. b)	40
Number of summaries presented in the dossier. c)	11
a) After all searches: Part 0, 1, 2, 3, 4, 5a&b, 6. b) All articles belonging to the category A, B, C. c) Summaries presented in the dossier: articles classified as relevant (EFSA GD, Point 5.4.1, category A) & reliable or relevant (EFSA GD, Point 5.4.1, category A) & reliable with restrictions.	

Assessment and conclusion by RMS:

From table B.7.8.1-2, it can be observed that 70 studies for residues have been checked in detail by the applicant. From these 70 studies, 11 studies have been summarized (Category A studies), and are presented in the respective dossier sections. Since one Category A study relates to the consumer risk assessment, which is not a separate dossier section in Vol. B.7, this one study is evaluated under **B.7.8.2**. In addition, one study was included for the ecotoxicology section, however, it appears that this study also contains residues information, and therefore, a summary of this publication has been presented in the respective residues dossier section (Ruuskanen et al, 2020; included in B.7.4).

It has been checked whether the RMS can agree on the 59 remaining publications: 19 articles are included by the applicant as Category B (relevant but supplementary); 10 publications are included as Category C; and 30 publications are considered by the applicant as not relevant after detailed assessment. Furthermore, 405 articles have been excluded by the applicant after the rapid assessment. These latter publications have been quickly checked whether we can agree on their exclusion based on their titles.

Since there are many publications which contain some monitoring results; or which contain analytical method development data, probably also containing some monitoring results; and the results from such publications are not directly expected to have impact on the risk assessment parameters; while such publications can be considered 'socially relevant', the RMS has made a selection for inclusion of such publications into the renewal dossier. In addition, the RMS proposes to include all publications related to the use of glyphosate as desiccant (i.e. pre-harvest application). Furthermore, the RMS has requested to include all Category C studies, which all relate to possible microbe-related effects. These study summaries have been requested, and these have been provided by the applicant.

Category A studies: 11 + 1 (from the ecotoxicology search) publications, which are presented in their respective dossier sections.

Author	**Year**	**Title**	**Source**	**Proposal by applicant**	**RMS conclusion**	**Reference for summary**

Shehata A. A. et al.	2014	Distribution of Glyphosate in Chicken Organs and its Reduction by Humic Acid Supplementation.	Journal of Poultry Science (2014), Vol. 51, No. 3, pp. 333	Reliable with restrictions	Supportive, no impact on existing risk assessment parameters	B.7.4.1.4	
Ruuskanen S. et al.	2020	Female Preference and Adverse Developmental Effects of Glyphosate-Based Herbicides on Ecologically Relevant Traits in Japanese Quails.	Environmental science & technology (2020), Vol. 54, No. 2, pp. 1128-1135	Excluded for ecotoxicology after detailed assessment, justification: Formulation used is not the representative formulation for the Annex I renewal.	Supportive, no impact on existing risk assessment parameters	B.7.4.1.5	
Schnabel K. et al.	2017	Effects of glyphosate residues and different concentrate feed proportions on performance, energy metabolism and health characteristics in lactating dairy cows.	Archives of animal nutrition (2017) Vol. 71, No. 6, pp. 413	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.4.2.5	
Shelver W. L. et al.	2018	Distribution of Chemical Residues among Fat, Skim, Curd, Whey, and Protein Fractions in Fortified, Pasteurized Milk	ACS Omega (2018), Vol. 3, No. 8, pp. 8697	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.4.2.4	
von Soosten D. et al.	2016	Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows.	Journal of dairy science (2016), Vol. 99, No. 7, pp. 5318	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.4.2.6	
Zoller O. et al.	2018	Glyphosate residues in Swiss market foods: monitoring and risk evaluation.	Food additives & contaminants. Part B, Surveillance (2018), Vol. 11, No. 2, pp. 83.	Uncertain reliability	Supportive, no impact on existing risk assessment parameters	B.7.8.2.1	
Berg C. J. et al.	2018	Glyphosate residue concentrations in honey attributed through geospatial analysis to proximity of large-scale agriculture and transfer off-site by bees.	PloS one (2018), Vol. 13, No. 7, pp. 0198876	Reliable with restrictions	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.4	
Chiesa L. M. et al.	2019	Detection of glyphosate and its metabolites in food of animal origin based on ion-chromatography-high resolution mass spectrometry (IC-HRMS).	Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment (2019), Vol. 36, No. 4, pp. 592	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.3	
El Agrebi N. et al.	2020	Honeybee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues	The Science of the total environment, (2020), Vol. 704, pp. 135312	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.1	

		in beebread, wax, and honey.				
Karise R. et al.	2017	Are pesticide residues in honey related to oilseed rape treatments?.	Chemosphere (2017), Vol. 188, pp. 389	Reliable with restrictions	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.5
Rubio F. et al.	2014	Survey of Glyphosate Residues in Honey, Corn and Soy Products	Journal of Environmental and Analytical Toxicology (2014), Vol. 5, pp. 249	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.6
Thompson T. S et al.	2019	Determination of glyphosate, AMPA, and glufosinate in honey by online solid-phase extraction-liquid chromatography-tandem mass spectrometry.	Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment (2019), Vol. 36, No. 3, pp. 434	Reliable	Supportive, no impact on existing risk assessment parameters	B.7.7.1.3.2

Category B studies: 9 out of the 19 publications have been requested to be summarized. These 9 studies are evaluated in **B.7.8.3**.

Author	**Year**	**Title**	**Source**	**Proposal by applicant**	**RMS conclusion**	**Reference for summary**
Tong M. et al.	2017	Uptake, Translocation, Metabolism, and Distribution of Glyphosate in Nontarget Tea Plant (Camellia sinensis L.).	Journal of agricultural and food chemistry (2017), Vol. 65, No. 35, pp. 7638	Relevant but supplementary information: Supplementary information on the uptake and metabolism of glyphosatephoste applied in nutrient solution totea plants.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.4
Wood L. J.	2019	The presence of glyphosate in forest plants with different life strategies one year after application.	Canadian Journal of Forest Research (2019), Vol. 49, No. 6, pp. 586	Relevant but supplementary information: In order to properly interpret the findings of the publication, it would be important to determine the residues in the non-target crops shortly after application. However, this information is only available indirectly from other studies. According to the publication : "Compared with levels detected in forest plants immediately after application by Feng and Thompson (1990), levels detected in this study are very low." This means that the residues shortly after application were extremely high, far above the levels that may occur in non-target plants in Europe due to contamination by spray-drift. For this reason and after full text review, the publication is considered to be of limited	Agree with proposal by applicant	-

				relevance to the EU renewal dossier. It only provides supplementary information.		
Tongo I. et al.	2015	Human health risks associated with residual pesticide levels in edible tissues of slaughtered cattle in Benin City, Southern Nigeria.	Toxicology Reports (2015), Vol. 2, pp. 1117	Relevant but supplementary information: Provides information on the relative residue levels in various edible cattle tissues but since the exposure of the cattle is not known no transfer factors can be derived.	Agree with proposal by applicant	-
Chiarello M. et al.	2019	Fast analysis of glufosinate, glyphosate and its main metabolite, aminomethylphosphonic acid, in edible oils, by liquid chromatography coupled with electrospray tandem mass spectrometry.	Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment (2019), Vol. 36, No. 9, pp. 1376	Relevant but supplementary information: Residue analytical method. Olive oil is relevant to the uses considered for renewal in the EU. But only few real samples analysed and all showed residues < LOQ which can be predicted from the physical-chemical properties of glyphosate and AMPA.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.5
Ehling S. et al.	2015	Analysis of Glyphosate and Aminomethylphosphonic Acid in Nutritional Ingredients and Milk by Derivatization with Fluorenylmethyloxycarbonyl Chloride and Liquid Chromatography-Mass Spectrometry.	Journal of agricultural and food chemistry (2015), Vol. 63, No. 48, pp. 10562	Relevant but supplementary information: Selected analysis of samples that provide confirmatory results.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.6
Jansons M. et al.	2018	Occurrence of glyphosate in beer from the Latvian market.	Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment (2018), Vol. 35, No. 9, pp. 1767	Relevant but supplementary information: Includes information on residues in beer. Not directly relevant to dietary risk assessment but provides supplemental information.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.7
Larsson M. O. et al.	2017	Quantifying dietary exposure to pesticide residues using spraying journal data	Food and Chemical Toxicology (2017), Vol. 105, pp. 407	Relevant but supplementary information: Estimate of glyphosate exposure based on spray data in DK. Supplemental to risk assessment.	Agree with proposal by applicant	-
Larsson M. O. et al.	2018	Refined assessment and perspectives on the cumulative risk resulting from the dietary exposure to pesticide residues in the Danish population	Food and Chemical Toxicology (2018), Vol. 111, pp. 207	Relevant but supplementary information: Refined dietary risk assessment for Danish population. Supplementary to DRA included in submission.	Agree with proposal by applicant	-

Liao Y. et al.	2018	Validation and application of analytical method for glyphosate and glufosinate in foods by liquid chromatography-tandem mass spectrometry.	Journal of chromatography. A (2018), Vol. 1549, pp. 31	Relevant but supplementary information: This is primarily an analytical method paper, but does include EU monitoring results on 136 food samples (only 2 residues detected).	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.8	
McQueen H. et al.	2012	Estimating maternal and prenatal exposure to glyphosate in the community setting.	International journal of hygiene and environmental health (2012), Vol. 215, No. 6, pp. 570	Relevant but supplementary information: Study estimated dietary exposure of pregnant women to glyphosate by survey and food analysis. Exposure is well within the National Estimated Daily Intake.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.9	
Poulsen M. E. et al.	2017	Results from the Danish monitoring programme for pesticide residues from the period 2004-2011	Food Control (2017), Vol. 74, pp. 25	Relevant but supplementary information: Summary of EU monitoring data.	Agree with proposal by applicant	-	
Skretteberg L. G. et al.	2015	Pesticide residues in food of plant origin from Southeast Asia - A Nordic project	Food Control (2015), Vol. 51, pp. 225	Relevant but supplementary information: Monitoring data that may be relevant to the actual exposure of EU consumers to glyphosate residues. But non EU data, therefore, not directly linked to the representative uses.	Agree with proposal by applicant	-	
Stephenson C. L. et al.	2016	An assessment of dietary exposure to glyphosate using refined deterministic and probabilistic methods.	Food and chemical toxicology (2016), Vol. 95, pp. 28	Relevant but supplementary information: Refined dietary risk assessment.	Agree with proposal by applicant	-	
Cebotari V. et al.	2018	Content of pesticide residues in the flowers of the acacia and linden trees from the Moldavian Codri area.	Scientific Papers, Series D. Animal Science (2018), Vol. 61, No. 2, pp. 235	Relevant but supplementary information: The publication is considered to only provide supplementary information that is not directly relevant to MRL setting and risk assessment. The residue levels found in linden flower would trigger the need for a honey residue study and cannot be used to directly estimate an MRL. The method used to determine the residues of glyphosate in flowers is not described in the publication and no validation data are provided.	Agree with proposal by applicant	-	
Ledoux M. L. et al.	2020	Penetration of glyphosate into the food supply and the incidental impact on the honey supply and bees.	Food Control (2020), Vol. 109, pp. 106859	Relevant but supplementary information: This publication is a review and does not provide any original data, but summarizes relevant data on honey.	Agree with proposal by applicant	-	
Pareja L. et al.	2019	Evaluation of glyphosate and AMPA in honey by water extraction	Analytical methods (2019), Vol.	Relevant but supplementary information: This is primarily an analytical method paper, but does include information	Study summary requested. Supportive,	B.7.8.3.1	

		followed by ion chromatography mass spectrometry. A pilot monitoring study	11, No. 16, pp. 2123	on analysis of collected samples.	no impact on existing risk assessment parameters	
Raimets R. et al.	2020	Pesticide residues in beehive matrices are dependent on collection time and matrix type but independent of proportion of foraged oilseed rape and agricultural land in foraging territory	Chemosphere (2020), Vol. 238, pp. 124555	Relevant but supplementary information: The data are over-summarized. Only the percentage of samples with detectable / quantifiable residues, the median and the maximum residues are provided and it is not clear how many samples were analysed. Furthermore, it seems that the same data were already published (with more details) in a previous article (Karise R. et al., 2017). Therefore, the publication is considered to only provide supplementary information that is not directly relevant to MRL setting and risk assessment.	Agree with proposal by applicant	-
Thompson H. M. et al.	2014	Evaluating exposure and potential effects on honeybee brood (Apis mellifera) development using glyphosate as an example.	Integrated environmental assessment and management (2014), Vol. 10, No. 3, pp. 463	Relevant but supplementary information: No MRLs are currently set for presented commodities and these commodities are not considered for dietary risk assessment either. Therefore, the findings do not directly impact the consumer risk assessment.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.2
Umsza-Guez M. A. et al.	2019	Herbicide determination in Brazilian propolis using high pressure liquid chromatography.	International journal of environmental health research (2019) pp. 1 (Ahead of print)	Relevant but supplementary information: Currently no EU MRL is set for propolis and since propolis is not taken into account for dietary risk assessment in the EU. Because of that and due to the reliability of the analytical method is not clearly established the publication is considered supplementary.	Study summary requested. Supportive, no impact on existing risk assessment parameters	B.7.8.3.3

Category C studies: all these publications have been requested to be summarized. These 10 studies are evaluated in **B.7.8.4**.

Author	**Year**	**Title**	**Source**	**Proposal by applicant**	**RMS conclusion**	**Reference for summary**
Ackermann W. et al.	2015	The influence of glyphosate on the microbiota and production of botulinum neurotoxin during ruminal fermentation.	Current microbiology (2015), Vol. 70, No. 3, pp. 374.	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. The system used in this study was not developed for microbiological research. Instead it was developed for comparing rates of digestion of feed. It is not a dynamic system like a rumen but a	Study summary requested. Agree with proposal by applicant	B.7.8.4.1

				batch culture system. In 48 hrs they showed that adding glyphosate resulted in greater drops in pH as a result of inadequate buffering. The endpoints are consistent with decreased pH. They are inconsistent with more sophisticated rumen simulation techniques that found no effects from glyphosate.			
Bote K. et al.	2019	Effect of a Glyphosate-Containing Herbicide on Escherichia coli and Salmonella Ser. Typhimurium in an In Vitro Rumen Simulation System.	European journal of microbiology & immunology, (2019), Vol. 9, No. 3, pp. 94	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. This study used a rumen simmulation technique that resonably replicated rumen conditions that allowed for dynamic effects of feeding and removal of waste products. In the absence of a suitable dossier datapoint it was allocated to point CA 6.4 as it concerns livestock. However, it is important to note that it is not a residue study and does not provide any data on the transfer of residues from feed to food of animal origin.	Study summary requested. Agree with proposal by applicant	B.7.8.4.2	
Gerlach H. et al.	2014	Oral application of charcoal and humic acids to dairy cows influences Clostridium botulinum blood serum antibody level and glyphosate excretion in urine.	Journal of Clinical Toxicology (2014), Vol. 4, No. 2, pp. 186	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. Additionally there significant difficiencies (lack of control group, treatments). Glyphosate concentrations in urine would be highly impacted by urine volume which is affected by milk production and environmental temperature. Interestingly, aerobes from feces are tested and ruminants rely on strict anaerobes in the rumen and colon.	Study summary requested. Agree with proposal by applicant	B.7.8.4.3	
Nielsen L. N. c. r. et al.	2017	Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels	Environmental pollution (2018), Vol. 233, pp. 364	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. This study shows that aromatic amino acids in culture conditions can negate impact on gut microbes from glyphosate because microbes with sensitive EPSPS can get these amino acids from the media.	Study summary requested. Agree with proposal by applicant	B.7.8.4.4	
Riede S. et al.	2016	Investigations on the possible impact of a glyphosate-containing herbicide on ruminal metabolism	Journal of applied microbiology (2016), Vol. 121, No. 3, pp. 644	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. In this study a system was developed for studying ruminal organisms that is dynamic, used mixed population of	Study summary requested. Agree with proposal by applicant	B.7.8.4.5	

		and bacteria in vitro by means of the 'Rumen Simulation Technique'.		microbes, and is periodically fed with removal of waste products. There were no impacts of glyphosate formulation to this system.			
Schrodl W. et al.	2014	Possible effects of glyphosate on Mucorales abundance in the rumen of dairy cows in Germany.	Current microbiology (2014), Vol. 69, No. 6, pp. 817	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. Methodological shortcomings of the approaches used reduce the significance of the results (rumen fungi are stricly anaerobic, but they use aerobic cultures; 2) spot-urine concentrations are highly affected by the level of milk production 3) the ELISA is not validated and the LOD ws not used, no validation is described for other assays.	Study summary requested. Agree with proposal by applicant	B.7.8.4.6	
Shehata A. A. et al.	2014	Neutralization of the antimicrobial effect of glyphosate by humic acid in vitro.	Chemosphere (2014), Vol. 104, pp. 258	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. In the absence of a suitable dossier datapoint it was allocated to point CA 6.4 as it concerns livestock. However, it is important to note that it is not a residue study and does not provide any data on the transfer of residues from feed to food of animal origin.	Study summary requested. Agree with proposal by applicant	B.7.8.4.7	
Shehata A. A. et al.	2013	The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro.	Current microbiology (2013), Vol. 66, No. 4, pp. 350	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. The publication does not provide new information (potential effects on microorganims with sensitive EPSPS are well known) and real world conditions of the gut are not replicated (study conducted on minimal media; microorganisms exposed to extremely high doses of glyphosate (1000x); aged cultures inducing additional stress).	Study summary requested. Agree with proposal by applicant	B.7.8.4.8	
Vicini J. L. et al.	2019	Glyphosate in livestock: feed residues and animal health.	Journal of animal science (2019), Vol. 97, No. 11, pp. 4509	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. Review article.	Study summary requested. Agree with proposal by applicant	B.7.8.4.9	
Clair E. et al.	2012	Effects of Roundup(®) and glyphosate on three food microorganisms: Geotrichum candidum,	Current microbiology (May), Vol. 64, No. 5, pp. 486	Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. However, based on the results presented, it is not possible to reach a scientifically sound conclusion that the	Study summary requested. Agree with proposal by applicant	B.7.8.4.10	

		Lactococcus lactis subsp. cremoris and Lactobacillus delbrueckii subsp. bulgaricus.		ability to make cheese using these organisms has been compromised by Roundup formulations. Application of dilutions (1%) of glyphosate were shown to inhibit a yeast-like organism, which is unsurprising. Surfactant solutions are routinely used to sanitize food processing equipment at concentrations at or above those tested by Clair et al. These concentrations are vastly higher than the concentrations of glyphosate or possible surfactant present (if any) in incoming milk.		

Studies, considered not relevant after detailed assessment: 5 out of the 30 publications have been requested to be summarized. These 5 studies are evaluated in **B.7.8.5**.
Studies, considered not relevant after rapid assessment: 18 out of the 405 publications have been requested to be summarized. These 5 studies are evaluated in **B.7.8.6**.

B.7.8.1.1. Top up literature search

A top up literature search has been conducted, covering the publication period of January 2020 to June 2020. In total 852 articles were identified upon removal of duplicates within the current search (January 2020 – June 2020) and articles found already in the previous search (January 2010 – December 2019, see B.7.8.1). All 852 articles were subsequently assessed for their relevance at title/abstract level ('rapid assessment'). A total of 774 of the 852 articles were identified as 'non-relevant' in the rapid assessment and excluded from further evaluation. For the remaining 78 articles, identified as potentially 'relevant' or of 'unclear relevance' in the rapid assessment, the full-text documents were reviewed in detail ('detailed assessment'). A total of 35 articles of the remaining 78 articles were identified as 'non-relevant' in the detailed assessment and were excluded from further evaluation. The remaining 43 articles identified as 'relevant' in the detailed assessment were classified as Category A, B or C.

Specifically for the residues section, the results of the literature search are shown in the following table (table B.7.8.1.1-1).

Table B.7.8.1.1-1: Results of the article selection process for residues

	Number
Total number of articles after manual removal of duplicates. [a)]	16
Number of articles excluded after rapid assessment (title / abstract).	15
Total number of full-text documents assessed in detail.	1
Number of articles excluded after detailed assessment (*i.e.* not relevant).	0
Number of articles not excluded after detailed assessment [b)]	1
Number of summaries presented in the dossier [c)]	1

[a)] After removal of duplicates within the current search (Jan 2020 – Jun 2020) and entries found already in the previous search (Jan 2010 – Dec 2019). Additional duplicates occurred due to different update frequencies within each database and entries of publications ahead of print.
[b)] All relevant articles by full-text belonging to the relevance Category A, B, C.
[c)] Summaries were compiled for relevant articles of Category A and classified either as reliable or reliable with restrictions.

Assessment and conclusion by RMS:
The one Category A study has been included in the respective dossier section. However, since this study relates to the consumer risk assessment, which is not a separate dossier section in Vol. B.7, this one study is evaluated under **B.7.8.2**.
No Category B and C studies were retrieved in the top up literature search, and also no articles were excluded as being not relevant after detailed assessment.
Furthermore, 15 articles have been excluded by the applicant after the rapid assessment. These latter publications have been quickly checked, and the RMS can agree on their exclusion based on their titles, and the provided justification.

B.7.8.2. Category A studies, related to the consumer risk assessment

B.7.8.2.1. Study 1

1. Information on the study

Data point	CA 6.9/001
Report author	Zoller, O. *et al.*
Report year	2018
Report title	Glyphosate residues in Swiss market foods: monitoring and risk evaluation
Document No.	DOI 10.1080/19393210.2017.1419509 E-ISSN 1939-3229
Guidelines followed in study	None stated
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Uncertain reliability

2. Full summary of the study according to OECD format

Executive Summary

A total of 243 samples of diverse foodstuffs were analysed for glyphosate and aminomethylphosphonic acid (AMPA) using a liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) method with a relatively low limit of quantification in the range of 0.0005 – 0.0025 mg/kg. Main contributors for dietary glyphosate and AMPA intake were cereals and pulses. The results suggest that pasta is a very important foodstuff for dietary glyphosate residue intake in Switzerland. Interestingly all samples of wine, fruit juice and nearly all samples of honey tested positive for glyphosate although at very low levels. A dietary risk assessment was conducted. Food products for analysis were not selected purely at random, rather products were selected for which high levels of glyphosate residues were suspected. However, even in samples where high residue levels were expected, no exceedances of maximum residue levels were found. Consequently, human exposure did not exceed neither acceptable daily intake nor acute reference dose. Therefore, glyphosate residues found in the sampled foodstuffs from the Swiss market were of no concern for human health.

Materials and Methods

Samples

In total, 243 samples were analysed. All samples were bought in retail stores with the aim to represent a wide range of food products. Usually a single consumer package of 500 – 2000 g was sampled, irrespective of the lot size. When necessary, samples were homogenised using different mills and mixing devices to a particle size of about 0.1 mm before further processing.

Chemicals, reagents, and consumables

All solvents were obtained in LC-MS grade (Chromasolv ®) from Sigma-Aldrich (Buchs, Switzerland), as well as formic acid. Ultrapure water, further referred to as water, was obtained from an Elga Purelab ultra-water purification system (Labtec Services, Villmergen, Switzerland). Glyphosate standards and AMPA were obtained from Sigma-Aldrich; glyphosate internal standard (IS) $^{13}C_3$-D_2-Glyphosate from Alsachim (Illkirch-Graffenstaden, France); AMPA IS ^{13}C-^{15}N-AMPA from Dr. Ehrenstorfer (LGC Standards, Teddington, UK). All dilutions of standard solutions were prepared in water except the last dilution for standards ready for injection where dilution solvent was used. These dilutions were made in 20 mL vials, which were rinsed with water and methanol before use.

The extraction solvent was a water/methanol 1/1 (v/v) mixture with 0.5 % formic acid; the dilution solvent was a water/acetonitrile 1/1 (v/v) mixture with 0.2 % formic acid; the glyphosate IS and the AMPA IS solutions were 5000 ng/mL in water; the glyphosate and the AMPA stock solutions were 250 ng/mL in water; the calibration working solutions were 0.004 mL each of glyphosate IS and of AMPA IS solutions, ranging 0 – 0.060 mL of both stock solutions, respectively and extraction solvent up to 0.500 mL. The calibration injection solutions for solid samples were 0.100 mL of calibration working solutions diluted with 0.400 mL of dilution solvent. Similar for liquid samples, but dilution with 0.200 mL of dilution solvent.

The applied consumables were 2 and 50 mL centrifuge vials, polypropylene (PP) tubes, high density polyethylene (PE) screw caps (Eppendorf, Hamburg, Germany); 20 mL super PE vials for liquid scintillation (PerkinElmer, Waltham, MA, USA); 0.6 mL PE autosampler vials (06-PESV, Chromacol, Thermo Fisher Scientific Inc., Waltham, MA, USA); PP pipet tips for microman (Gilson Inc., Middleton, WI, USA); solid-phase extraction (SPE) cartridges Oasis HLB, 3 cc, 60 mg sorbent (Waters, Milford, MA, USA).

Sample preparation
Solid samples
Five gram of the homogenous or homogenised sample was weighed (rounded to the next 10 mg) into a 50 mL centrifuge vial and 20 mL of extraction solvent and 0.160 mL each of IS solutions were added. The tube was vigorously shaken by hand, then treated for 10 min in an ultrasound bath and shaken for 30 min on a shaker (Innova 2000, Eppendorf, Hamburg, Germany) at 400 rpm. The mixture was then centrifuged for 10 min at 2500 relative centrifugal force (RCF) and 10°C. Two times 1.5 mL of the supernatant was transferred into a 2 mL centrifuge vial and centrifuged for 10 min at 20,000 RCF. The combined supernatants were the final extract. Clean-up was performed on a SPE cartridge, which was first activated with 2 mL of methanol, conditioned with 2 mL of extraction solvent and pre-rinsed with 0.5 mL of extract. The eluate was discarded up to this step. A further 0.4 mL of extract was loaded onto the cartridge, the eluate collected in a 2 mL centrifuge vial and 0.100 mL of this eluate was diluted with 0.400 mL of dilution solvent in an autosampler vial.

Liquid samples
Five millitre of degassed (20 s in an ultrasound bath) beverage was transferred into a 50 mL centrifuge vial and 5 mL of extraction solvent and 0.080 mL each of IS solutions were added. The tube was shaken by hand. The SPE cartridge clean-up was performed as described above, only differing in the last step where 0.100 mL of the final eluate was diluted with 0.200 mL of dilution solvent in an autosampler vial.

Calibration
A 6-point calibration curve, corresponding to a range of 0 – 0.120 mg/kg for solid samples and a range of 0 – 0.060 mg/L for liquid samples, was constructed. If a sample contained a higher concentration, an extract using a lower amount of sample was prepared or further calibration points were introduced..

LC/MS/MS conditions
LC-system and conditions
A Symbiosis-System (Spark Holland B.V., Emmen, The Netherlands) was used with the following parameters: injection volume 10 µL; column BioRad Micro-Guard Cation H Refill Cartridge 30 × 4.6 mm (BioRad, Hercules, CA, USA); column oven at 40°C; elution solvent A: water; elution solvent B: acetonitrile with 0.2 % formic acid; program: 0:00 flow rate 0.5 mL/min 60 % A; 1:00 flow rate 0.5 mL/min 60 % A; 1:30 flow rate 0.5 mL/min 99 % A; 3:30 flow rate 0.5 mL/min 99 % A; 3:35 flow rate 0.8 mL/min 99 % A; 7:50 flow rate 0.8 mL/min 99 % A; 8:00 flow rate 0.8 mL/min 60 % A; 10:00 flow rate 0.5 mL/min 60 % A; 10:10 flow rate 0.5 mL/min 60 % A. The use of a specific rinsing procedure was important to minimise carryover and contamination. Needle rinsing was performed as follows: 500 µl water/methanol/acetonitrile 8/1/1 (v/v) followed by 700 µl water/methanol 1/1 (v/v) with 0.1 % phosphoric acid 85 % and finishing with 500 µl water/acetonitrile 6/4 (v/v) with 0.1 % formic acid. After each sample, a blank run was carried out.

MS/MS-system and conditions
An API 5000 (AB Sciex Netherlands B.V., Nieuwerkerk aan den Ijssel, The Netherlands) with electrospray ionisation in negative mode was used and scheduled multiple reaction monitoring was applied. The eluent in the first 1.5 min was diverted into waste. The optimised ionisation source parameters were source temperature, 650°C; ionisation voltage − 4500 V; curtain gas, 25 units; collision gas, 5 units; gas 1, 60 units; gas 2, 50 units; Dwell time, 50 ms. The transitions measured were the following (quantifier in bold): glyphosate, 168 → 150, 168 → 124, 168 → 79, **168 → 63**; glyphosate IS, 173 → 128, 173 → 81, **173 → 63**; AMPA, 110 → 81, 110 → 79, **110 → 63**; AMPA IS, 112 → 81, 112 → 79, **112 → 63**..

Method validation
The applied anion exchange method was based on the methods published by Guo *et al.* (2016) and Jensen *et al.* (2016). Validation of the analytical method was based on repeated experiments verifying limit of detection (LOD), LOQ, repeatability, and recovery in different matrices. Internal reference materials were used in each run. For the LOQ, the signal-to-noise threshold was set at 10 for the quantifier and at 7 for the two qualifiers. In addition, two external reference materials of wheat flour and rapeseed and the respective blank materials were analysed on a regular basis: reference material P1601-RMWh, wheat flour spiked with glyphosate, AMPA, glufosinate; blank material P1601-BLWh, wheat flour; reference material P1601-RMRape, rapeseed spiked with glyphosate, AMPA, glufosinate; blank material P1601-BLRape, rapeseed; all from PROOF-ACS GmbH (Hamburg, Germany). Further details of these reference materials are given in the explanation to **Table 1**. A Food Analysis Performance Assessment Scheme (FAPAS 2017) proficiency test on oat test material with chlormequat, mepiquat, and glyphosate was also completed, of which only glyphosate was analysed.

Results and Discussion

Method quality assurance

The method showed to be very robust and can be applied for nearly all kind of foodstuffs. It turned out that it is not necessary to use matrix-matched calibration. The absolute recovery was estimated using the absolute peak area of the IS. The absolute recovery was always better than 70 % for liquid samples and for solid samples it was always better than 50 % and in most cases also better than 70 %. Dilution experiments with naturally contaminated samples with concentrations above 0.05 mg/kg showed identical quantitative results. There was no indication for disturbing matrix effects in the undiluted sample. The LOQ for solid samples was generally 0.001 and 0.0025 mg/kg for glyphosate and AMPA, respectively. For liquid samples (i.e. beverages like wine and beer), the LOQ was 0.0005 mg/kg for glyphosate and 0.0005 – 0.001 mg/kg for AMPA. Details of the performance data of the method are given in **Table 1**. The FAPAS proficiency test (2017) was successfully passed with a z-score of 0.9 at the assigned value for glyphosate of 0.483 mg/kg. This level was appropriate for the validation of the higher levels that were measured, for instance in durum wheat and pasta, but not optimal for the lower levels around and below 0.05 mg/kg. For these levels, the wheat and rapeseed reference materials (PROOF-ACS GmbH) with assigned values for glyphosate of 0.034 and 0.086 mg/kg, respectively, were more appropriate. In **Table 1** it is shown that our measurements were in good agreement with the assigned values and also with the spiked values. In the FAPAS 09109b, oats blank material, 0.0057 mg/kg of glyphosate was measured.

The measurement uncertainty which is indicated in the supporting information is an estimate for the expanded uncertainty with a confidence level of 95 %. The values are roughly estimated with the help of the method performance data given in **Table 1**. Twenty percent is set as minimum value for the uncertainty. A more conservative approach would be to take the uncertainty from the proficiency tests of the mentioned FAPAS test and PROOF-ACS reference materials. The range of ±2 for z-scores is a good estimate for the confidence interval of 95 %. In this case, the uncertainty would generally be set at 45 % as the uncertainty for all values from the PROOF-ACS materials were between 43.3 % and 44.7 %. The respective uncertainty for glyphosate in the FAPAS test was 35.6 %.

In a few cases where it was suspected that the sample might not be sufficiently homogeneous, another two subsamples were analysed. In all cases, the difference to the first result was well below 10 %. In the case of the gram flour with a concentration of 2.756 mg/kg of glyphosate, which is discussed further down in the text, a package of the same lot could be purchased 6 months later. The measured concentration in the second package differed less than 2 % from the first result.

Table 1: Method performance data.

Analyte	Matrix	LOD [mg kg^{-1}]	LOQ [mg kg^{-1}]	concentration [mg kg^{-1}]	Repetitions (n)	Recovery (%)	RSD (%)	Comments and applied reference materials
Glyphosate	Wheat, white flour	0.0003	0.001	0.001	5	94	9.5	s, st
AMPA	Wheat, white flour	0.001	0.0025	0.005	5	101	6.5	s, st
Glyphosate	Beer	0.0002	0.0005	0.001	5	103	2.2	s, st
AMPA	Beer	0.0005	0.001	0.001	5	97	6.6	s, st
Glyphosate	Beer	0.0002	0.0005	0.010	3	98	7.1	s, st, d
AMPA	Beer	0.0005	0.001	0.010	3	102	0.6	s, st, d
Glyphosate	Wine	0.0002	0.0005	0.010	2	92	9.2	s, st, d
AMPA	Wine	0.0005	0.001	0.010	2	99	5.0	s, st, d
Glyphosate	Milk	0.0002	0.0005	0.004	2	96	1.8	s, st, d
AMPA	Milk	0.0005	0.001	0.004	2	111	1.6	s, st, d
Glyphosate	Honey	0.0003	0.001	0.005	5	92	13.9	s, st, d
AMPA	Honey	0.001	0.0025	0.005	5	115	3.5	s, st, d
Glyphosate	Vegetable oil	0.0004	0.001	0.010	2	102	2.8	s, st, d
AMPA	Vegetable oil	0.001	0.0025	0.010	2	92	6.1	s, st, d
Glyphosate	Smoked salmon	0.0004	0.001	0.010	1	95	N/A	s
AMPA	Smoked salmon	0.001	0.0025	0.010	1	97	N/A	s
Glyphosate	Poultry meat	0.0003	0.001	0.050	3	102	1.3	s, st, d
AMPA	Poultry meat	0.001	0.0025	0.050	3	100	1.3	s, st, d
Glyphosate	Red wine	0.0002	0.0005	0.0132	7	N/A	3.6	nc, lt
AMPA	Red wine	0.0005	0.001	<0.001	7	N/A	N/A	nc, lt
Glyphosate	Whole meal flour	0.0003	0.001	0.051	5	N/A	3.7	nc, st
AMPA	Whole meal flour	0.001	0.0025	0.0036	5	N/A	8.4	nc, st
Glyphosate	Whole meal flour	0.0003	0.001	0.051	22	N/A	5.4	nc, lt
AMPA	Whole meal flour	0.001	0.0025	0.0024	22	N/A	12.5	nc, lt
Glyphosate	Wheat	0.0003	0.001	<0.001	19	N/A	N/A	P1601-BLWh, lt
AMPA	Wheat	0.001	0.0025	<0.0025	19	N/A	N/A	P1601-BLWh, lt
Glyphosate	Wheat	0.0003	0.001	0.0376	21	N/A	8.4	P1601-RMWh, lt
AMPA	Wheat	0.001	0.0025	0.0577	21	N/A	9.1	P1601-RMWh, lt
Glyphosate	Rapeseed	0.0003	0.001	<0.001	3	N/A	N/A	P1601-BLRape, lt
AMPA	Rapeseed	0.001	0.0025	<0.0025	3	N/A	N/A	P1601-BLRape, lt
Glyphosate	Rapeseed	0.0003	0.001	0.0925	3	N/A	2.2	P1601-RMRape, lt
AMPA	Rapeseed	0.001	0.0025	0.0778	3	N/A	3.1	P1601-RMRape, lt

N/A: not applicable; s: spiked; nc: naturally contaminated; st: repetitions within 1 day; lt: repetitions over a time period of 7 months; d: different products; P1601-BLWh; wheat blank material; P1601-RMWh: wheat reference material, spiked level for glyphosate 0.037 mg kg^{-1} and assigned value by proficiency test 0.034 mg kg^{-1}, spiked level for AMPA 0.055 mg kg^{-1} and assigned value by proficiency test 0.050 mg kg^{-1}; P1601-BLRape: rapeseed blank material; P1601-RMRape: rapeseed reference material, spiked level for glyphosate 0.098 mg kg^{-1} and assigned value by proficiency test 0.0859 mg kg^{-1}, spiked level for AMPA 0.088 mg kg^{-1} and assigned value by proficiency test 0.0739 mg kg^{-1}.

Another peak showing quite similar ion transitions as glyphosate, eluting just after glyphosate, was often observed. This peak was identified as 2-amino-3-phosphonopropionic acid, a substance with identical sum formula and similar functional groups as glyphosate. This compound seems to occur in many products in the range of 0.001 – 0.5 mg/kg. For this reason, it can be recommended to check if 2-amino-3-phosphonopropionic acid is properly distinguished from glyphosate in the chromatograms, as to avoid the risk of too high results when analysing glyphosate. 2-Amino-3-phosphonopropionic acid was analysed semi-quantitatively and seems to occur in many products, especially in cereals, in the range of 0.001 – 0.9 mg/kg. There was no correlation between the concentration of 2-amino-3-phosphono-propionic acid and glyphosate. From the chemical structure point of view, it seems unlikely that 2-amino-3-phosphonopropionic acid is a metabolite of glyphosate. 2-Amino-3-phosphonopropionic acid may be a natural compound. Its occurrence in the ciliate *Tetrahymena pyriformis* is described by Horsman and Zechel (2017); however, no reference on the occurrence in higher plants is available. This issue will be examined in more detail in the context of another project.

Table 2: Concentrations of glyphosate and AMPA in different food categories.

	Glyphosate								AMPA							
Food category	Number of samples	Number of samples above the LOQ	Proportion of samples above the LOQ	LOQ (mg kg^{-1})	Min (mg kg^{-1})	Median (mg kg^{-1})	Arithmetic mean (mg kg^{-1})	Max (mg kg^{-1})	Number of samples above the LOQ	Proportion of samples above the LOQ	LOQ (mg kg^{-1})	Min (mg kg^{-1})	Median (mg kg^{-1})	Arithmetic mean (mg kg^{-1})	Max (mg kg^{-1})
Beer	15	2	13%	0.0005	<0.0005	<0.0005	0.0006	0.0068	0	0%	0.001	<0.001	<0.001	<0.001	<0.001
Wine	21	21	100%	0.0005	0.0006	0.0031	0.0048	0.0189	4	19%	0.0007	<0.0007	<0.0007	0.0005	0.0034
Mineral water	2	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005
Milk	3	0	0%	0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Fruit juice	11	11	100%	0.0005	0.0005	0.0016	0.0019	0.0035	2	18%	0.0006	<0.0006	<0.0006	0.0002	0.0006
Baby food	11	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Potatoes and vegetables	10	3	30%	0.001	<0.001	<0.001	0.0013	0.0077	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Honey	16	15	94%	0.001	<0.001	0.0030	0.0046	0.0159	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Eggs	1	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Meat and fish	13	3	23%	0.001	<0.001	<0.001	0.0008	0.0049	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Pulses	41	21	51%	0.001	<0.001	0.0012	0.1733	2.948	10	24%	0.0025	<0.0025	<0.0025	0.0031	0.025
Oilseeds and vegetable oil	6	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Pseudo cereals	3	0	0%	0.001	<0.001	<0.001	<0.001	<0.001	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Breakfast cereals	10	8	80%	0.001	<0.001	0.0036	0.0508	0.291	3	30%	0.0025	<0.0025	<0.0025	0.0025	0.010
Durum wheat	18	16	89%	0.001	<0.001	0.139	0.1349	0.421	15	83%	0.0025	<0.0025	0.0107	0.0110	0.0247
Pastry and snacks	11	4	36%	0.001	<0.001	<0.001	0.0037	0.0179	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Bread	10	7	70%	0.001	<0.001	0.0019	0.0069	0.0458	0	0%	0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Flour and baking mixtures	28	8	29%	0.001	<0.001	<0.001	0.0106	0.133	2	7%	0.0025	<0.0025	<0.0025	0.0007	0.0027
Other cereal products	13	2	15%	0.001	<0.001	<0.001	0.0012	0.0124	1	8%	0.0025	<0.0025	<0.0025	0.0007	0.0052

Description of categories: *pulses*: including products thereof like tofu and soy sauce, etc.; *breakfast cereals*: processed breakfast cereals like corn flakes, pops, etc. Rolled oats are placed in the category of other cereal products; *durum wheat*: all products with durum wheat as main ingredient as for instance pasta; *pastry and snacks*: all dry bake goods, sweet or salty, and also tortilla chips and potato chips (crisps); *bread*: also special bread that may contain minor amounts of oilseeds or pulses; *flour and baking mixtures*: flour and baking mixtures for bread making, the main ingredients are bread cereals like wheat, rye, and spelt, but they may also contain minor amounts of other cereals, oilseeds, and pulses; *other cereal products*: category with a wide variety of products like rolled oats, popcorn, semolina of maize (polenta), pasta with wheat instead of durum wheat, etc.; for beverages as beer, wine, milk, fruit juices, and mineral water, the measurement unit is mg L^{-1} instead of mg kg^{-1}. For the calculation of the arithmetic mean, all samples below the LOD were taken as zero; for samples between the LOQ and the LOD, the estimated value was used.

Concentrations in foodstuffs

Food products were sampled with the aim to determine the relevant foodstuffs for glyphosate intake. Samples with higher residue concentrations are probably over-represented to some extent, because categories like pulses and durum wheat were more frequently sampled, since these were suspect to reveal more glyphosate positive results. Additionally, every time when food samples turned out to contain more than 0.01 mg/kg, a few similar food items were collected. All together survey results are probably not representative for the residue levels in all foodstuffs on the market, as to achieve this goal analysis of a few thousand samples would have been necessary. The results for glyphosate and AMPA are summarised in **Table 2** and grouped into different food categories. Detailed data is available as supporting information.

For cereals and pulses, the contamination rate for glyphosate on the level above 0.1 mg/kg is comparable with data from Germany (Scherbaum *et al.* 2012) and a bit lower as in the United Kingdom (Stephenson and Harris 2016). The two samples with the highest glyphosate concentration were chickpeas originating from Canada with 2.948 mg/kg and gram flour (chickpea flour) with 2.756 mg/kg produced in the United Kingdom with unknown origin of the processed chickpeas. In 24 samples, glyphosate was measured above 0.1 mg/kg, but all AMPA values were below 0.1 mg/kg and usually much lower than the respective glyphosate values. Thirteen of 24 samples were durum wheat products like pasta and semolina, 8 samples were pulses and products thereof, 2 further samples were breakfast cereals and the last product was a bread baking mix containing seeds. It could be shown that the main contributor for glyphosate residue in this mix was linseed. There was no hint that 1 of these 24 products contained relevant ingredients of Swiss origin. Pulses are not consumed very often in Switzerland; however, pasta is an important dish of the regional diet. As nearly 100 % of durum wheat for the production of pasta is imported, this might be an important commodity regarding glyphosate residues. All samples of wine and fruit juice and all except one sample of honey were positive for glyphosate but all in the low ng/g range.

Of all analysed samples, 38 were clearly indicated as made of Swiss ingredients. The product with the highest glyphosate concentration of this category was a red wine containing 0.0132 mg/kg. All cereal products of this category contained undetectable or low amounts. The highest value found was 0.0025 mg/kg glyphosate in a wholegrain wheat flour. The number of 38 samples with ingredients of Swiss origin is not large enough as to guarantee that Swiss regulations on the use of glyphosate in agricultural practice are not violated, but at least do not indicate unregistered use of glyphosate, since not one single high contamination was found in food items containing raw products originating from Switzerland.

Also, all products labelled as organic had no or only low residues. In 37 of totally 43 organic samples, the concentration was below the LOQ and only 6 samples showed quantifiable amounts. In three of these six samples the concentration was just above the LOQ and only one sample showed a concentration above 0.01 mg/kg. This organic sample with the highest glyphosate concentration was a pasta product (spaghetti) containing 0.0123 mg/kg of glyphosate and 0.0024 mg/kg of AMPA. On the label, it was indicated that the durum wheat originated from North America, Europe and the eggs from Europe. Carryover during transport and production is conceivable. No detailed data are available to what extent such a contamination is avoidable by using adequate practices. As far as we know there is not yet a binding agreement on how low the residues in organic products should be, but a value of 0.01 mg/kg is at least under discussion or maybe already partially implemented.

Risk assessment

Based on the measured residues (**Table 2**), simple exposure estimates were derived (**Table 3**) and compared to the ARfD and the ADI, both amounting to 0.5 mg/kg bw/day, as recently established by EFSA's revaluation (EFSA 2015). Food consumption values applied in the exposure estimation were chosen at a level to overestimate actual daily average consumption. It seems plausible that these amounts of the respective food items are consumed at least occasionally during a single day. Risk assessments, i.e. comparison of estimated residue intake with the ADI and ARfD, were conducted for both the measured median and MRLs found per food item.

None of the median residues found in any food item resulted in an exposure greater than 0.5 % of the ADI/ARfD and virtually all are significantly below 0.5 % of the ADI/ARfD. If measured MRLs were applied, substantial exposures (ca. 5 % of ADI/ARfD in adults and ca. 10 % of ADI/ARfD in children) resulted for pulses, exclusively. All other MRLs resulted in exposures that were mostly significantly lower than 1 % of the ADI/ARfD. It is concluded that none of the residue levels identified in any of the food categories are of any health concern. This is not surprising, as none of the measured residue levels exceeded the legally tolerated MRL.

Table 3: Exposure to median and maximum glyphosate residue levels and expected urine glyphosate concentrations (nr: not relevant).

	Child of 15 kg body weight				Adult of 60 kg body weight			
		Exposure as % of ADI or ARfD				Exposure of % of ADI or ARfD		
Food category	Consumption (kg or L/day)	At median residue level	At maximum residue level	Expected urine concentration (µg L^{-1})	Consumption (kg or L per day)	At median residue level	At maximum residue level	Expected urine concentration (µg L^{-1})
Beer	nr	nr	nr	nr	0.50	0.0008	0.0113	0.340
Wine	nr	nr	nr	nr	0.25	0.0026	0.0158	0.473
Mineral water	1.00	0.0067	0.0067	0.067	2.00	0.0033	0.0033	0.100
Milk	0.50	0.0033	0.0033	0.033	1.00	0.0017	0.0017	0.050
Fruit juice	0.50	0.0107	0.0233	0.233	1.00	0.0053	0.0117	0.350
Potatoes and vegetables	0.25	0.0033	0.0257	0.257	0.50	0.0017	0.0128	0.385
Honey	0.03	0.0010	0.0053	0.053	0.05	0.0005	0.0027	0.080
Eggs	0.10	0.0013	0.0013	0.013	0.20	0.0007	0.0007	0.020
Meat and fish	0.25	0.0033	0.0163	0.163	0.50	0.0017	0.0082	0.245
Pulses	0.25	0.0033	9.8267	98.27	0.50	0.0017	4.9133	147.4
Oilseeds	0.05	0.0007	0.0007	0.007	0.10	0.0003	0.0003	0.010
Pseudo cereals	0.10	0.0013	0.0013	0.013	0.20	0.0007	0.0007	0.020
Breakfast cereals	0.10	0.0048	0.3880	3.880	0.20	0.0024	0.1940	5.820
Durum wheat	0.25	0.4633	1.4033	14.03	0.50	0.2317	0.7017	21.05
Pastry and snacks	0.05	0.0007	0.0119	0.119	0.10	0.0003	0.0060	0.179
Bread	0.25	0.0063	0.1527	1.527	0.50	0.0032	0.0763	2.290
Flour and baking mixtures	0.25	0.0033	0.4433	4.433	0.50	0.0017	0.2217	6.650
Other cereal products	0.10	0.0013	0.0165	0.165	0.20	0.0007	0.0083	0.248

Exposure per kg body weight is calculated by multiplying the residue concentration in food by the assumed food consumption and dividing the result by body weight (15 kg for children and 60 kg for adults). Risk is expressed by calculating exposure as per cent ADI or ARfD (both amounting to 0.5 mg kg^{-1} bw). Maximally expected urine concentrations are calculated by multiplying maximum residue concentrations in food by the assumed consumption and by the fraction of orally ingested glyphosate excreted by the urine (20%). The obtained result is divided by an assumed daily urine volume of 1.5 L for a child and 2 L for an adult. If residues were below LOQ, the LOQ value was used for risk assessment.

The exposure estimates for maximum residues derived as described above were also used to predict probable urine concentrations. It was assumed that the amount indicated in Table 3 of the respective food item was ingested and this food item contained the measured MRL of glyphosate (**Table 2**). Based on toxicokinetic studies, the amount of an orally ingested single dose of glyphosate excreted with the urine was assumed to equal 20 % (EFSA 2015). Further, it was assumed that daily urine volumes of 1.5 and 2.0 L are excreted by children and adults, respectively. For glyphosate residues at the maximally measured levels, predicted urine concentrations would be greater than 0.5 µg/L only for a few commodities. Again, only for the maximum residues found in pulses substantial amounts were predicted in urine of adults (ca. 147 µg/L). Overall, the predicted urine concentrations correspond very well with actually measured glyphosate urine levels in samples of the human population: Conrad *et al.* (2017) reported median levels well below 0.5 µg/L in samples of the German population, while maximum values slightly exceeded 0.5 µg/L. Also Niemann *et al.* (2015) concluded that urine concentrations of glyphosate corresponded well with levels in food; however, urine levels of AMPA were somewhat too high and not in good agreement with reported levels in foodstuffs. In a report of glyphosate urine levels in a small, not representative survey of the Swiss population, values in the range of 0.1 – 1.5 µg/L (RTS 2015) were measured.

Conclusion

In this market survey, food products for analysis were not selected purely randomly, rather products were selected for which measurable levels of glyphosate residues were suspected. However, even in samples where high residues were expected, no exceedances of MRLs were detected. Consequently, exposures did not exceed neither ADI nor ARfD. Therefore, glyphosate residues found in the sampled foodstuffs from the Swiss market are of no health concern for the consumer. This conclusion may be valid for all food products on the Swiss food market, considering that products for which high residue levels were suspected were over-represented in this survey.

Chromatographic conditions

Chromatograph:	Symbiosis
Column:	BioRad Micro-Guard Cation H Refill Cartridge (30 mm x 4.6 mm)
Column oven temperature:	40°C
Injection volume:	10 µL

Mobile phases:	(A) Water (B) Acetonitrile with 0.2 % formic acid			
Gradient:	Time (Min)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)
	0:00	60	40	0.5
	1:00	60	40	0.5
	1:30	99	1	0.5
	3:30	99	1	0.5
	3:35	99	1	0.8
	7:50	99	1	0.8
	8:00	60	40	0.8
	10:00	60	40	0.5
	10:10	60	40	0.5
Retention time:	Not provided			

Detector:	Sciex API 5000 triple quadrupole mass spectrometer		
Scan type:	MRM		
Ion source:	ESI negative		
Source gas 1:	60 units	Source gas 2:	50 units
Collision gas:	5 units	Source temperature:	650°C
Curtain gas:	25 units	Source voltage:	−4500 V

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Declustering potential (V)	Collision energy (eV)	Scan time (ms)
Primary transition (quantification)					
Glyphosate	168	63	–	–	50
Glyphosate (IS)	173	63	–	–	50
AMPA	110	63	–	–	50
AMPA (IS)	112	63	–	–	50
Secondary transition (confirmation)					
Glyphosate	168	79	–	–	50
Glyphosate (IS)	173	81	–	–	50
AMPA	110	79	–	–	50
AMPA (IS)	112	79	–	–	50

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes the results of monitoring analyses for residues of glyphosate and AMPA in food conducted by Swiss authorities between 2012 and 2017. A total of 243 samples of diverse food commodities were analysed for glyphosate and AMPA using an LC-MS/MS method that was developed specifically by the Swiss monitoring laboratory. According to the authors the method has a limit of quantification of 0.001 mg/kg for parent glyphosate and 0.0025 mg/kg for AMPA in solid matrices and 0.0005 mg/kg and 0.001 mg/kg, respectively, in liquid matrices (beer, fruit juice, wine). While it seems that these LOQs were established according to recognised procedures, details are missing and it is, therefore, difficult to evaluate the reliability of the provided analytical results. This

would be especially important since the reported LOQs are far below the LOQs achieved by most of the other official monitoring laboratories.
As stated by the authors the publication is not intended to provide a representative picture of the residues of glyphosate and AMPA in food commodities placed on the market in Switzerland since the commodities showing high residues were over-represented. In spite of that, the samples relevant to the uses supported in the renewal dossier (e.g. fruits, vegetables, fruit juice, wine, food of animal origin) all showed residues of glyphosate and AMPA far below 0.05 mg/kg (LOQ of most enforcement method so far).
In total, 16 honey samples from Europe and the Americas were analysed. They showed residues of parent glyphosate between < 0.001 mg/kg and 0.0159 mg/kg while the residues of AMPA were always < 0.0025 mg/kg (details are provided as supplementary data). Since according to SANTE/11956/2016 rev. 9 it is possible to derive EU MRLs in honey based on monitoring data and since honey marketed in Switzerland is likely to be also marketed in the EU, these results are deemed relevant to the setting of an EU MRL for glyphosate in honey. The fact that all the samples showed residues of AMPA < 0.0025 mg/kg is in contrast to another publication in which the analyses were also conducted with a very sensitive analytical method and where the residues of AMPA were often found at levels comparable to or even greater than the levels of parent glyphosate residues.

Assessment and conclusion by RMS:
In the current public article, glyphosate and AMPA have been analysed and detected in several foodstuffs. Subsequently, a dietary risk assessment was conducted, showing no concern for human health. The observed residue levels in honey could be useful for the calculation of an MRL based on monitoring data (see B.7.7.1), since the lab can be considered as an official monitoring lab. However, since the reliability of the analytical method cannot be fully evaluated, these results are not included in the calculations in B.7.7.1. No further impact expected on the existing risk assessment parameters.

B.7.8.2.2. Study 2

1. Information on the study

Data point:	CA 6.9
Report author	Panseri, S. *et al.*
Report year	2020
Report title	Occurrence of perchlorate, chlorate and polar herbicides in different baby food commodities
Document No	Food chemistry, (2020) Vol. 330, Art. No. 127205 DOI 10.1016/j foodchem.2020.127205
Guidelines followed in study	SANTE/11813/2017: Method validation & quality control procedures for pesticide residues analysis in food & feed US FDA FVM (2015): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data within the Office of Foods and Veterinary Medicine. US FDA FVM (2019): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition. (Analytical methods)
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable

2. Full summary of the study according to OECD format
The incidence of glyphosate, its metabolite aminomethylphosphonic acid (AMPA) and other substances was estimated in baby food commodities (meat, fish, cheese, vegetable and fruit). Ion chromatography coupled to high resolution mass spectrometry analysis of the 105 samples did not show traces of glyphosate or its metabolite AMPA.

Materials and methods
Chemicals and reagents

Glyphosate and aminomethylphosphonic acid (AMPA) (certificated standards) and the internal standard (IS) *N*-Acetyl-D3-glufosinate were purchased from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Formic acid (98-100 %) was from Riedel-de Haën (Sigma-Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions
The principal standard solutions of each compound (1 mg/mL) were prepared in water and stored as recommended by the EU Reference Laboratory for pesticides (Anastassiades *et al.*, 2015). The working solution was kept in 4 °C plastic flasks to avoid pesticide interaction with glass-surfaces.

Sample collection
A total of 105 baby food samples were collected; all details are specified in Table 1. They were from different commercial brands, present in international markets, and bought in different Italian supermarkets (94 samples) and in some Serbian supermarkets (11 samples). In particular, all products are commercialized as homogenized food, packaged in sterile conditions (jar of 80 g) made from vegetable, fruits, meats, fish, cheese or combining different of these matrices, directly ready-for-eat. No sample processing (rehydration/mixing) is necessary before consumption.

Table 1 Sample collection details according to the different matrix typology

Meat / meat+vegetables n=43[#]	**Fish / fish+vegetable n=13**	**Fruit / vegetables n=42**	**Cheese / cheese+ham n=7**
veal	plaice	apple	cheese (bovine milk)
swine	hake	plum	cheese and ham**
horse	plaice and potatoes*	pear	
lamb	trout and vegetables*	pear and blueberry	
rabbit	bream and vegetables*	apple and blueberry	
chicken	bream and potatoes*	apple and banana	
turkey	bass and vegetables*	apple and peach	
veal and ham	cod and potatoes*	apple and apricot	
chicken and carrots*	cod and vegetables*	banana and kiwi	
chicken with green beans and zucchini*	salmon and vegetables*	zucchini mix fruit	
turkey, corn and potatoes*		carrot and apple	
veal and carrots*		legumes	
veal and potatoes*		sweet potato and carrots	
veal, potatoes and mushrooms*		broccoli	
veal, broccoli and carrots*		carrots, potatoes and zucchini	
veal and vegetables*		mixed vegetables	
		tomato and vegetables	
		peas and spinach	

[#]- number of samples per category
*for mixed categories, meat and fish represented the major component as declared in the label
**for mixed categories, cheese is the dominant component as declared in the label

Sample extraction
The samples (1 g) were extracted as designated by Chiesa, Nobile, Panseri, and Arioli (2019). The only changes were the internal standard (IS), *N*-Acetyl-D3-glufosinate, and its concentration (0.1 µg/g) for each matrix category. Briefly, 1 g, representative of each single purchased sample, was spiked with the IS, extracted with a mixture of 3 mL of methanol and 7 mL of 1 % formic acidified water, vortexed and sonicated for 15 min, after centrifugation (4 °C, 10 min, 2500 ×g), 1 mL of the supernatant was filtered in a vial, ready for the analysis.

IC-HRMS Orbitrap parameters
Ion chromatography high resolution mass spectrometry (IC-HRMS) instrumentations, parameters and software are described in Chiesa *et al.* (2019). Briefly, the instrumental analysis was performed by an Ion Chromatography Dionex

ICS-5000 + system (Sunnyvale, CA, USA) made up of Dual Pump, a Conductivity Detector and an Autosampler. The column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm) kept at 30 °C. The column was chosen on the basis of preliminary trials. In fact, the Thermo Scientific Dionex IonPac AS11 column provided similar results, as evaluated also by Rajski, Díaz Galiano, Cutillas, and Fernández-Alba (2018).
The KOH eluent was converted to water by a Dionex AERS 144 500, 2 mm suppressor (Thermo Scientific). The eluent flow rate (0.3 mL/min), chromatographic run duration (30 min) gradient and injection volume were the same as described by Chiesa *et al.* (2019). Chromatographical separation started with an isocratic 15 mM $KOH_{(aq)}$ elution for the first 8 min, than increased linearly (from 8 to 20 min) up to 55 mM $KOH_{(aq)}$, and was held in these conditions for next 4 min. The initial KOH concentration was brought back at 24.1 min, which was followed by 6 min equilibrium time. The injection volume was 50 µL.
Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), high resolution mass spectrometer equipped with heated electrospray ionization (HESI) source operating in negative mode was used for characterisation of compounds / anions of interest with the same operative conditions described in our original method (Chiesa *et al.*, 2019). Briefly, capillary temperature and vaporizer temperature were set at 330 °C and 280 °C, while the HESI voltage was 3.50 kV. Sheath and auxiliary gas were adjusted at 35 and 15 arbitrary units, with S lens RF level of 60. Instrument calibration was done every analytical session with a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan (FS, resolution – 70,000 FWHM) was accompanied by a Data-Independent Acquisition (DIA resolution – 35,000 FWHM). DIA method recorded the MS/MS fragmentation events for all compounds/ anions enrolled in this study. On the basis of our compound list, a scan range of *m*/*z* 50–250 was chosen in FS with the automatic gain control (AGC) of 1×10^{-6}, while maximum injection time was 100 ms. For the DIA segment the AGC target was set to 5×10^{-4}, with an auto regulated maximum injection time. The precursor ions were filtered by the quadrupole which operated at an isolation window of 1 *m*/*z*. Fragmentation of precursors was optimised as three-stepped normalized collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was achieved by comparing the retention time at which negative molecular ions with exact *m*/*z* value appeared, accompanied with two specific characteristic fragments. The chemical formulas, retention times, the theoretical mass of the precursors and the corresponding diagnostic fragments / isotopic ratios applied for confirmation purposes are reported in Table 2.
Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while XcaliburTM 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) to control the HRMS system, the exact mass of the compounds, record and elaborate data.

Method validation
Validation was assessed according to the European Commission (2017) SANTE/11813/2017 Guidance document on method validation & quality control procedures for pesticide residue analysis in food & feed, as described in our recently published papers (Chiesa *et al.*, 2019). Moreover, the method follows the US Food and Drug Administration Foods Veterinary Medicine Research Steering Committee (US FDA FVM) recommendations that regard the validation of chemical methods (US FDA FVM, 2019) and criteria for confirmation of identity of chemical residues using Exact Mass Data (US FDA FVM, 2015). The method was validated for four different blank samples (baby food based on meat, fish, vegetables and cheese) to assess selectivity / specificity, linearity, recovery, precision (as coefficient of variation, CV %) and limit of quantification (LOQ). Besides quantitative validation aspects, also the identification parameters were assessed e.g. ion ratio and retention time. A number of 6 replicates were analysed to check the recovery and precision at two concentration levels (LOQ and 50 ng/g). The LOQ was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤20 %. The matrix effect was also evaluated by comparing the response produced from the analyte in a solvent solution with that obtained from the same quantity of analyte in the sample extract, expressed as percentage. The matrix-matched calibration curves were made of 5 calibration points in triplicate at 5, 10, 20, 50 and 100 ng/g for all analytes to cover the high concentrations found in the samples.

Table 2 IC-HRMS data of the studied compounds acquisitioned in negative ionisation mode: formulas, retention time (RT) exact mass, of parent pseudo-molecular anion confirmation fragments and isotopic pattern

Compound	Formula	R_t (min)	Precursor (*m*/*z*)	Main products (*m*/*z*)		
AMPA	CH_6NO_3P	14.15	110.00125	62.96417	78.95904	80.97468
Glyphosate	$C_3H_8NO_5P$	23.66	168.00673	62.96417	124.01687	149.99612
N-Acetyl-glufosinate-D3 (IS)	$C_7D_3H_{11}NO_5P$	13.98	225.07251	62.96413	137.05953	181.08277

Results

The method, already described in a previous study (Chiesa *et al.*, 2019), confirmed the satisfactory validation parameters for all compounds analysed, in Table 3. Briefly, the validated method for different baby food typology showed high specificity, without any interference close to the retention time of selected analytes; good selectivity with a S/N ≥3 and ions with mass accuracy ≤5 ppm since the lowest concentration. Our LOQs were 5 ng/g, recoveries were from 90 to 102 %, linearities demonstrated a good fit with a R^2 >0.99 and precisions expressed as CVs % were lower than 11 %, for all the matrices investigated. The ion ratio was always compliant with the validation guidelines within ±30 % and the matrix effect was always within ±20 % signal suppression or enhancement, as recommended. The internal standard glyphosate-2-^{13}C, ^{15}N used in the previous work (Chiesa *et al.*, 2019), was substituted by *N*-acetyl-glufosinate-D3, more stable and consistent with an absolute recovery ranging from 98 to 102 % in the four different investigated matrices. The proposed method, in comparison to the original QuPPE one (Anastassiades *et al.*, 2015) shows better analytical limits and the protocol is the identical for matrices, not only for vegetables but also for those of animal origin.

The European Union has always paid attention to baby food components and contaminants, glyphosate and its metabolites, as shown by the directives and the regulations on the subject. Regulation (EU) No. 609/2013 (European Regulation, 2013) defines a 'baby food' as a "food intended to fulfil the particular requirements of infants in good health while they are being weaned, and of young children in good health as a supplement to their diet and/ or for their progressive adaptation to ordinary food". The same document specifies that follow-up on the use of pesticides in baby food shall be updated regularly and suggests limiting the use of pesticides as much as possible. The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. The absence of these analytes is therefore an important result, given the worldwide alert and debates on the glyphosate issue, as well as decisions that will be taken in coming years. The lack of data in literature on screening that covers all types of baby food matrices and the encouraging results of this study, also considering the different origin context of our samples, show that the products of the most common brands present on the Italian and international market are safe for infants. Maybe the attention or the presence of glyphosate is mostly linked to soy-based products or cereals, in particular from American products, differently regulated, as can also be seen from the results of Rodrigues and de Souza (2018).

Table 3 Validation parameters about all selected compounds in the four different baby food analysed by IC-HRMS

Vegetable baby food	LOQ (ng g^{-1})	Matrix effects % at LOQ	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Linearity R^2
AMPA	5	92	7, 5	95, 97	0.9906
Glyphosate	5	95	5, 4	97, 100	0.9915
Cheese baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	90	9, 8	90, 93	0.9996
Glyphosate	5	94	5, 3	94, 97	0.9984
Fish baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	95	9, 8	96, 98	0.9931
Glyphosate	5	93	5, 3	93, 95	0.9965
Meat baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	99	10, 9	97, 99	0.9936
Glyphosate	5	107	6, 4	102, 100	0.9973

*The 2 concentration levels were LOQ and 50 ng/g.

Conclusions
The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. Generally summarising, the levels reported for glyphosate in exanimated baby food commodities indicate compliance with existing / forthcoming legislation.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article describes a monitoring of residues of glyphosate and AMPA in 105 commercially available baby food samples. The article is well described, the samples were analysed using validated analytical methods and the methodology is considered as reliable. The publications shows clearly absence of residues of glyphosate and AMPA in all tested commercially available baby food. This finding indicates that infants and toddlers that are mainly fed with ready-to-eat baby food are not exposed to significant levels of glyphosate or AMPA residues. However the finding cannot be directly related to the supported representative uses of glyphosate for renewal.

Assessment and conclusion by RMS:
In this article, it has been investigated whether glyphosate and AMPA can be detected in commercially available baby food commodities. No glyphosate and AMPA were detected. No further impact expected on the existing risk assessment parameters.

B.7.8.3. Category B studies, included in the renewal dossier as supplementary information

B.7.8.3.1. Study 1

1. Information on the study

Data point:	CA 6.10.1
Report author	Pareja L. *et al.*
Report year	2019
Report title	Evaluation of glyphosate and AMPA in honey by water extraction followed by ion chromatography mass spectrometry. A pilot monitoring study
Document No	Analytical Methods, (2019), Vol. 11. Pp. 2123-2128
Guidelines followed in study	SANTE/11813/2017 (for calculation of recovery percentage)
Deviations from current test guideline	None stated
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format
Two straightforward methods for glyphosate and AMPA analysis in honey through ion chromatography coupled to Q-Orbitrap accurate high-resolution mass spectrometry as well as their application to real samples are presented. The sample preparation step is accomplished through dilution with methanol/water or water. No pH adjustment is necessary. Recoveries were in the range 80 to 110 % with RSDs < 20 %. The LOQ of glyphosate was 0.005 mg/kg, far below the allowed EU-MRL (0.05 mg/kg) in honey, showing a linear range of 0.005–0.5 mg/kg and medium matrix effect. LOQ of AMPA was 0.02 mg/kg. Glyphosate quantitation was performed with internal or external calibration yielding identical results, thus broadening the applicability of the method, as isotopically labelled standards are no longer mandatory. The method was applied to 32 honey samples from different origins. Glyphosate was found in 81% of the samples and 41 % were above the EU-MRL showing its applicability in routine work. AMPA was not detected in any of the samples.

Materials and methods
Reagents and materials
High-purity glyphosate (98 %) and AMPA (95 %) standards were obtained from LGC Standards (Wesel, Germany) and were stored at -30 ºC. Glyphosate solution (2000 mg/L) was prepared in water and were stored in plastic vials in the dark at -20 ºC. A mixed-standards solution was prepared from the stock standards. LC-MS grade water was

obtained from Fisher Scientific (Fair Lawn, NJ), and LS-MS grade methanol was obtained from Fluka Analytical (Steinheim, Germany). A 98 % formic acid was purchased from Sigma-Aldrich. Thermo Fisher Scientific (Waltham, MA) provided Pierce LTQ Velos ESI Negative Ion Calibration Solution.

Spiking procedure
For recovery studies, samples obtained from the local market were analyzed to ensure that did not contain glyphosate nor AMPA, and those sample in which the target analytes were not detected was selected as a blank for spiking, calibration curves, and recovery purposes. A 30 g portion of honey matrix was weighed and transferred to a beaker, fortified with a 150 mL aliquot of the analytes and placed in a water bath at 45 ºC and homogenized for 30 min. The sample was allowed to stand at room temperature before analysis. The final spiking concentration levels in the sample used for recovery studies were 0.005 and 0.05 mg/kg for glyphosate and 0.005, 0.020 and 0.050 mg/kg for AMPA.

Sample preparation
Modified quick polar pesticides (QuPPe) method
A 5 g portion of homogenized sample was weighed into a 50 mL PTFE centrifuge tube. Next, 9 mL water was added, the mixture was shaken and let stand for 5 minutes. Afterwards, 10 mL methanol were added, and the mixture was vortexed for 30 s. The samples were shaken in an automatic axial extractor (AGYTAX®; Cirta Lab S.L., Madrid, Spain) for 5 min at 40 ºC. The extract was then centrifuged (3220 g) for 5 min. The extracts were filtered using a 0.22 µm nylon filter and transferred to a plastic vial. Samples were diluted 5 times with water before the injection.

Dilute and shoot
Honey (5 g) accurately weighted was diluted with 19 mL of water, shaken vigorously for 5 min and centrifuged at 3220 g for 5 min. Then it was filtrated using a 0.22 µm nylon filter and transferred to a plastic vial before injection.

IC-MS analysis
For the IC separation, Dionex Integrion IC (Thermo Scientific, San Jose, USA) system was used. Mobile phase was water. Gradient was created by increasing the concentration of KOH. Separation was carried out on a Dionex IonPac AS19 column. The length, diameter and particle size were 250 mm, 2 mm and 4 mm, respectively. To protect the column a guard column was used (Dionex IonPac AG19). The length, diameter and particle size of the guard column were 50 mm, 2 mm and 4 mm, respectively. The column was thermostatted at 40 ºC. The gradient started from 5 mM of KOH and increased to 20 mM in 8 minutes, from 8 to 12 minutes increased to 60 mM and maintained until 22 minutes. In 22.1 minutes decreased to 5 mM and constant over 4 minutes for re-equilibration. The injection volume was 50 mL. The autosampler was thermostatted at 15 ºC. The mobile phase flow was 0.35 mL/min. To neutralize the column effluent AERS 500es 2 mm suppressor was used. Suppressor current was set to 52 mA. The regenerating water flow was 0.6 mL/min. Post column organic solvent (acetonitrile) flow was 0.2 mL/min.
A QExactive Focus (Thermo Scientific, Bremen, Germany) mass spectrometer was equipped with Heated Electrospray Ionization Source (HESI II). The spectrometer was operated in negative polarity. The HESI parameters were as follows: sheath gas flow rate: 32; auxiliary gas flow rate: 10; sweep gas flow rate: 0; spray voltage: 3.50 kV; capillary temperature: 380 ºC; S-lens RF level: 55.0; heater temperature: 350 ºC. MS analysis was carried out simultaneously in MS (full scan and selected ion monitoring) and MS2 (parallel reaction monitoring, PRM) mode. In PRM mode a targeted precursor ion is isolated in the quadrupole then fragmented in the collision cell and finally all product ions are analyzed in the Orbitrap. In MS two mass ranges m/z 78–212 and 109.5–110.5 were acquired. Resolution was set to 35 000 (for m/z 200), AGC target to 1 x 10 ^{6} and max IT to auto. For MS2 resolution of 17 500 (for m/z 200) was selected. AGC target and max IT were 1 x 10^{6} and auto, respectively. Precursor ions were filtered with isolation window of 1 Da (precursor mass ±0.5 Da). After optimization a collision energy of 25 eV was selected for both glyphosate and AMPA. The following ions were used for detection and identification of glyphosate: m/z 62.9641, m/z 78.9587, m/z 80.9744. All of them were fragment ions obtained in PRM MS2 mode. Precursor ion acquired in full scan MS was not useful because of high amount of interferences. AMPA was detected in SIM mode (m/z 100.0013) and identified in PRM MS2 (m/z 62.9641, m/z 78.9587).
The external mass calibration was carried out weekly. For the calibration, a mixture containing ultramark 1621, sodium dodecyl sulphate and sodium taurocholate (Pierce LTQ Velos ESI Negative Ion Calibration Solution) was used. The lowest mass present in the mixture was 265.14790. Chloride anion (82.95414) was added to the calibration mixture to improve mass accuracy for low masses. Trace Finder 4.1 (Thermo Scientific, San Jose, USA) was used for qualitative and quantitative analysis. Automatic detection and quantification were followed up by a manual verification.

Methods validation
The validation was carried out on a commercial sample of honey at two different concentration levels for glyphosate (0.005 and 0.050 mg/kg) and at three concentration levels for AMPA (0.005, 0.020 and 0.050 mg/kg) in five replicates

for each level. For the modified QuPPe method, trueness, expressed in terms of recovery percentages (% Rec), was calculated by the ratio of the areas of each analyte in the spiked sample before and after the extraction according to DG-SANTE guidelines.
Intra-day and inter-day precision were determined from quintuplicate analyses of samples spiked at the studied levels during the same day (repeatability-RSDr) and in different days by different operators (reproducibility-RSDwR). These two parameters were expressed as relative standard deviation (% RSD). Limit of quantification (LOQ) was considered as the lowest concentration assayed which presented acceptable recovery percentage and reproducibility (mean % Rec in the range 70 – 120 % with an RSD ≤20 %). The linearity of the methodology was evaluated in the range 0.005 – 0.50 mg/kg using a matrix matched calibration curve in honey extract as well as in solvent. Linearity was assumed when the variation coefficient was higher than 0.99 and residuals were below 20%. Also, variation of back-calculated concentrations was lower than 20 %. Matrix effects were also evaluated by comparing the slopes of the calibration curve prepared in solvent and matrix-matched calibration. The matrix effect was calculated according to eqn (1).

Matrix effect definition:

$$ME\ (\%) = \frac{(slope\ in\ matrix) - (slope\ in\ solvent)}{slope\ in\ solvent} \times 100 \quad (1)$$

Retention times of both analytes were stable (glyphosate 15.2 min and AMPA 9.7 min). The observed variation was lower than 0.1 min. For the dilute and shoot method the validation parameters were evaluated as it was described for the modified QuPPe methodology.

Results

Methods performance

In this study, two different approaches seeking the simplification for the sample preparation step were studied. The methods precision and accuracy, evaluated as recoveries and RSD were in accordance to DG-SANTE guidelines (**Table 1**).

Table 1: Results of recovery and repeatability obtained for glyphosate and AMPA in honey with the two evaluated methods

Concentration level (mg kg^{-1})	Glyphosate, MeOH + water		Glyphosate, water		AMPA, water	
	% Rec	% RSD	% Rec	% RSD	% Rec	% RSD
0.005	113	5	76	7	—	—
0.020	Not analysed				78	11
0.050	91	2	87	1	81	2

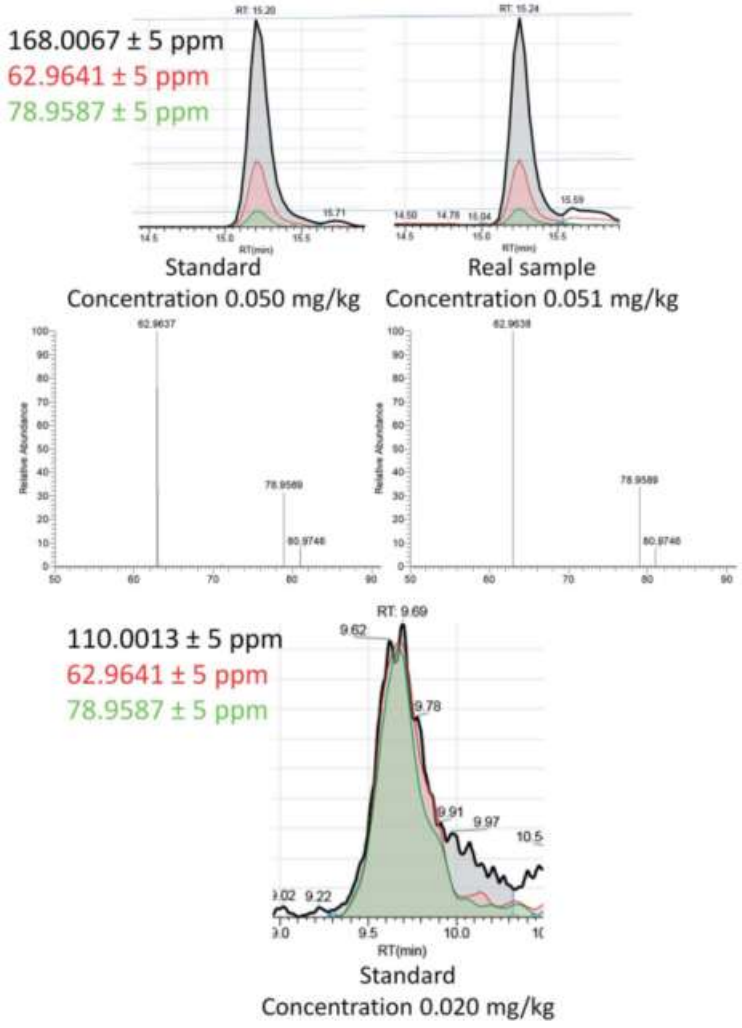
Due to the low *m/z* values of glyphosate transitions, the use of isotopically labelled glyphosate as internal standard is advised, in order to avoid matrix effects and isobaric interferences. However, as this compound is investigated in ESI negative mode, the possible interferences are minimized, despite the inherent complexity of the honey matrix. Comparing to ESI positive mode, less compounds can ionize in negative ESI thus this polarity provides higher selectivity because less interferences is present. The quantification of glyphosate residues was assayed using both, internal and external standard calibration. The results obtained using the two different calibration procedures were similar (**Table 2**), considering the accepted uncertainties of pesticide residue determinations. Both methods presented % Rec in the acceptable range (70 – 120 %) at the different fortification levels and the precision results showed a dispersion below 20 %, as required by SANTE guidelines.

Table 2: Results of recovery and repeatability obtained for glyphosate with and without isotopically labelled internal standard

Concentration level (mg kg^{-1})	External calibration		Internal standard	
	% Rec	% RSD	% Rec	% RSD
0.01	113	5	109	2
0.05	91	2	104	1
0.20	91	2	101	2

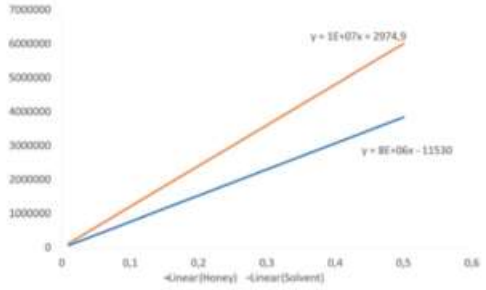
Glyphosate peaks had Gaussian shape. An example of peak shape and ion ratio stability is depicted on **Figure 1**. The ion ratio from sample extracts was very stable, below ± 30 %.

Figure 1: Peak shape of glyphosate and ion ratio stability (upper chromatogram) for standard and one of the positive real samples. Peak shape of AMPA (bottom chromatogram).



Glyphosate calibration curves in solvent and in matrix, were linear between 0.005 and 0.5 mg/kg. The matrix effect led to signal suppression. Slope of the calibration curve in matrix was 36 % smaller than the one obtained in pure solvent. Both calibration curves are shown in the **Figure 2**. The need of using labelled standards for glyphosate determination was studied. The calculations using external curve calibration and labelled internal standard showed that no significant differences between both calculation methods could be noticed. Therefore, IC allows the use of external calibration with no labeled compounds, making the determinations much cheaper. Subsequently, the dilute and shoot method was employed for AMPA analysis. AMPA showed less sensitivity than glyphosate. It was not detected at 0.005 mg/kg. And it was successfully validated only at 0.020 and 0.050 mg/kg level.

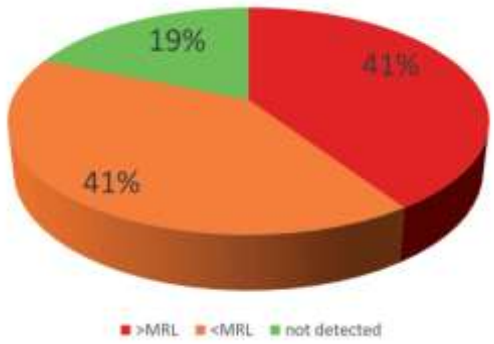
Figure 2: Calibration curves obtained in pure solvent (orange line) and in honey (blue line). Matrix effects calculated by comparison of the slops indicated 36% of signal suppression.



Real sample analysis

The methods were successfully applied to the analysis of 32 real samples from Europe and South America. The universe of the analyzed samples were as follows: 16 commercial honey samples from Uruguay and Europe, and 16 were raw honey samples, directly taken from the beehives. Glyphosate was detected in 26 samples (81 %). In 13 (41 %) samples, the levels of this herbicide were above the MRL for honey. The summary of these results is shown in **Figure 3**. None of the analyzed samples contained AMPA.

Figure 3: Graphical representation for the real samples analyses. Total number of samples was 36, in 6 the glyphosate was not detected, in 13 it was present in the concentration below the MRL established by the European Union, whereas 13 samples present a concentration level above the MRL.



Conclusions

Two simple and straightforward sample preparation methods for glyphosate analysis in honey using IC-Q-Orbitrap in the determination step were presented; a modification of QuPPe method without the pH adjustment and a simple water dilution and injection in the LC. It was demonstrated that IC greatly facilitate the application of the analysis of polar herbicides such as glyphosate. The LOQ of both methods is below the EU-MRL for glyphosate in honey. They presented the same linear range (0.005 –0.50 mg/kg) and suffered of medium matrix effect. In both cases, the quantitation could be performed without the addition of labelled standards. AMPA was validated using the dilute and shot method. The LOQ was 0.020 mg/kg. The methods were applied to real sample analysis, showing their applicability for the routine analysis of the target herbicide in honey. In our case, most of the samples contained glyphosate at different concentration levels. AMPA was not detected in any of the real samples, justifying not being included in the EU residue definition for glyphosate in honey-includes only the parent compound. The methods described here allow high sample throughput useful for monitoring purposes.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study compares two sample preparation methods (modification of QuPPe method without the pH adjustment and a simple water dilution) for the analysis of glyphosate and AMPA in honey by using ion chromatography coupled to Q-Orbitrap accurate high-resolution mass spectrometry. Moreover, it assesses the need of an internal standard for the analysis.

The methods were applied to real sample analysis. In total, 32 samples of honey from Europe and Uruguay were analysed, but the exact distribution of sample per country is not given. Glyphosate was detected in 26 samples (81 %). In 13 (41 %) samples, the levels of this herbicide were above the MRL for honey. None of the analysed samples contained AMPA.

The study provides some information on the residue level of glyphosate and AMPA in honey samples, but it is primarily an analytical method paper. The information is relevant, but supplementary.

Assessment and conclusion by RMS:
The article describes primarily analytical methods to quantify glyphosate and AMPA in honey. In addition, some real honey samples were analysed. However, since it does not concern an official monitoring lab, these honey results cannot be used for the calculation of a monitoring MRL (see B.7.7.1). No further impact expected on the existing risk assessment parameters.

B.7.8.3.2. Study 2

1. Information on the study

Data point:	CA 6.10.1
Report author	Thompson H. M. *et al.*
Report year	2014
Report title	Evaluating Exposure and Potential Effects on Honeybee Brood (*Apis mellifera*) Development Using Glyphosate as an Example
Document No	Integr. Environ. Assess. Manag. (2014), Vol. 10, pp. 463-470.
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

This study aimed to develop an approach to evaluate potential effects of plant protection products on honeybee brood with colonies at realistic worst-case exposure rates. The approach comprised 2 stages. In the first stage, honeybee colonies were exposed to a commercial formulation of glyphosate applied to flowering *Phacelia tanacetifolia* with glyphosate residues quantified in relevant matrices (pollen and nectar) collected by foraging bees on days 1, 2, 3, 4, and 7 post application and glyphosate levels in larvae were measured on days 4 and 7. Glyphosate levels in pollen were approximately 10 times higher than in nectar and glyphosate demonstrated rapid decline in both matrices. Residue data along with foraging rates and food requirements of the colony were then used to set dose rates in the effects study. In the second stage, the toxicity of technical glyphosate to developing honeybee larvae and pupae, and residues in larvae, were then determined by feeding treated sucrose directly to honeybee colonies at dose rates that reflect worst-case exposure scenarios. There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies. This study has developed a versatile and predictive approach for use in higher tier honeybee toxicity studies. It can be used to realistically quantify exposure of colonies to pesticides to allow the appropriate dose rates to be determined, based on realistic worst-case residues in pollen and nectar and estimated intake by the colony, as shown by the residue analysis. Previous studies have used the standard methodology developed primarily to identify pesticides with insect-growth disrupting properties of pesticide formulations, which are less reliant on identifying realistic exposure scenarios. However, this adaptation of the method can be used to determine dose–response effects of colony level exposure to pesticides with a wide range of properties. This approach would limit the number of replicated tunnel or field-scale studies that need to be undertaken to assess effects on honeybee brood and may be of particular benefit where residues in pollen and nectar are crop- and/or formulation-specific, such as systemic seed treatments and granular applications.

Materials and methods

Technical grade glyphosate (62.27% w/w glyphosate isopropylamine [IPA] salt corresponding to 46.14% w/w glyphosate acid equivalent [a.e.]) and the soluble concentrate formulation of glyphosate (MON 52276) (30.68%

glyphosate a.e. as the IPA salt, batch no GLP-0810-19515-A), supplied by Monsanto (St. Louis, MO) were used in the study. All honeybee colonies were obtained from National Bee Unit, FERA, (York, UK) apiaries and were confirmed as having low incidence of adult bee diseases, viruses, and varroa with no clinical signs of brood diseases.

Exposure assessment

Two 180 m^2 well-ventilated but insect-proof glasshouses were used for the study so as to be as representative as possible of the outdoor situation (e.g., polytunnel) but without direct rainfall. *Phacelia* was planted directly into the soil in the glasshouses and no pesticides were used during its cultivation. Application was performed when *Phacelia* flowers were at 100% of full bloom.

Three days before the application, 2 small honeybee colonies comprised of 4 to 6 frames of brood and 6000 to 12 000 adult bees were located on opposite sides of each glasshouse and allowed to fly freely. At the time of installation, each colony was fitted with a pollen trap and provided with a limited amount of stores to ensure that feeding on the crop was encouraged. This was done by removing as many frames as possible which contain only nectar or pollen, while ensuring survival and a maximum foraging activity. A supply of clean water, with provision to prevent bees from drowning, i.e., a sponge, was provided and replenished as required (it was removed during spray application).

To confirm that bees were foraging on the flowering *Phacelia*, foraging assessments were carried out each day during times when peak activity was expected. The assessments were performed by marking a 5 m × 1 m wide transect within the crop and counting the number of bees foraging within the marked area during a 1 min period once each day during the peak activity period (between 10.00–15.00 h in this study, based on previous experience). In addition, the number of bees returning to each hive and the number carrying pollen loads were counted during a 30 s period. These 2 counts provided information on the level of foraging activity of each hive within each glasshouse. Daily assessments of the crop were undertaken by visual assessment of the quality of the forage available, e.g., % plants with wilted flowers, wilted leaves.

The glyphosate formulation was applied at a rate equivalent to 8 L/ha (2.88 kg a.e./ha) in 400 L water/ha achieving an application efficiency of between 102% to 104% of the target rate, in both glasshouses. The application rate of 2.88 kg a.e./ha is the highest single application rate recommended for glyphosate, whereas the typical single application rate is 2.16 kg a.e./ha. The final treatment solution was prepared by adding the required quantities of test item—measured by weight, to measured volumes of tap water and thoroughly mixing in the field immediately before use to give the final treatment solution. The application was made during a period when the bees were actively foraging, using a 3 nozzle lunch box sprayer unit with a hand-held boom fitted with Lurmark 03 F110 nozzles. Direct spray drift onto the colonies was avoided by directing the spray away from the hives, and no direct overspray of the colonies occurred.

Pollen traps were activated 24 h before pollen collection, and the content of the pollen trap fitted to each hive was collected on days –1 (i.e., the day before application), 1, 2, 3, 4, and 7 after the application. The content of the traps was discarded on day 6 so as to only collect a sample from days 6 to 7. Each day and hive sample was kept separate unless they were too small for residue analysis, in which case samples from the same glasshouse were combined. All samples of pollen, nectar, and larvae were stored at –20° C.

On days 0 (before application), 1, 2, 3, 4, and 7 after the application samples of approximately 40 returning forager bees were collected from each colony by blocking the entrance of the hives with a foam bung and collecting returning foraging bees directly into collection jars. The nectar was collected from the honey stomachs of individual honeybees by removal of the stomach by dissection and placed in a preweighed tube. Samples were combined to produce samples large enough for residue analysis (minimum 200 mg).

On days 4 and 7 after the application, samples of 10 4–5-day-old larvae were taken from each colony using a forceps and stored at –20° C. Each day and hive sample was kept separate. On day 7, an additional sample of nectar was taken from the combs using a syringe in each colony and each hive sample was kept separate.

Residue analysis

Residues of glyphosate were extracted from larvae, pollen, nectar, and sucrose solution samples with acetonitrile/water (1:4, v/v). Recovery samples were fortified by spiking blank samples after weighing. For larvae, pollen, and nectar, the whole sample was accurately weighed into a single-use centrifugation tube. The sample was then homogenized, extracted with acetonitrile–water (1:4) with a high speed laboratory mixer, separated by centrifugation followed by solid-phase extraction of the supernate using a C18 column. All samples were then derivatized with fluorenylmethyl-chloroformate (FMOC-Cl). For derivatization, internal standard (1.0 µg/mL), borate buffer (0.2 mol/L sodium tetraborate decahydrate in water), and FMOC-Cl (5 g/L in acetonitrile) were added to the diluted extract. The samples were closed, mixed, and incubated at ambient temperature for at least 1 h. Finally, pH 3 water was added.

A second cleanup was carried out by applying the derivatized product to an Oasis HLB SPE column (equilibrated with dichloromethane followed by methanol and pH 3 water) and then rinsed with dichloromethane and the glyphosate-FMOC was eluted with methanol. The eluate was evaporated to dryness using a vacuum rotary evaporator.

The residue was reconstituted in 5% acetonitrile solution and transferred into a glass vial for high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) analysis.
The samples were analyzed using high-pressure liquid chromatography (Shimadzu LC-System) coupled with a triple quadrupole mass spectrometry detector (Sciex API4000). A Phenomenex Synergi column 2.5 µm Max-RP, 20 × 2.0 mm, 2.5 µm (No. 00M-4372-B0-CE) + 4 mm guard column was used. The column temperature was 40°C and a 30 µL injection volume was used. The mobile phase comprised A: water + 0.1% acetic acid (80%), B: methanol + 0.1% acetic acid (15%), and C: 100 mM ammonium acetate solution in methanol (5%) with a linear gradient over 5 min to comprise A: water + 0.1% acetic acid (0%); B: methanol + 0.1% acetic acid (95%) and C: 100 mM ammonium acetate solution in methanol (5%). Glyphosate-FMOC was quantified using the transition 390.0 to 149.8 with an internal standard glyphosate 1,2-^{13}C2 15N-FMOC transition 393.0 to 152.8.
At the start of the analytical sequence, the detector linearity was confirmed over the calibration range of interest by constructing a calibration function of peak area versus concentration within the range from 2.0 ng/mL to 5000 ng/mL for larvae and nectar samples, 1.0 ng/mL to 3500 ng/mL for pollen samples, and from 2.0 ng/mL to 4000 ng/mL for sucrose solution samples. Injections of sample extracts were interspersed with injections of quality control standards after 2 to 4 samples to verify the detector response.
The methods were validated before use and showed 92%–102% recovery with relative standard deviation (RSD) <15% with sucrose samples spiked at 1 and 400 mg a.e./kg, larval samples spiked at 1 and 200 mg a.e./kg, pollen samples spiked at 1, 500 and 700 mg a.e./kg and nectar samples spiked at 1 and 500 mg a.e./kg. Calibrations were linear within the range. Unless otherwise specified the limit of detection (LOD) was 0.3 mg a.e./kg, denoted as not detected (n.d.), and the limit of quantitation (LOQ) was 1.0 mg a.e./kg. Where data were used to generate mean values residues less than the LOQ were ascribed a value of 0.6 mg a.e./kg.

Effects assessment
Two approaches were made to assess exposure levels to be used in the effects study: one based on generic published data on the requirements for nectar and pollen by larvae (generic data) and the other based on the observations made in the exposure study (study data).

Generic data
The calculations were based on a daily brood requirement of 30 mg nectar (based on 40% sugar in nectar) and 1mg pollen per worker larva (Rortais et al. 2005). Based on a brood frame being 3600 cells (British Standard frame) and 5 frames of brood (4–6 were used in this study), there are 18 000 brood cells. The brood is unsealed for 25% of the time (hatch day 3 to sealed day 8 with emergence day 21, empirically determined in this study) therefore 4500 larvae have a requirement for 135 g/d nectar and 4.5 g/d pollen.

Study data
The second approach was to assess the amount of pollen and nectar returning to the hive over the time course of exposure using the data on the numbers of returning foragers in the study and the amounts of pollen and nectar collected from bees by using the pollen trap and individual bee samples.
The maximum pollen collected per colony was 2.9 g on day 1 and the traps were estimated to be approximately 50% efficient based on calculated pollen collection (Levin and Loper 1984; Delaplane et al. 2013). Thus 6 g of pollen per day was returned to the hive (the colony was using approximately 4.5 g of this based on the study by Rortais et al. [2005]).
The nectar collection was more difficult to directly assess but with a mean of 18 foragers returning to the hive per 30 s (observed in this study) and approximately 50 µL per load (max) this gives 18 trips/30 s × 60 s/min × 60 min/h × 12 h max foraging/d = 25 920 trips/d × 0.050 mL = 1296 mL/day (of which the colony was using 135 g, based on Rortais et al. [2005]). Because the assessment is brood exposure, the conservative collection estimate is justified. Therefore, as a worst case example considering the colony size used in the exposure study, the colony collected 6 g pollen and 1296 mL (i.e., 518 g sugar, assuming 40% sugar content) nectar and of this the brood consumes 4.5 g pollen and 135 g nectar (Rortais et al. 2005) that allowed the excess to be stored for later consumption.
Considering that bee colonies used in the brood study were up to 50% bigger than those used in the residue study, an additional calculation for the expected total daily intake of glyphosate residues was undertaken assuming that such colonies would collect 9 g pollen and 1944 mL nectar (Table 2). Furthermore, the determined residue content based on a worst-case application rate of 2.88 kg a.e./ha for spot treatments in orchards and vines and was adjusted to reflect the more realistic maximum application rate of 2.16 kg a.e./ha for preplanting, preemergence of crops, and preharvest applications.
The brood feeding study was undertaken using glyphosate as the technical grade IPA salt. Three dose levels of the test item were used based on the residues identified in pollen and nectar in a glass house study performed before the initiation of the bee brood study (Table 1). The lowest dose was based on the mean residue concentrations achieved over the first 3 days following the residue study spray application (75 mg glyphosate a.e./L). The mid-dose was based

on the highest residue concentrations following the spray application (150 mg glyphosate a.e./L) and the highest dose was equivalent to twice this latter rate (301 mg glyphosate a.e./L). The test item was introduced into each hive in equivalent volumes of 50% sucrose (w/v) solution (1 L) for each treatment group. Hence, the range could also be expressed in terms of concentration in the introduced dosing solution (mg glyphosate a.e./L and mg glyphosate a.e./kg). Control colonies were supplied with 50% w/v sucrose solution in deionized water and the toxic reference, fenoxycarb, (750 mg a.s./L as the formulation Insegar WG 250 g a.s./kg, batch no SM01A406) reported to have significant adverse effects on honeybee brood, was used to ensure that the study had the ability to detect effects of the test substance if they occurred (de Ruijter and van der Steen 1987).
Twenty standardized honeybee colonies each consisting of a single wooden Smith hive with British Standard frames and a queen were used; each of the queens used in the study was of similar age and lineage. The colonies were divided into 5 groups of 4 colonies. Each colony had a dead bee trap fitted to the front and the contents were counted daily during the brood assessment period (Imdorf et al. 1987). The colonies contained a mean of 14 250 to 19 500 adult bees, 1.5 to 2.5 frames of brood, 1.0 to 1.9 frames of stores, and 0.2 to 0.7 frames of pollen. The test colonies were allowed to fly freely, there were no nearby flowering crops and few flowering weeds (clover). Colonies were assembled according to treatment and groups were placed at least 20 m apart from each other. Two colonies (one control colony and one of the highest exposure rate colonies) (301 mg glyphosate a.e./L) became queenless after dosing but were retained in the study as the marked brood was viable and this was therefore not considered to have a significant impact on the study. All colonies were generally assessed within 1 week before dosing and again within weeks 1, 2, and 3 after dosing (day 0). Each assessment was carried out on every frame within each colony, and included counts of the number of combs of adults, brood (sealed and unsealed), and stores (nectar and pollen) as well as any behavioral or physical abnormalities.

Table 1 Summary of residue analyses of nectar collected from hive combs and larvae during the exposure study

		[mg glyphosate acid equivalent/kg]	
Matrix	**Hive**	**4 days after treatment**	**7 days after treatment**
Nectar directly from hive	A	—	<LOQ (<0.6)
	B	—	1.30
	C	—	1.06
	D	—	1.00
	Mean ± SE	—	0.99 ± 0.15
Larvae from hive[a]	A	8.32	2.54
	B	16.70	10.6
	C	19.50	6.72
	D	2.88	1.23
	Mean ± SE	11.9 ± 3.8	5.3 ± 2.1

[a]LOQ = 0.3 mg a.e./kg for 4-day-old larvae and LOQ = 1.0 mg a.e./kg for 7-day-old larvae.

Table 2 Exposure assessment of a brood study colony to glyphosate residues under 2 scenarios used to establish low- and mid-dose levels in bee brood study

Scenario	Daily intake of glyphosate residues in nectar (1944 g nectar/day) [mg]	Daily intake of glyphosate residues in pollen (9 g pollen/day) [mg]	Total daily intake of glyphosate residues [mg]	Uptake over 3 days [mg]	Adjustment from 2.88 kg a.e./ha to 2.16 kg a.e./ha [mg] [g]
Day 1 maximum mean residues (31.3 μg a.e./g in nectar; 573.5 μg a.e./g in pollen)	60.8[a]	5.2[b]	66.0	198	148.5[c]
Mean residues over days 1–3 (15.6 μg a.e./g in nectar; 310.1 μg a.e./g in pollen)	30.3[d]	2.8[e]	33.1	99.3	74.5[f]

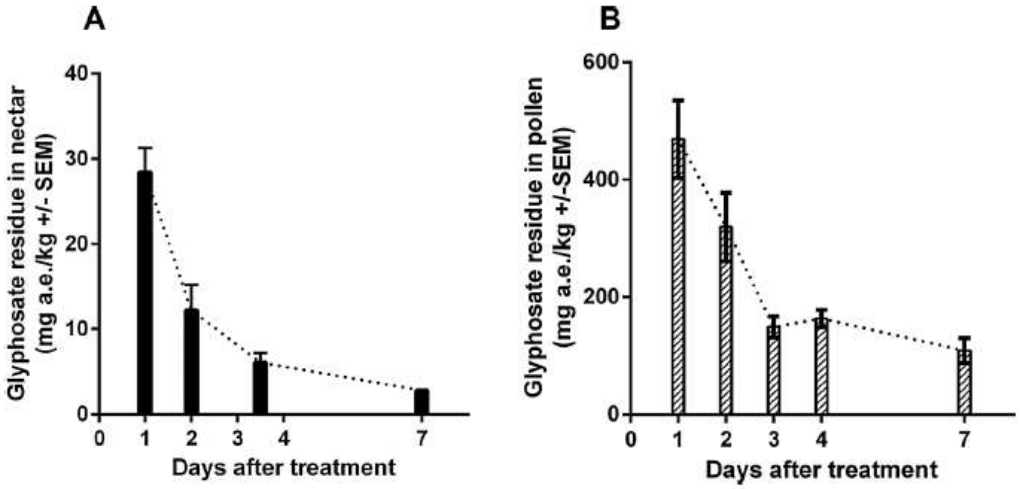
The high dose for the study reflects twice the mid-dose level.
[a] Derived from 1.944 kg nectar consumed/d × 31.3 mg a.e./kg = 60.8 mg glyphosate a.e.
[b] Derived from 0.009 kg pollen consumed/d × 573.5 mg a.e./kg = 5.2 mg glyphosate a.e.
[c] Value of 148.5 mg was rounded to 150 mg to achieve the nominal mid-dose concentration in brood study.
[d] Derived from 1.944 kg nectar consumed/d × 15.6 mg a.e./kg = 30.3 mg glyphosate a.e.
[e] Derived from 0.009 kg pollen consumed/d × 310.1 mg a.e./kg = 2.8 mg glyphosate a.e.
[f] Value of 74.5 was rounded to 75 mg to achieve the nominal low-dose concentration in brood study.
[g] The determined residue content based on an application rate of 2.88 kg a.e./ha was adjusted to reflect the lower application to the rate of 2.16 kg a.e./ha.

The processes during the study followed the method for honeybee brood feeding test with insect growth regulating compounds (Oomen et al. 1992). Up to 24 h before dosing, 100 brood cells containing eggs, 100 cells containing 1- to 2-day-old larvae and 100 cells containing 3- to 4-day-old larvae were selected in each colony and marked using the standard Oomen et al. (1992) acetate overlay sheet method.
On day 0, one group was an untreated control, i.e., fed 1 L 50% sucrose solution, 3 groups were treated with glyphosate IPA salt (added to 1 L of 50% sucrose to achieve doses of 301, 150 and 75 mg glyphosate a.e./L), and one group was treated with the toxic reference, fenoxycarb, dispersed in 1 L of 50% w/v sucrose (750 mg a.s./L). Doses were administered by removing frames of stores from the colonies and placing a 1 L glass container containing the treated or control sucrose within the brood chamber. The container contained a cork float to allow access to the sucrose solution. Samples of each concentration of test item treated sucrose solution were retained for analysis by subsampling 5 mL from each of the prepared solutions and combining to a single sample (total 4 samples; control and 3 doses of glyphosate). The uptake of each sucrose solution was checked daily and the container removed when empty or after 5 days whichever was later.
On day 7, the marked brood cells (eggs, young, and old larvae) were assessed for mortality and appearance in each test colony. The final assessment for each larval was undertaken at day 13 for brood cells marked as containing old larvae, day 15 for cells containing young larvae, and day 16 for cells containing eggs. The cells were uncapped, the bee removed carefully with forceps, and the age of the bee assessed, weighed, and any deformities noted.
On days 4 and 7 (when the marked brood cells were assessed), samples of ten 4- to 5-day-old larvae were sampled from each treated colony (not from an area in which marked brood cells were located) for residue analysis. For the purpose of this study, mortality was defined as the total number of cells in any one group at any one observation period that were empty (other than recently emerged), contained dead larvae or pupae or contained larvae or pupae that were considered unhealthy (sick) and unlikely to survive. Brood mortality was statistically analyzed using a generalized linear model linked to a logit distribution for the brood mortality data and an analysis of variance for pupae weight data to determine the no observed effect concentration (NOEC) (equivalent to the no observed adverse effect level [NOAEL]) statistically, using the software Genstat v12 (VSN International). The study was considered valid if there were significant effects of the toxic reference (>40% effects on all stages) during the detailed brood assessment when compared to the control. The performance of the colonies in the control group were comparable with historical control data for the testing facility (10%–30% larval mortality overall), and demonstrate that the control colonies had performed correctly.

Results
Exposure study
Daily assessments were made of the percentage of the plants that had wilted leaves or flowers. The crop started to show significant effects of the treatment from day 4 onward in both glasshouses and this coincided with the decreased foraging activity in glasshouse 2 although less pronounced effects on foraging were observed in glasshouse 1.
Foraging assessments showed foraging activity on the crop at the start of the study and this continued throughout the exposure period in glasshouse 1 with a peak on day 4; lowest foraging activity was on day 5 at 38% of the mean prespray activity. In glasshouse 2, the foraging activity declined throughout the assessment period and reached <10% of the mean prespray activity on days 5 to 7. The weights of pollen collected from the traps fitted to each hive ranged from 0.37 to 1.8 g per colony per day.
Samples of honeybee products (nectar and pollen) and larvae were analyzed for residues of glyphosate acid equivalents. Glyphosate residues in nectar samples taken from forager bees before the application were not detectable

(<0.3 mg a.e./kg). Residues in nectar samples taken at various time points after the application and originating from forager honeybees ranged from 2.78 to 31.3 mg a.e./kg and declined over time (Figure 1A). Residues in nectar samples taken from the colonies 7 days after the application ranged from below the LOQ (1.0 mg a.e./kg) to 1.30 mg a.e./kg (Table 2).
Residues in pollen samples taken from the pollen trap before the application were not detectable (<0.3 mg a.e./kg). Residues in pollen samples taken at various time points after the application and originating from the trap ranged from 87.2 mg a.e./kg to 629 mg a.e./kg and declined over time (Figure 1B). Residues in larvae samples at 2 time points (day 4 and day 7) after the application ranged from 1.23 mg a.e./kg to 19.50 mg a.e./kg (Table 2).

Fig. 1. Decline of glyphosate residues (mg a.e./kg ± SE). (A) Nectar collected from foragers. The nectar sample from days 3 and 4 were combined due to the small amount collected for analysis. (B) Pollen collected in pollen traps in mg a.e./kg matrix.



Effects study
Consumption of treated sucrose. Analysis of the dosing solutions showed they were within 11% of the nominal doses. The control colonies consumed between 0.63 and 1.0 L of untreated sucrose. In the glyphosate-treated colonies, at least 3 of the 4 colonies in each group consumed the total volume of treated sucrose fed to each of them. There was no statistically significant difference in sucrose consumption in comparison to control for the 301 mg a.i./L group (p = 0.438), 150 mg a.i./L group (p = 0.212), the 75 mg a.i./L group (p = 0.054), which was slightly higher than the control, and the positive control fenoxycarb (p = 0.151).
In the 301 mg glyphosate a.e./L group, one colony consumed 0.39 L and the other 3 each consumed 1.0 L resulting in mean exposure to 255 ± 26 mg glyphosate a.e. In the 150 mg glyphosate a.e./L group, one colony consumed 0.67 L and the other 3 each consumed 1.0 L resulting in mean exposure to 130 ± 12 mg glyphosate a.e. In the 75 mg glyphosate a.e./L group one colony consumed 0.90 L and the other 3 each consumed 1.0 L resulting in mean exposure to 73 ± 2 mg glyphosate a.e. In the fenoxycarb treated colonies, consumption rates ranged from 0.45 to 0.88 L resulting in mean exposure to 510 ± 72 mg fenoxycarb. Exposure at the 150 mg a.i./L dose was significantly lower than at the 301 mg a.i./L dose (p = 0.049) and exposure at the 75 mg a.i./L dose was significantly lower than at 150 mg a.i./L dose (p = 0.002).

Brood mortality
Figure 2 summarizes the survival of marked brood stages at day 7 after dosing and just before emergence. There were no significant treatment-related effects except in the fenoxycarb toxic reference treated colonies, in which overall survival of marked cells was 20% for marked eggs (p < 0.001), 0% for marked young larvae (p < 0.001) and 12% for marked old larvae (p < 0.001), meeting the established validity criterion for the toxic reference (>40% effects at all stages). This can be compared with overall survival of 85% for marked eggs, 96% for marked young larvae, and 96% for marked old larvae in controls and 82%–87% for marked eggs (300 mg a.i./L: p = 0.435, 150 mg a.i./L: p = 0.310, 75 mg a.i./L: p = 0.250), 87%–94% for marked young larvae (300 mg a.i./L: p = 0.185, 150 mg a.i./L: p = 0.060, 75 mg a.i./L: p = 0.254), and 94%–95% for marked old larvae (300 mg a.i./L: p = 0.434, 150 mg a.i./L: p = 0.202, 75 mg a.i./L: p = 0.291) in the glyphosate-treated colonies. The control mortality is similar to historical levels in studies conducted at the Food and Environmental Research Agency (FERA) (10%–30%). Deformities were observed in the fenoxycarb-treated colonies where discolored heads, thorax, and abdomens were noted. No deformities were observed in of the control or any glyphosate-treated colonies. Additionally, there were no significant effects on the mean weight

of the exposed pupae (Table 3) compared to controls in the 300 mg a.i./L group (p = 0.424), the 150 mg a.i./L (p = 0.207), or the 75 mg a.i./L (p = 0.292). The fenoxycarb-treated colonies showed significant effects on weight of surviving pupae marked as old larvae (p = 0.003). The only dead pupae observed in any significant number were those in the fenoxycarb treated group where a mean of up to 190 pupae/day was observed and a mean of 600 pupae were recovered from the colonies over the 17-day period after dosing compared with 2.0 pupae/d in the control and 1.3 to 1.8 pupae/d in the glyphosate-treated colonies. The only adverse effects on colony development were observed in the fenoxycarb-treated colonies where declines in the numbers of bees and brood were observed in the latter stages of the study compared to controls for the 300 mg a.i./L group (p = 0.401), the 150 mg a.i./L group (p = 0.414), the 75 mg a.i./L group (p = 0.360), or the positive control fenoxycarb (p = 0.070).

Fig. 2. Survival (% ± SE) of Eggs (7 and 16 Days After Treatment, DAT), Young Larvae (7 and 15 DAT) and Old Larvae (7 and 13 DAT) for treatment groups (mean consumption) Control (0 mg glyphosate a.e.), A (255 ± 46 mg glyphosate a.e.), B (138 ± 12 mg a.e.), C (73 ± 2 mg glyphosate a.e.), and Fenoxycarb (510 ± 72 mg). Different letters above the bars indicate statistical difference (p < 0.05) from the respective control. # no statistical analysis as no variance due to 100% mortality.

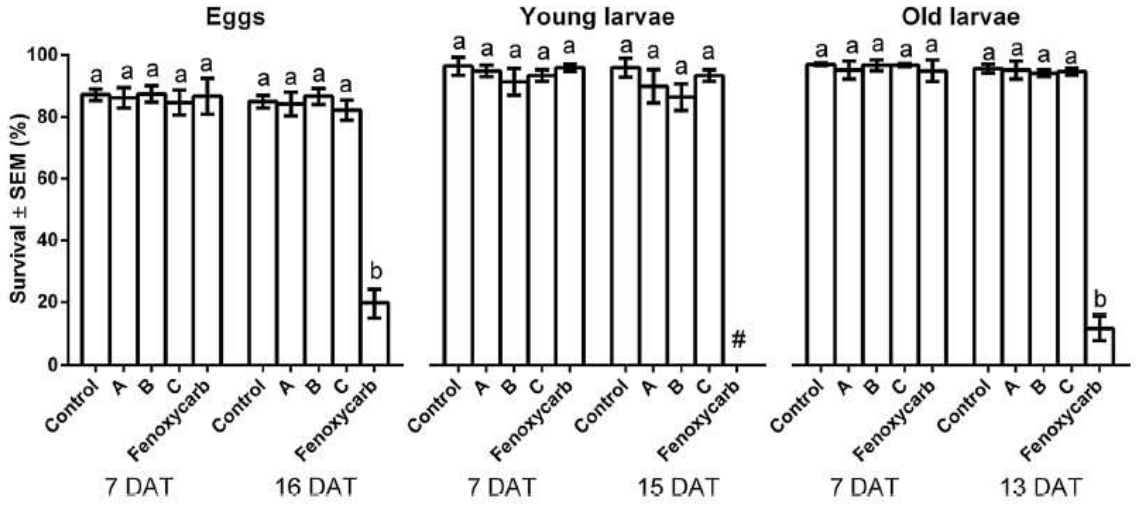


Table 3. Mean pupae weight with SE at final assessment including dead and sick in the fenoxycarb treatment

Treatment	Dose rate mg/L	Mean dose consumed mg (SE)	Weight-surviving pupae marked as eggs (mg)	Weight-surviving pupae marked as young larvae (mg)	Weight-surviving pupae marked as old larvae (mg)
Control	0	0	127.5 ± 0.7	128.4 ± 0.6	128.9 ± 0.4
Glyphosate	301	255 ± 46	135.7 ± 0.6	125.4 ± 0.6	125.6 ± 0.4
Glyphosate	150	138 ± 12	126.7 ± 0.6	124.4 ± 0.8	122.6 ± 0.5
Glyphosate	75	73 ± 2	124.7 ± 0.8	128.3 ± 1.0	121.2 ± 0.5
Fenoxycarb	750	510 ± 72	125.9 ± 0.9	128.8 ± 1.3	115.4 ± 1.0[a]

SE = standard error.
[a]Statistically different effect (p < 0.01).

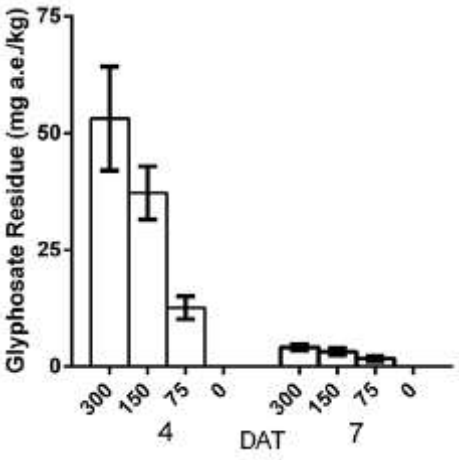
Adult bee mortality

No biologically significant adult mortality was observed in any treatment group with a mean total of 73 to 25 dead adult workers were recovered from dead bee traps over the entire 17-day period after dosing.

Residue analysis

The residues in larvae sampled at 2 time points (day 4 and day 7) after dosing of the colonies (Figure 3) ranged from below the LOQ (1.0 mg a.e./kg) to 82.1 mg a.e./kg (at the highest dose rate) confirming that larvae were exposed to test item provided in the sucrose solution and consumed it. There was a linear relationship between dose level and glyphosate levels in larvae on days 4 and 7. Levels of day 7 were considerably lower than on day 4 and are likely the result of larval growth and glyphosate exposure ending after 5 days of exposure. Notably, these residue levels are comparable with values from the exposure study which ranged from 2.9 to 19.5 mg a.e./kg with a mean of 11.5 mg a.e./kg on day 4 to 1.2 to 10.6 mg a.e./kg with a mean of 5.3 mg a.e./kg on day 7 after the glyphosate application.

Fig. 3. Residues (mg a.e./kg ± SE) in larvae 4 and 7 days after treatment (DAT) for dose groups with dose rate of 300, 150, 75, and 0 mg a.e./kg sucrose solution.



Conclusion

There were no significant effects from glyphosate observed in brood survival, development, and mean pupal weight. Additionally, there were no biologically significant levels of adult mortality observed in any glyphosate treatment group. Significant effects were observed only in the fenoxycarb toxic reference group and included increased brood mortality and a decline in the numbers of bees and brood. Mean glyphosate residues in larvae were comparable at 4 days after spray application in the exposure study and also following dosing at a level calculated from the mean measured levels in pollen and nectar, showing the applicability and robustness of the approach for dose setting with honeybee brood studies.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Although only limited information is given about the validation of the method for the determination of glyphosate residues, the analytical results are deemed reliable. The exposure part of the study follows an approach similar to that outlined in SANTE/11956/2016 rev. 9. From that point of view the residue findings for glyphosate in nectar and pollen may be considered relevant.
Nevertheless, no MRLs are currently set for these commodities and these commodities are not considered for dietary risk assessment either. Therefore, the findings do not directly impact the consumer risk assessment. The article is relevant but supplementary.

Assessment and conclusion by RMS:

The study is primarily investigating the effect of glyphosate on honeybee brood development. Glyphosate is used as an example pesticide to develop an approach to study such effects on honeybee brood. In addition, nectar and pollen were analysed for glyphosate residues. However, both nectar and pollen are currently not considered in Annex I of Reg. (EU) 396/2005 as relevant commodities to be investigated for MRL setting. Also for dietary risk assessment, these commodities are not considered. Therefore, no further impact expected on the existing risk assessment parameters.

B.7.8.3.3. Study 3

1. Information on the study

Data point	CA 6.10.1
Report author	Umsza-Guez M.A. *et al.*
Report year	2019
Report title	Herbicide determination in Brazilian propolis using high pressure liquid chromatography
Document No.	International Journal of Environmental Health Research (2019) (DOI 10.1080/09603123.2019.1670335)
Guidelines followed in study	None

Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

Propolis is a widely used medicinal product sourced by bees from vegetation that may be frequently irrigated with herbicides. Exposure to herbicides can affect bees' health and the quality of commercial propolis. The objective of this study was to calculate the concentrations of glyphosate, aminomethylphosphonic acid (AMPA), picloram and atrazine in different types of propolis from Brazil using high-performance liquid chromatography (HPLC). Four types of propolis (brown, green, red, and yellow) were evaluated for a total of 19 samples. Of these types of propolis, 47% tested positive for the herbicides atrazine (5 to 17.4 µg/g) and AMPA (10.2 to 11.3 µg/g). No samples were reported to be positive for glyphosate; however, the presence of AMPA indicates its existence. The concentrations observed in this study are less than international maximum-residue-level standards.

Materials and Methods

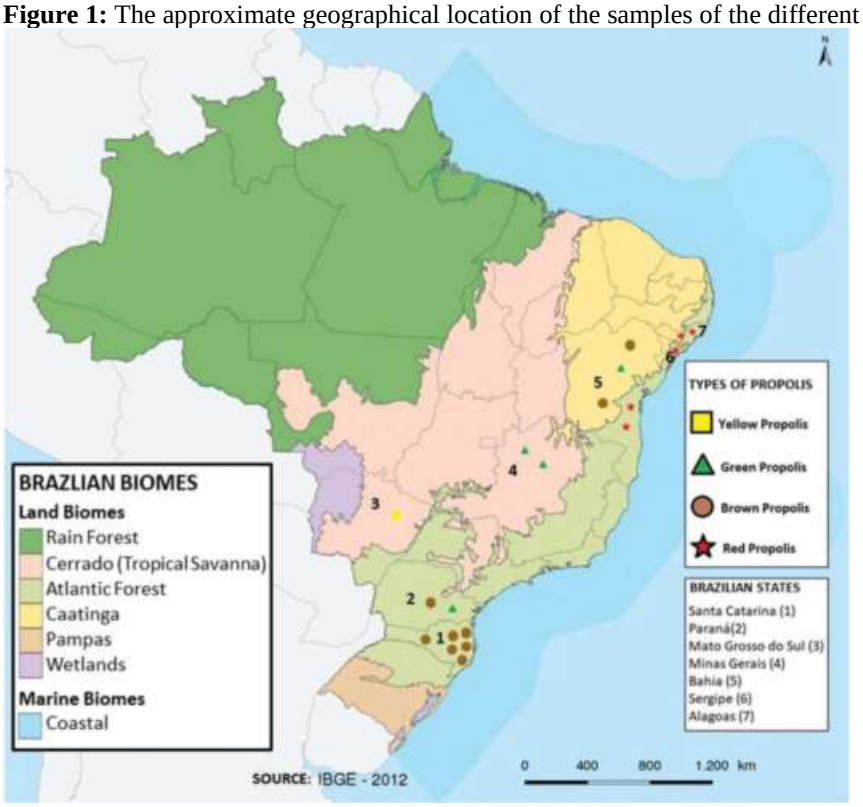
Materials and reagents

In this study, 19 propolis samples (9 brown, 5 red, 4 green and 1 yellow), all native to specific regions of Brazil (**Figure 1** and **Table 1**), were used. These samples were donated by beekeepers from seven different Brazilian states.

Table 1: Botanical origin of the propolis samples.

Sample code	Propolis tipe (color)	State of Brazil	Geographical location
1	Red	Bahia (Northeast)	15° 39′ 1″ S, 38° 57′ 42″ W
2	Red	Bahia (Northeast)	14° 51′ 53″ S, 40° 50′ 13″ W
3	Red	Alagoas (Northeast)	9°41'59.7" S, 36°20'10.7" W
4	Red	Alagoas (Northeast)	9°41'59.7" S, 36°20'10.7" W
5	Red	Sergipe (Northeast)	10°28'10.5" S, 37°17'47.3" W
6	Brown	Bahia (Northeast)	10° 26′ 19″ S, 39° 19′ 51″ W
7	Brown	Bahia (Northeast)	14° 51′ 53″ S, 40° 50′ 13″ W
8	Brown	Santa Catarina (Southern)	26° 10′ 38″ S, 50° 23′ 24″ W
9	Brown	Paraná (Southern)	26° 9′ 41″ S, 51° 33′ 11″ W
10	Brown	Santa Catarina (Southern)	28° 11′ 06″ S, 49° 12′ 53″ W
11	Brown	Santa Catarina (Southern)	28° 11′ 06″ S, 49° 12′ 53″ W
12	Brown	Santa Catarina (Southern)	28° 11′ 06″ S, 49° 12′ 53″ W
13	Brown	Santa Catarina (Southern)	28° 11′ 06″ S, 49° 12′ 53″ W
14	Brown	Santa Catarina (Southern)	10° 26′ 19″ S, 39° 19′ 51″ W
15	Green	Bahia (Northeast)	14° 51′ 53″ S, 40° 50′ 13″ W
16	Green	Minas Gerais (Southeast)	16° 45′ 17″ S, 42° 54′ 30″ W
17	Green	Minas Gerais (Southeast)	20° 7′ 6″ S, 44° 12′ 4″ W
18	Green	Paraná (Southern)	26° 9′ 41″ S, 51° 33′ 11″ W
19	Yellow	Mato Grosso do Sul (Central-west)	-

Figure 1: The approximate geographical location of the samples of the different propolis evaluated.



Sample preparation

The extraction method for the determination of glyphosate, picloram and AMPA was the following: Propolis (1 g) was weighed in a glass flask and suspended with 6 mL of methanol for 24 h at 25°C; after this time, the samples were shaken for three minutes and centrifuged at 2031x g for 10 min. The supernatants were purified by filtration through a cellulose acetate membrane of 0.45 µm (MF-Millipore™); next, the extract obtained was diluted 1:100 of HPLC water (Sigma-Aldrich, 270,733). Derivatization was performed by mixing 3 mL of purified sample and 2 mL of 9-fluorenylmethyl chloroformate (FMOC-Cl) (0.005 M) (23,186 Sigma-Aldrich, MO, U.S.A.) prepared with chloroform (650,498 Sigma-Aldrich, MO, U.S.A.). The solution was maintained for 45 min in the dark. After the reaction, 3-mL methyl chloride (270,997 Sigma-Aldrich, MO, U.S.A.) was added to remove excess of FMOC-Cl; subsequently, the supernatant was filtered using solid-phase extraction (SPE) polymeric columns (Strata C-18 Phenomex, 8B-S001-HCH-T, Torrance, CA, U.S.A.) for the extraction of polar organic substances in aqueous matrices (Olivo et al. 2015), and the C18 cartridges were preconditioned with 5 mL methanol followed by 5 mL of water (prefiltered with 20 µm cellulose acetate membrane). The fluid obtained was reserved for the HPLC analysis.

The sample preparation for determination of atrazine in the propolis was as follows: a mass of 1.0 g of propolis was weighed in the glass flasks and suspended with 3 mL of acetonitrile (HPLC grade, Sigma Aldrich, USA) and shaken for 30 min in a rotary shaker (Thomas Scientific, No. 1171T45). The suspensions were centrifuged at 2031 x g for 15 min, and the supernatant phase was retained. The extraction step was performed three times, and the respective supernatant phases were combined. The extracts were transferred to 10.0-mL volumetric flasks and filtered using 0.45-µm cellulose acetate membranes (MF-Millipore™) before HPLC determination (Sanchez *et al.* 2017).

HPLC conditions

The collected filtered solutions were used for HPLC (Agilent 1200 series, Santa Clara, CA, U.S.A.). Modifications to the chromatographic conditions were established as follows:

(1) Glyphosate, AMPA and picloram analysis: Agilent 250 × 4.6 mm column (Zorbax SB-C18), injection volume of 20 µL, isocratic method at room temperature, HPLC water as the mobile phase 1 mL/min, UV 240 nm and FL excitation 266 nm emission 315 nm (Leyva- Soto *et al.* 2018).

(2) Atrazine analysis: 250 × 4.6 mm column (C18 of 5 µm, Zorbax Bonus-RP) maintained at 35°C, injection volume of 10 µL, linear gradient methanol (Sigma-Aldrich, 646,377)-HPLC water of 50/50 v/v from 0 to 5 min, 80/20 v/v from 5 to 15 min and finally 50:50 from 15 to 17 min, water flux mobile phase 1 mL/min, UV 223 nm (Sanchez *et al.* 2017).

Calibration methods
The calibration parameters involved the linearity range and recovery rate. Internal standards were used for the herbicide quantification. Linearity ranges were calculated by implementing standard curves using one blank run and different aqueous concentration of glyphosate (Pestanal, analytical standard, Sigma-Aldrich, MO, U.S.A.), AMPA (analytical standard, Sigma-Aldrich), atrazine (Supelco, analytical standard, Sigma-Aldrich), and picloram (Pestanal, analytical standard, Sigma-Aldrich). The following concentrations (µg/g) were tested: 5–25 for glyphosate, 25–150 for atrazine, and 15–90 for AMPA and picloram. These concentrations were previously used in the detection of herbicides by HPLC (Olivo *et al.* 2015; Sanchez *et al.* 2017; Leyva-Soto *et al.* 2018). The retention times of glyphosate, AMPA, picloram, and atrazine were 8.69, 26, 3.5 and 17 min, respectively. The analyses were performed in technical and biological triplicate.
The recovery rates were calculated using fortified samples (5 µg of the herbicides; the experiments were carried out in triplicate for glyphosate, AMPA, picloram, and atrazine. The quantification of the recovery through fortified samples that undergo the same treatment process and subsequent chromatographic determination of the problem samples are the best conditions. In this mode, the recovery is not relevant when it is quantified with a standard (Hidalgo 1999). The following equation was used for the calculation of the percentage (Olivo *et al.* 2015):

$$Recovery\ rates = \frac{EAC}{TAV} \times 100 \quad (1)$$

EAC is the experimental average concentration (result of the experimental sample after treatment), and TAV is the theoretical average concentration (5 µg/g).

Human-health risk
Propolis is not usually consumed in raw form, but it is important to consider that the samples do not pose a health risk for human consumption. The exposure values were calculated using the following formula:

$$E = \frac{CXI}{BW} \quad (2)$$

where C is the herbicide concentration, I is the daily ingestion that we considered the consumption of 1 g of raw propolis/day/60 kg based on suggested medical doses (Pasupuleti *et al.* 2017) and BW is the body weight (60 kg). For the calculation of the Hazard Index (HI),

$$HI = \frac{E}{ADI} \quad (3)$$

where E is the exposure and ADI is the Admissible Daily Ingestion.
The sum of the hazard index for each evaluated herbicide was not calculated because the ADI for the herbicides is different: atrazine is 700 µg/kg body weight (WHO/IPCS 1990), and glyphosate/AMPA is 1000 µg/kg body weight (FAO/WHO 2016).

Results
From the total propolis samples, 47% were positive for herbicides atrazine and AMPA (**Table 2**). None of the samples had detectable glyphosate or picloram. AMPA, the degradation product of glyphosate, was present in 10% of the samples, which means that propolis was exposed to glyphosate. Brown propolis had the highest contamination; out of the nine samples, six were contaminated with atrazine or AMPA, and both herbicides were verified in one sample (Sample 10). Four of these samples came from the South region, and two came from the Northeast region. Atrazine was verified in two of the five samples of red propolis.
Only a sample of green propolis (Southeast region) showed contamination with atrazine. The yellow propolis sample (the one with little known commercial availability) showed no evidence of contamination.

Table 2: Concentrations (µg/g) of herbicides in propolis samples from various regions of Brazil.

		µg/g			
Sample code	Propolis type (colour)	AMPA		Atrazine	
1	Red	-		-	
2	Red	-		-	
3	Red	-		-	
4	Red	-		13.7	(±3.41)
5	Red	-		9.7	(±0.11)
6	Brown	-		+	+
7	Brown	-		+	+
8	Brown	10.2	(±1.39)	-	
9	Brown	-		-	
10	Brown	11.3	(±2.65)	+	+
11	Brown	-		-	
12	Brown	-		-	
13	Brown	-		17.4	(±2.60)
14	Brown	-		11.5	(±0.65)
15	Green	-		-	
16	Green	-		+	
17	Green	-		-	
18	Green	-		-	
19	Yellow	-		-	

Symbol (+) refers to having a signal ≤ 5

The estimated values of exposure and HI considering the daily consumption of 1 g of raw propolis an adult with a body weight of 60 kg are shown in **Table 3**.

Table 3: Estimated values of exposure and hazard index considering the daily consumption of 1 g of raw propolis an adult with a body weight of 60 kg.

Herbicide	Sample code	Exposure (µg/kg body weight)	Hazard Index
AMPA	8 (brown)	0.17	$1.7X10^{-4}$
	10 (brown)	0.18	$1.8X10^{-4}$
Atrazina	4 (red)	0.22	$3.2X10^{-4}$
	5 (red)	0.16	$2.3X10^{-4}$
	13 (brown)	0.29	$4.1X10^{-4}$
	14 (brown)	0.19	$2.7X10^{-4}$

$ADI_{Glyphosate\ and\ AMPA}$ = 1000 µg/kg body weight (FAO, 2016)
$ADI_{Atrazine}$ = 700 µg/kg body weight (WHO/IPCS 1990)

Conclusions

The impact of the presence of herbicides on propolis is important for the health of humans and bees. For humans, this impact may represent an increase in daily doses of pesticides, and for bees, the impact may represent a real risk, since herbicide-contaminated plants are the raw material normally used by them. This work presents evidence of exposure of bees to herbicides, and these results can be used in future assessments of the risk of contamination of bee products and the safety of the bees themselves.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The article describes a monitoring survey for glyphosate, AMPA, picloram and atrazine residues in propolis samples (n = 19) from Brazil. While the analytical method and validation procedure are described in detail, the validation results are not provided and the limit of quantification (presumably 5 mg/kg) is not clearly reported and established. It is, therefore, not possible to assess the accuracy of the residue determination. Glyphosate residues were not detected while AMPA residues were detected in 2 samples at 10.2 and 11.3 mg/kg, respectively. This in contrast to most of the data for honey which tend to show higher residues of parent glyphosate than of AMPA.

Currently no EU MRL is set for propolis and since propolis is not taken into account for dietary risk assessment in the EU. Because of that and due to the fact, that reliability of the analytical method is not clearly established, the publication is considered relevant but supplementary.

Assessment and conclusion by RMS:

The article provides data for the measurement of glyphosate and AMPA in propolis. However, propolis is currently not considered in Annex I of Reg. (EU) 396/2005 as relevant commodity to be investigated for MRL setting. Also for dietary risk assessment, propolis is not considered. In addition, it concerns propolis samples from outside the EU. Therefore, no further impact expected on the existing risk assessment parameters.

B.7.8.3.4. Study 4

1. Information on the study

Data point	CA 6.2.1
Report author	Tong M. *et al.*
Report year	2017
Report title	Uptake, translocation, metabolism, and distribution of glyphosate in nontarget tea Plant (*Camellia sinensis* L.)
Document No.	J. Agric. Food Chem, (2017), Vol. 65, No. 35, pp. 7638-7646
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

The uptake, translocation, metabolism, and distribution behavior of glyphosate in nontarget tea plant were investigated. The negative effects appeared to grown tea saplings when the nutrient solution contained glyphosate above 200 mg/L. Glyphosate was highest in the roots of the tea plant, where it was also metabolized to aminomethyl phosphonic acid (AMPA). The glyphosate and AMPA in the roots were transported through the xylem or phloem to the stems and leaves. The amount of AMPA in the entire tea plant was less than 6.0% of the amount of glyphosate. The glyphosate level in fresh tea shoots was less than that in mature leaves at each day. These results indicated that free glyphosate in the soil can be continuously absorbed by, metabolized in, and transported from the roots of the tea tree into edible leaves, and therefore, free glyphosate residues in the soil should be controlled to produce teas free of glyphosate.

Materials and Methods

Chemicals and reagents

Chromatography-grade acetonitrile and dichloromethane (CH_2Cl_2) were obtained from Tedia Company, OH, USA. Water used for LC − MS/MS was produced in the laboratory with a Milli-Q water purification system (Millipore, Bedford, MA). Graphitized Carbon Black (GCB, 120/400 Mesh) and C_{18} (230 − 400 Mesh, 60 Å; SiliCycle, Canada) were obtained from ANPEL Scientific Instrument Co., Ltd. (Shanghai, China), and polyvinylpolypyrrolidone (PVPP) was purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). An Oasis HLB cartridge for SPE (Oasis HLB, 3 mL/60 mg) was obtained from Waters Corporation (Milford, MA). KOH, acetone, sodium tetraborate decahydrate, and ammonium acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HCl was purchased from Shanghai SuYi Chemical Reagent Co., Ltd. (Shanghai, China). The FMOC-Cl (9-fluorenylmethyl chloroformate) was purchased from Alfa Aesar (Tianjin, China). Formic acid was obtained from Aladdin Industrial Corporation (Shanghai, China). Glyphosate (98.0%), glufosinate ammonium (97.5%), and aminomethylphosphonic acid (99.0%) were received from Dr. Ehrenstorfer (Augsburg, Germany). Glyphosate isopropylammonium salt (41%) was obtained from Anhui Sanonda Biological Technology Co., Ltd. (Anhui, China). Standard stock solutions of glyphosate (PMG) and aminomethylphosphonic acid (AMPA) were prepared by weighing 10 mg of each analyte and dissolving in 10 mL of water. Working standard solutions were prepared by diluting the standard stock solution with water. All solutions were stored at - 4°C.

FMOC-Cl was dissolved in acetone at concentrations of 0.5, 1.0, 10, 20, and 40 g/L. Borate buffer consisted of 5 g of $Na_2B_4O_7$ 10 H_2O dissolved in 100 mL of water with the pH adjusted to 9 using 5 mol/L HCl.

Annual cuttings of *Camellia sinensis* cultivar Shu Cha Zao (Shucheng County, Anhui province, China) were cultured for six months in an automated hydroponic system (Anhui Agricultural University, Hefei, China). The nutrient solution contained (in mg/L) 30 NH_4^+, 10 NO_3^-, 3.1 PO_4^-, 40 K^+, 20 Ca^{2+}, 25 Mg^{2+}, 0.35 Fe^{2+}, 0.1 B^{3+}, 1.0 Mn^{2+}, 0.1 Zn^{2+}, 0.025 Cu^{2+}, 0.05 Mo^+, and 10 Al^{3+}. All tea saplings displayed the same growth rate and were 15 − 20 cm in height. All cultivation experiments were done in the greenhouse at Anhui Agricultural University (China).

Sample preparation and LC−MS/MS Analysis

Tea Sapling Sample Preparation. About 5 g of leaves, stems, and roots from tea plants was picked, cut into pieces, and mixed homogeneity, and then a 0.25 g aliquot of the samples was put into mortar, to which was added 10 mL of water, and then ground. After that, the roots or stem samples mixture was sonicated for 10 min and leaves sonicated

for 30 min, respectively. After ultrasonic extraction, the samples were centrifuged at 5000 rpm for 5 min. The aqueous supernatant was transferred to a new centrifuge tube, mixed with 2.5 mL of CH_2Cl_2 by vortex for 2 min, and centrifuged at 5000 rpm for 5 min. A 2 mL aliquot of supernatant was transferred into a 5 mL centrifuge tube to which 5 mg of GCB and 50 mg of PVPP were added. The mixture was shaken by vortex for 2 min and then centrifuged at 10000 rpm for 10 min. The supernatant (1.0 mL) was transferred into a 5 mL centrifuge tube and mixed with 1 mL of borate buffer a by vortex for 2 min. FMOC-Cl (1.0 mL of 20 g/L) was added to the mixture and allowed to react overnight at room temperature. The reaction solution was filtered through a 0.22 μm, hydrophilic PTFE needle filter for subsequent UPLC−MS/MS analysis.

LC−MS/MS Analysis
The LC−MS/MS system included an Agilent Series 1290 ultraperformance liquid chromatography system (UPLC) and an Agilent 6460 triple quadrupole mass spectrometer (QQQ; Agilent Technologies, Palo Alto, CA, USA). The UPLC system was equipped with a quaternary pump, a vacuum solvent degasser, a column oven, and an autosampler. A Waters HSS T3 column (particle size, 1.8 μm; length, 100 mm; internal diameter, 2.1 mm) was used with a solvent flow rate of 0.3 mL/min. The column compartment temperature was set at 40°C, and the injection volume was set at 5 μL. Mobile phase A was 0.1% formic acid in water containing 2 mmol/L ammonium acetate and B was 0.1% formic acid in acetonitrile. The solvent gradient was as follows: 0−0.5 min 5% B, 0.5−6 min 50% B, 6−7 min 95% B, 7−9 min 5% B, and 9−14 min 5% B, according to the Chinese standard method SN/T 1923–2007 [34].
The mass spectra were acquired using electrospray ionization (ESI) in the positive ionization mode. Analyses of glyphosate and AMPA were performed in multiple reaction monitoring (MRM) mode. The settings were: a drying gas flow of 6 L/min with a drying gas temperature of 325°C, a nebulizer pressure of 45 psi, a sheath gas temp of 350°C, and a sheath gas flow of 11.0 L/min. The fragmentor voltage for PMG-FMOC and AMPA-FMOC was 135 V, cell accelerator voltage was 7 V, and collision energies were 11 and 15 eV, respectively. The mass transition ion-pair of PMG-FMOC and AMPA-FMOC were m/z 392 → 88 and 334 → 179, respectively.

Validation of Analytical Procedure
Evaluation of the method included fitting to linear equations and determining the matrix effect, recovery rate, and limit of quantification (LOQ). The standards produced a linear result between 5 and 500 μg/L. The matrix effect (ME), the change of ionization efficiency in the presence of other compounds, was expressed as the responses of FMOC derivatives of PMG and AMPA in matrix compared to the signal in solvent, calculated by the following equation:

$$ME\ (\%) = \left(\frac{Peak\ area\ (spiked\ extract)}{Peak\ area\ (solvent\ standard)} - 1\right) \times 100$$

An ME value equal to 0% means that no matrix effect was detected, while positive and negative values indicate enhancements and suppressions, respectively, of the analyte signal by matrix compounds. Matrix effects were classified into different categories based on the value of this percentage. The matrix effect was not obvious when the values was within ± 20% [36]. The matrix effect in this proposed method was evaluated in fresh tea leaves spiked with 0.5 mg/kg compared with the same concentration of standard sample. Leaves were used because they are a more complex matrix than roots or stems. The recoveries of glyphosate and AMPA in roots, stems, and leaves at spiked levels of 0.5 or 2 mg/kg using standard calibration, each concentration level repeated six times. The limit of quantitation (LOQ) was calculated as a signal-to-noise ratio of 10 (S/N = 10) using the lowest responding concentration for each pesticide at the primary ion transition (quantitation ion transition) obtained from the MS/MS mode.

Uptake, transport, metabolism, and distribution of glyphosate in tea saplings

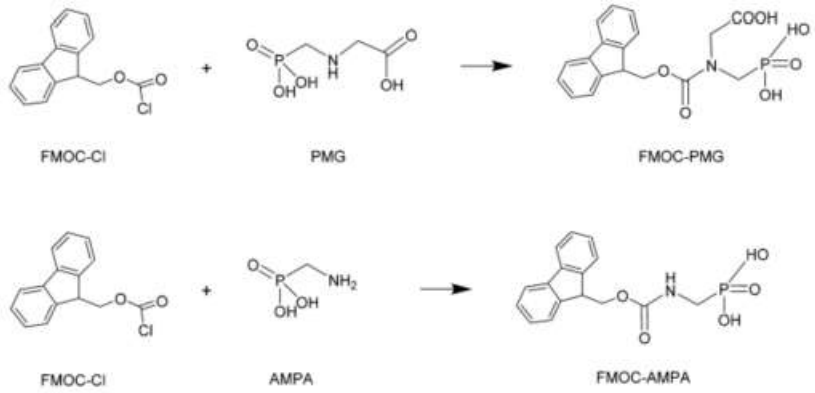
Phytotoxicity of glyphosate to tea saplings
Tea saplings were selected from the hydroponic system and moved into one of fivered plastic buckets. Five tea saplings were cultivated in 1.2 L of nutrient solution containing 0, 5, 50, 200, or 2000 mg/L glyphosate (41% glyphosate isopropylammonium salt) in different buckets. After 1, 3, 5, 7, 10, and 14 days, growth, wilting, and phytotoxicity of the tea saplings were noted.

Uptake, transport, metabolism, and distribution of glyphosate
Tea saplings were transferred from the hydroponic system into blue plastic buckets, with 30 tea saplings cultivated in 6 L of nutrient solution containing 5 mg/L glyphosate. After 0, 1, 3, 5, 7, 10, 14, and 21 days, different parts of the saplings or the whole sapling were collected for determination of the content of glyphosate and AMPA.

Results

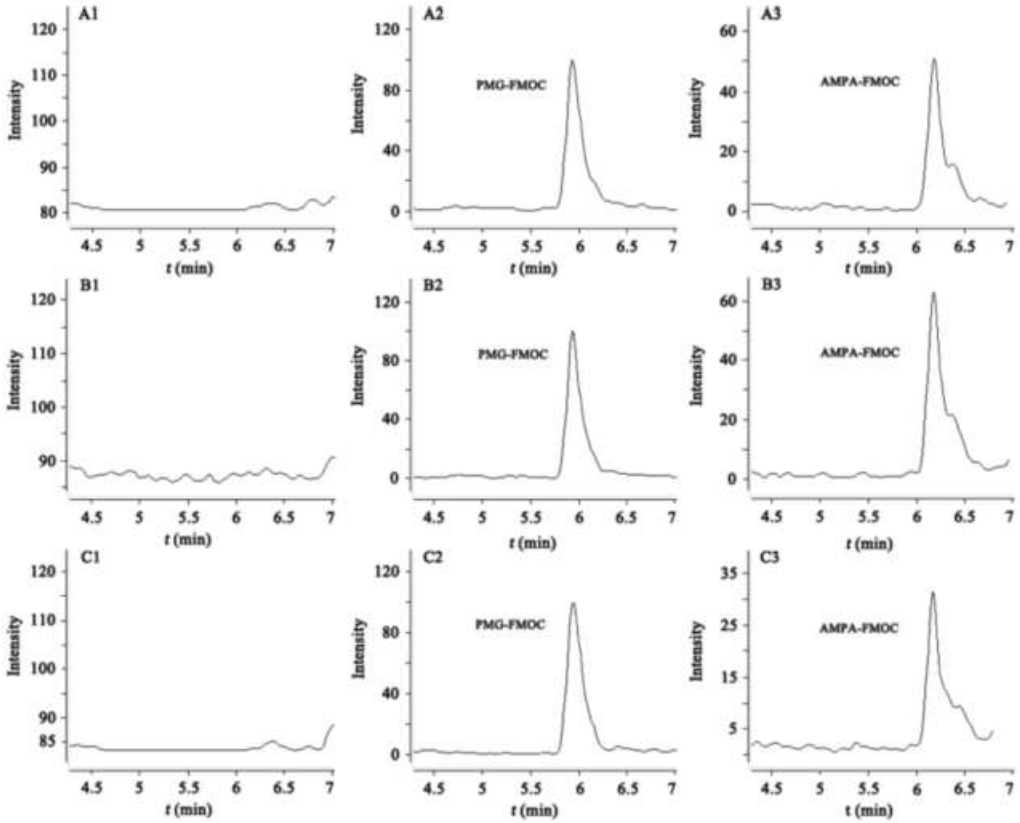
Sample preparation.
Derivatization reaction
The derivatization reaction is shown in **Figure 1**. Among the published methods for derivatization of PMG and AMPA, the concentration of FMOC-Cl in solvent differed significantly. One study used 0.1 mL of 10 g/L FMOC-Cl for derivatization [18], another used 1.0 mL of 20 g/L FMOC-Cl [27], and the latest research used 0.3 mL of 1.5 g/L FMOC-Cl [37]. In this study, different concentrations (1.0 mL) of the derivation regent FMOC-Cl (0.5, 1.0, 10, 20, or 40 g/L) were mixed with 0.05 mg/L of the pesticide standards. The average peak areas of glyphosate were 188.7, 214.7, 186.3, 212, and 205.7 and of AMPA were 202, 234.3, 220.7, 231.7, and 239.7 with the different concentrations of FMOC-Cl. Perhaps 1.0 g/L is the economical choice, but 20 g/L slightly increased the response of them in tea samples compared with the standard samples. Thus, 20 g L FMOC-Cl was used in our proposed method.

Figure 1: Derivatization of glyphosate (PMG) and its metabolite AMPA with 9-fluorenylmethyl chloroformate (FMOC-Cl).



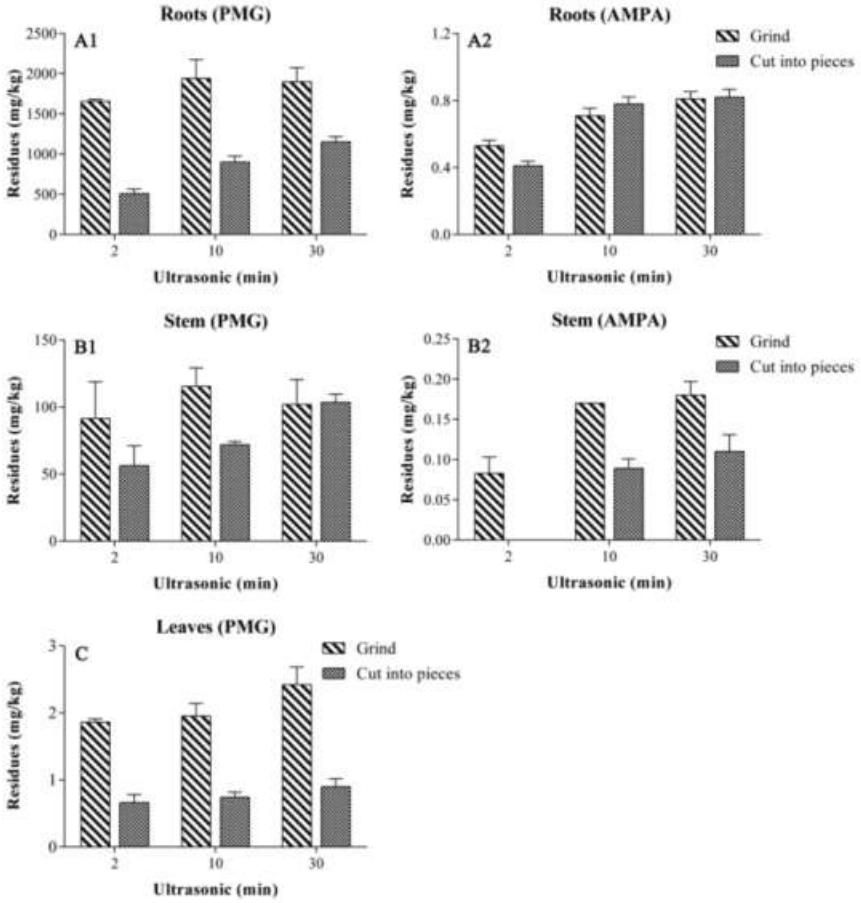
Extraction
Glyphosate and AMPA are strongly polar, water-soluble compounds. Water has been used as the extraction solvent in most published methods, whereas different extract methods and treatment times were used, such as sonication or grinding. In this study, glyphosate-treated samples (about 3–5 g) were cut to small pieces. A 0.25 g aliquot of the cut pieces of sample was ground in mortar and then sonicated for 2, 10, or 30 min to compare the extraction amounts for glyphosate and AMPA. The MS/MS total ion chromatograms for the derived compounds in blank matrix and 0.5 mg/kg spiked samples were shown in **Figure 2**. The results showed that there is a baseline separation of glyphosate and AMPA and little interference in the trace of AMPA.

Figure 2: MS/MS total ion chromatograms of derived compounds in blank matrix (first column) and from (A) leaves, (B) stems, or (C) roots spiked (0.5 mg/kg) with PMG (second column) or AMPA (third column).



The extraction amount of different method was shown in **Figure 3**. The amounts of glyphosate and AMPA recovered were significantly lower with the cutting method than with the grinding method. A longer sonication time of 10 min increased the extraction of glyphosate in all samples. However, sonication for longer than 10 min caused a slight decrease in the extraction of PMG from roots and stems, although extraction from leaves still increased with increase sonication time. There were no significant differences in extraction of APMA from roots or stems when sonication increased from 10 to 30 min. From these preliminary extraction tests, the optimized extraction used in the study consisted of grinding all samples and extracting root and stem samples with sonication for 10 min and the fresh leaf samples for 30 min.

Figure 3: Extraction contents of PMG and AMPA of different part of tea plant by two extraction methods of cutting and grinding. (A1, A2) Roots; (B1, B2) stem; (C) leaves.

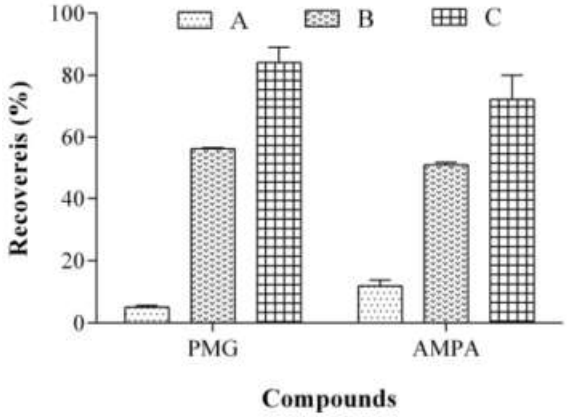


Cleanup method

Strategies used to minimize matrix interference include improvement of chromatographic selectivity, to avoid interference of coextracted matrix components, and modification of sample preparation. Tea represents a complex matrix, containing high amounts of free amino acid (1−2%), polyphenols (18−36%), and alkaloids (2−4%), which can easily be coextracted with target pesticides and may interfere with the subsequent derivatization reaction, especially the free amino acids [38]. A method has used liquid extraction with CH_2Cl_2 as solvent combined C_{18} column (alkylsilane bonded to silica gel) to minimize matrix disturbance during determination of glyphosate and AMPA in commercial tea [36]. The absorbents in HLB cartridges (m-divinylbenzene and N-vinylpyrrolidone copolymer) have similar characteristics to C_{18}. For example, glyphosate and AMPA residues in tea were determined by alkaline solution extraction and HLB column purification 18. The difference between these methods was whether the first water extraction was followed by a re-extraction containing or lacking CH_2Cl_2. Other studies have shown that the purification effect of CH_2Cl_2 is better for fresh agricultural products extraction such as soybean [26], rice, maize and soybean [30], olive, and other plant materials [39]. Until now, there is no report about the fresh tea plant. Some coextracted unknown compounds may greatly affect the pesticide derivatization that follows. Our recent research showed that a QuEChERS extraction method using PVPP combined with GCB effectively and efficiently cleans up tea samples for detection of pesticide residues [40]. To find a suitable cleanup method for fresh samples taken from different parts of the tea plant, three preparation methods were compared, as follows: (A) cleanup the aqueous extract with HLB; (B) re-extract the aqueous extract with CH_2Cl_2 as solvent and then cleanup with HLB cartridge; and (C) re-extract the aqueous extract with CH_2Cl_2 as solvent and then cleanup with PVPP and GCB as sorbents. As the most complex matrix of our three tissues, tea leaf was chosen to verify the effectiveness of these three cleanup methods. The pesticide standard (4 mg/kg) was added to the fresh leaf extract after aqueous solution extraction and sonication for 30 min. This mixture was cleaned up by three methods, derivatized with FMOC-Cl, and analyzed by UPLC−MS/MS. To quickly compare the recoveries between the cleanup methods, the standard solutions of glyphosate and AMPA were used rather than matrix match calibration (**Figure 4**). The lowest recovery of both glyphosate and AMPA resulted from using HLB only. This MS signal decreased possibly because the matrix effect was higher than the other two methods. When the leaf extract was re-extracted with CH_2Cl_2, the recoveries of glyphosate and AMPA increased. The highest recoveries occurred when the re-extracted sample was mixed with PVPP and GCB. This QuEChERS method resulted in 84.2% and 72.3% recovery rates for glyphosate and AMPA, respectively. It was

encouraging to see that the best recovery rate was achieved with the quickest and least expensive sample preparation method.

Figure 4: Effects of different clean-up methods following aqueous extraction of fresh tea leaves on the recoveries of spiked PMG and AMPA (4 mg/kg). Clean-up methods were: (A) HLB; (B) CH_2Cl_2 + HLB; (C) CH_2Cl_2 + PVPP + GCB.



Method validation

The standards ranged from 5 to 500 mg/L as described in the LC−MS/MS analysis section. The peak areas for each standard concentration were plotted and fit to a linear equation, from which the correlation coefficients of the two compounds were obtained. As shown in **Table 1**, the linearities of the standard curves of the two compounds were good, and the r^2 values were higher than 0.999.

Table 1: Linear equations and correlation coefficients of PMG and AMPA; Matrix effect (ME), recovery, and relative standard deviation (RSD) of PMG and AMPA compounds in fresh tea leaf extract (n=6) at spiked with 0.5 mg/kg of PMG and AMPA.

Compound	Linear equations	Correlation Coefficients (r^2)	Spiked level (0.5 mg kg^{-1}) ME(%)	Recovery	RSD
PMG	y =9807.6x-15.536	0.9999	13.95	84.2	5.87
AMPA	y =10821x+0.0647	0.9998	18.84	72.3	10.74

The recovery rates and relative standard deviation (RSD) of the two compounds from the roots, stems, and leaves of tea plant are as shown in **Table 2**. The recovery rate of glyphosate ranged from 82.3 to 116.0% and of AMPA from 72.3 to 94.6%. The RSD (n = 6) values were 4.70−12.99% and 6.50−13.43%, respectively. The recovery rate and RSD values meet the requirement for pesticide analysis. The LOQ of glyphosate was 0.1 mg/kg for both glyphosate and AMPA in leaf samples and 0.05 mg/kg for stem and root samples.

Table 2: Average recovery rates, relative standard deviation (RSD), and limit of quantification (LOQ) of PMG and AMPA compounds from leaves, stems, and roots (n = 6).

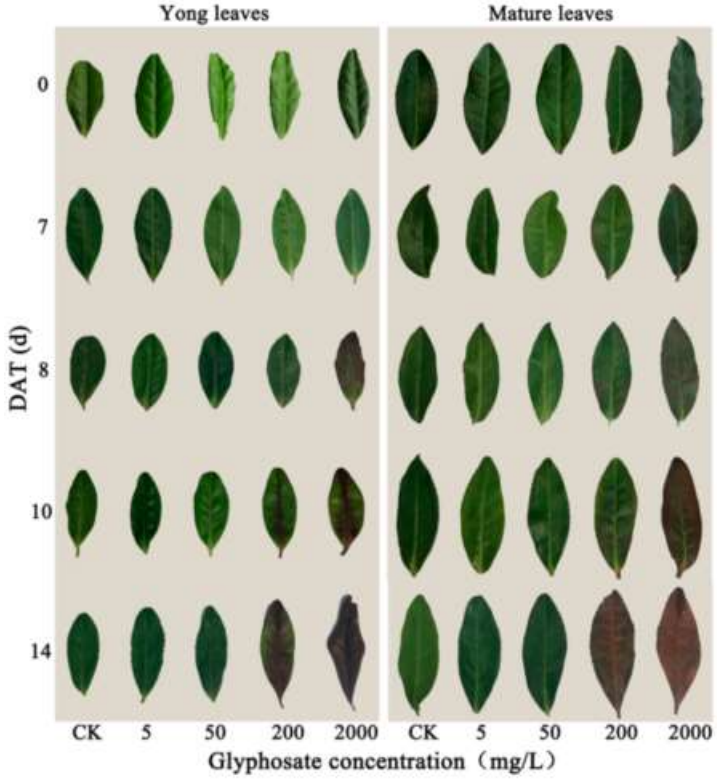
		spiked level				
		0.5 mg/kg		2 mg/kg		
matrix	compound	recovery (%)	RSD (%, *n* = 6)	recovery (%)	RSD (%, *n* = 6)	LOQ (mg/kg)
leaves	PMG	84.2	5.87	82.3	10.82	0.1
	AMPA	72.3	10.14	94.6	13.06	0.1
stem	PMG	91.7	5.95	84.9	4.70	0.05
	AMPA	91.8	6.50	93.2	8.68	0.05
roots	PMG	116.0	12.99	96.7	7.34	0.05
	AMPA	74.8	13.43	85.8	8.73	0.05

Phytotoxicity of glyphosate to tea plant

To investigate the phytotoxicity of glyphosate to tea plants, tea saplings were cultivated in a nutrient solution containing different concentrations of glyphosate (0, 5, 50, 200, or 2000 mg/L). The tea leaves were observed at different times (from 0 to 21 days; **Figure 5**). When grown in 2000 mg/L glyphosate, mature leaves on 7 DAT and

young leaves on 8 DAT showed some dark brown spots. This browning gradually spread over the entire leaves, which began to fall off on 14 DAT. For tea plants cultivated in 200 mg/L glyphosate, the mature leaves began to develop dark brown spots on 8 DAT, and the young leaves began to appear dark brown on 10 DAT. The browning increased gradually and the leaves fell off eventually. This negative effect can come from inhibited acquisition of micronutrients such as Mn, Zn, Fe, and B, which are involved in plant disease resistance mechanisms [13, 41].
However, with the concentrations of 5 mg/L and 50 mg/L glyphosate, the tea leaves did not show any phytotoxicity over 14 days and showed no significant difference to the control sample. These data indicate that the application of glyphosate needs to be controlled under 50 mg/L to avoid toxicity to the tea plants.

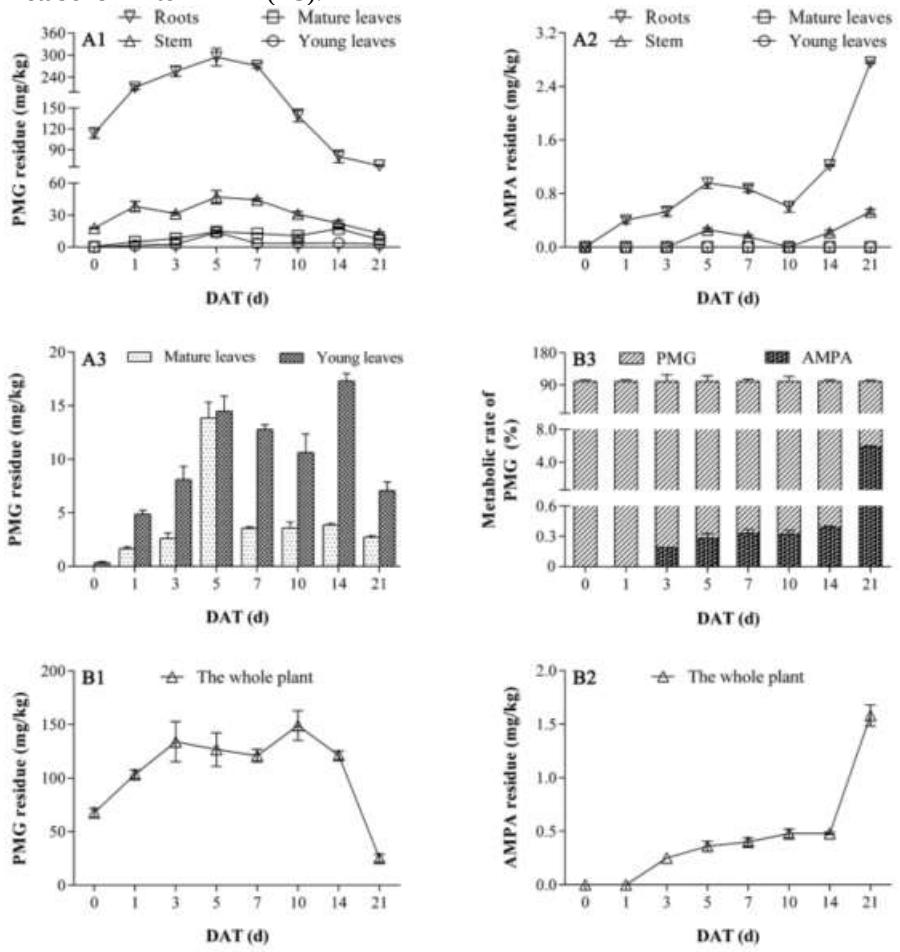
Figure 5: Visual phytotoxicity on tea leaves of different ages, young leaves (leaves 1−3) or mature leaves (leaves 4−6) at different days after treatment (DAT) with different concentrations of glyphosate (0, 5, 50, 200, or 2000 mg/L) delivered in the hydroponic nutrient solution.



Uptake, transport, metabolism, and distribution of glyphosate in tea plant
Afterward, the tea saplings were treated with water nutrient solution containing 5 mg/L glyphosate for 21 days in blue box. The uptake, transport, metabolism, and distribution of glyphosate and AMPA in tea plants were investigated. Results shown in **Figure 6 A1** indicate that the amount of glyphosate in the roots increased gradually with time, from 113.54 mg/kg on day 0 (2 h) to 294.87 mg/kg on day 5, which marked the highest accumulation level, and then decreased to 66.94 mg/kg on day 21. Compared to the concentration of glyphosate (5 mg/L) in the nutrient solution, the accumulation coefficient of roots was about 3.5 mg at 2 h. The amount of glyphosate in stems increased from 17.80 mg/kg on day 0 to 46.83 mg/kg on day 5 and then decreased to 12.78 mg/kg on day 21. The amount of glyphosate in mature tea leaves increased from 0.35 mg/kg on day 0 to 14.49 mg/kg on day 5 and then decreased to 7.07 mg/kg on day 21. The amount of glyphosate in young leaves increased from 0 mg/kg on day 0 (after 2 h) to 13.84 mg/kg on day 5 and then decreased to 2.17 mg/kg on day 21. After glyphosate absorption by the roots, the young leaves accumulated 1.65 mg/kg glyphosate on the first day. This indicated that glyphosate was transferred from roots to leaves through transpiration pull [42]. The cumulative amount of glyphosate in each part of the tea sapling decreased gradually from the fifth day because glyphosate was gradually degraded to AMPA and other metabolites. On days 1, 3, 7, 10, 14 and 21, the cumulative amounts of glyphosate in the young leaves (leaves 1−3) were 1.65, 2.61, 3.55, 3.57, 3.86, and 2.71 mg/kg, respectively, amounts that were all less than half of the amounts in mature leaves (4.87, 8.09, 12.78, 10.63, 17.31, and 7.07 mg/kg, respectively) (**Figure 6 A3**). Interestingly, the glyphosate level in young and mature leaves was almost equal on day 5 (13.84 and 14.49 mg/kg). From these results, it seems that teas prepared with young leaves would not only be of higher grade, but also would also have lower glyphosate content, making these products relatively safer than those made from mature leaves.

The levels of the glyphosate metabolite AMPA were determined in roots, stems, and leaves of tea saplings (**Figure 6 A2**). AMPA was not detected in the young or mature leaves from day 0 to 21 but did increase in stems and roots with time (in roots, from 0 to 2.76 mg/kg and in stems from 0 to 0.53 mg/kg). The absorption of glyphosate and the production of AMPA in the whole plant was also quantified (**Figure 6 B1**, **B2**). The cumulative amount of glyphosate in the plant as a whole ranged from 67.70−133.99 mg/kg from 0 to 3 days, remained relatively constant during days 3 to 10, and then decreased to 25.26 mg/kg by day 21. Meanwhile, the AMPA increased gradually from 0 to 1.58 mg/kg from 0 to 21 days. The metabolic rate of glyphosate transformation into AMPA in whole plant was calculated (**Figure 6 B3**). The rate of glyphosate metabolism into AMPA increased from 0.19% on the 3st day to 5.89% on the 21st day. AMPA represented a very small portion of the PMG/AMPA pool, and so had a lesser impact on tea or tea product safety than did glyphosate.

Figure 6: Concentration of PMG and AMPA in di ff erent parts of tea saplings (A1, A2) and the whole plant (B1, B2) with sustained treatment of 5 mg/L glyphosate in nutrient solution. Comparison of the content of glyphosate in young leaves (leaves 1−3) and mature leaves (leaves 4−6) of tea saplings (A3) and the metabolic rate of glyphosate metabolism into AMPA (B3).



Conclusion

This report represents an investigation of the uptake, transport, metabolism, and distribution of a systemic pesticide in tea plants over time. This study informs growers that by controlling the free glyphosate residues in the soil, one can produce tea product free of glyphosate. It also reminds researchers and growers alike that systemic pesticides such as glyphosate can transfer through the root to leaf. This represents another route generating potential exposure to consumers that differs from the direct contact of pesticide residues applied to edible parts.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The article describes the development and validation of an analytical method for the determination of glyphosate and AMPA in tea matrices (leaves, stems, roots). Using this method uptake and translocation of glyphosate and AMPA from nutrient solution was assessed.

Glyphosate was highest in the roots of the tea plant, where it was also metabolized to AMPA. The glyphosate and AMPA in the roots were transported through the xylem or phloem to the stems and leaves. The amount of AMPA in the entire tea plant was less than 6% of the amount of glyphosate. The results indicate that glyphosate in the soil can be absorbed by the roots and translocated to leaves of tea plants.
The article does not provide relevant regulatory endpoints, however provides supportive information for the analysis of glyphosate and AMPA in tea leaves, which is a matrix difficult to analyze. Therefore the article is relevant but supplementary.

Assessment and conclusion by RMS:
Uptake and translocation of glyphosate from nutrient solution in tea plants was investigated. The article provides supportive information on the metabolism of glyphosate in tea. No further impact expected on the existing risk assessment parameters.

B.7.8.3.5. Study 5

1. Information on the study

Data point:	CA 6.5.3
Report author	Chiarello M. *et al.*
Report year	2019
Report title	Fast analysis of glufosinate, glyphosate and its main metabolite, aminomethylphosphonic acid, in edible oils, by liquid chromatography coupled with electrospray tandem mass spectrometry.
Document No	Food additives & contaminants: Part A (2019), Vol. 36, No. 9, pp. 1376-1384
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	None stated
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format
A method has been developed for the rapid, specific, accurate, precise and sensitive determination of glufosinate, glyphosate and its major metabolite, aminomethylphosphonic acid, in edible oils, by liquid chromatography coupled to tandem mass spectrometry. Oils were extracted with acidified water (1 % formic acid), and the extracts were directly injected into an LC using a Hypercarb column as the stationary phase. The analytes were eluted by a mobile phase of methanol and water containing 1% acetic acid, and they were ionised by electrospray ionization in negative ion mode. The method was validated and limits of quantification ranged from 5 µg/kg (aminomethylphosphonic acid) to 10 µg/kg (glyphosate and glufosinate). Three concentrations (10, 50 and 100 µg/kg) were selected to perform recovery studies. Mean recoveries ranged from 81.4 % to 119.4 %. Intra and inter-day precision were lower than 19 %. Different edible oils were analysed, and no residues of the studied herbicides were detected above limits of quantification.

Materials and methods
Chemicals and standards
Herbicide standards, glyphosate (99.7 %) and glyphosate metabolite, AMPA (99.0 %) were acquired from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol, acetonitrile, formic acid, glacial acetic acid and ammonium formate were purchased from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q water system (Milli-Q, Millipore, Bedford, USA), and it was used for the preparation of mobile phase and extraction solvent. For sample clean-up, zirconia-coated silica (Z-Sep+) and octadecyl silica (C18) sorbents were purchased from Supelco (Bellefonte, PA, USA), whereas primary secondary amine (PSA) was obtained from Scharlab (Barcelona, Spain). Standard solutions of the individual compounds were prepared by exact weighing of the substance (10 mg powder), which was dissolved in 50 mL of an aqueous solution containing 10 % of acetonitrile. These samples were stored at 4 °C in plastic bottles and stoppers in order to avoid interaction with glass surfaces. Standard solutions were diluted with water containing 10 % of acetonitrile to prepare a multicompound solution at 2 mg/L.

Extraction of herbicide residues from oil samples
Sample treatment was based on the QuPPe method. Briefly, the procedure was as follows: 10 g of edible oil was weighed into a 50 mL centrifuge tube. Then, 10 mL of acidified water (1 % of formic acid) was added. After that, the tubes were shaken for 1 min by vortex and then they were centrifuged for 10 min at 3700 rpm (3060 g). Finally, 1 mL of the aqueous phase was filtered through a syringe filter (0.45 µm) and 5 µL of the extract was injected into the LC-MS/MS.

LC-MS/MS analysis
To perform chromatographic analyses, an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA) was used, and a Hypercarb column (100 × 2.1 mm i.d., 5 µm particle size) from Thermo Scientific (Waltham, MA, USA) was selected as the stationary phase. The column temperature was set at 25 °C. During the optimisation of the method, other stationary phases were tested: ODS Hypersil (250 × 4.6 mm i.d., 5 µm particle size) from Thermo Scientific, and Zorbax RRHD Eclipse Plus C18 (100 × 2.1 mm i.d., 1.8 µm particle size) and Zorbax HILIC Plus (100 × 2.1 mm i.d., 3.5 µm) from Agilent Technologies. Chromatographic analyses were performed using a gradient elution, with eluent as methanol acidified with acetic acid (1 %, v/v) and eluent B, water/methanol/acetic acid (94/5/1, v/v/v). The elution started at 100 % of eluent B, and it was linearly decreased to 70 % in 5 min. This was held for 1 min, and then decreased to 10% in 1 min, and kept constant for 1 min. After that, the composition of the mobile phase returned to 100% of eluent B in 0.5 min, and a re-equilibration step of 1.5 min was added. The total running time was 10 min. Flow rate was set at 0.4 mL/min, and 5 µL of extracted sample was injected. Mass spectrometric detection was carried out using a triple quadrupole mass spectrometer from Agilent (6460A). Negative electrospray ionization mode was used for the ionisation of the targeted compounds. Capillary voltage was set at 3500 V and drying and sheath gas flow were set at 5 and 11 L/min, respectively. Drying gas temperature was set at 325 °C, whereas sheath gas temperature was kept at 400 °C. Data acquisition was carried out using the MassHunter (Agilent) software.

Method validation
The developed method was validated testing the following parameters: specificity, matrix effect, linearity, trueness, precision and limit of quantification (LOQ) according to SANTE guideline (European Commission Directorate General for Health and Food Safety 2017). Specificity was evaluated analysing several blanks of different types of edible oils (extra virgin olive oil, virgin olive oil 1, virgin olive oil 2, olive pomace oil, soy oil and sunflower oil). Matrix effect was tested performing calibration curves in six types of edible oils (extra virgin olive oil, virgin olive oil 1, virgin olive oil 2, olive pomace oil, soy oil and sunflower oil) and solvent at concentrations from 10 to 250 µg/L, and matrix effect was estimated according to equation 1:

$$ME(\%) = \left[\frac{slopematrix}{slopesolvent} - 1\right] \times 100 \quad (1)$$

The linearity of the method was tested preparing solvent standard calibration at different concentration levels (from 5 to 250 µg/L). Peak area was selected as an analytical signal, and linear least square regression analysis was performed, evaluating the determination coefficients (R^2), as well as the standard deviation of the residuals. For recovery studies, blank samples from six different edible oils were spiked at three concentration levels, 10, 50 and 100 µg/kg, and five replicates were analysed per level. Precision, expressed as relative standard deviation (RSD), was evaluated as intra-day (repeatability) and inter-day precision (intermediate precision). For the estimation of repeatability, samples spiked at the same levels used for recovery study were tested (five replicates). Intermediate precision was checked at lower (10 µg/g) and higher (100 µg/kg) concentration by extracting spiked samples in five different days. LOQs were estimated by analysing spiked samples at low concentration levels (from 1 to 10 µg/kg), and they were set as the minimum concentration that provides recovery between 70 % and 120 % and RSD values lower than 20 %.

Oil samples
Different olive oil types (extra virgin olive oil, virgin olive oil, olive pomace oil), as well as soy and sunflower oils, were obtained from stores located in Almería, Spain. During the optimisation of the method as well as validation steps, those samples showing the absence of the target compounds were used as blank samples.

Results
Optimisation of LC-MS/MS methodology
For the analysis of target pesticides by MS/MS, a multiple reaction monitoring (MRM) mode acquisition method was developed. For that, individual solutions of each analyte (10 mg/L) were injected by an external syringe pump into the QqQ using electrospray ionisation in negative mode, using a mixture of acetonitrile and water (50/50, v/v) as mobile phase at a flow rate of 0.2 mL/min. Full scan mass spectra and product ion scan were recorded to select the

precursor ion and corresponding production ions. The deprotonated molecule $[M-H]^-$ was the most intense ion for the three compounds. Then, the collision energy of the two most abundant product ions was optimised. Fragmentation of the deprotonated molecular ion at *m/z* 168.0 of glyphosate yielded two product ions at *m/z* 150.0 and 63.1. The most abundant product ion at *m/z* 150.0 corresponds to deprotonated molecular ion at *m/z* 168.0 with loss of a hydroxyl group and the second ion corresponds to $[M\text{-}CO_2CH_2\ NHCH_2\text{-}OH]^-$. Fragmentation of the deprotonated molecular ion of AMPA provided two product ions at *m/z* 78.9 and 63.0. **Table 1** shows the UHPLC-MS/MS parameters used for the identification of the target compounds.

Table 1: Optimised MRM parameters for target compounds for their analysis by UHPLC-MS/MS.

Compound	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)[b]	Ion ratio %
Glyphosate	1.00	168.0 (90)[a]	**150.0** (10)[c]	
			63.1 (20)	96
AMPA	0.77	110.0 (130)	**63.0** (15)	
			78.9 (35)	83
Glufosinate	0.94	180.1 (130)	**94.8** (15)	
			84.9 (15)	75

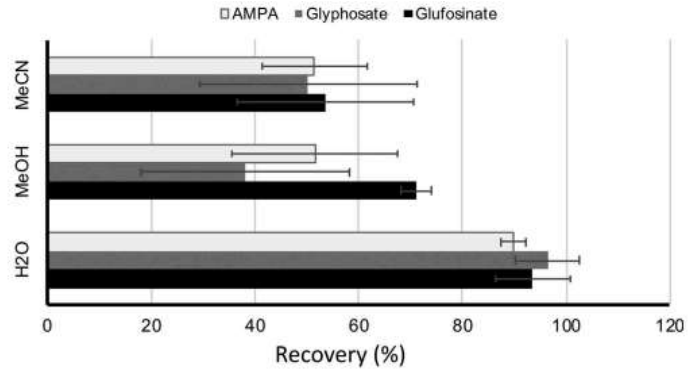
[a] Fragmentor voltage (V) is given in brackets.
[b] Transition in bold was used for quantification.
[c] Collision energy (eV) is given in brackets.

Then, the chromatographic conditions were tested. First, four stationary phases described in Section LC-MS/MS analysis were assessed: ODS Hypersil, Hypercarb, Zorbax RRHD Eclipse Plus C18 and Zorbax HILIC Plus. For the optimisation of this stage, a mixed solution containing 100 µg/L of each compound was injected, using as mobile phase acetonitrile and water and the gradient described in Section LC-MS/MS analysis. Best results were obtained when Hypercarb was used, whereas poor peaks were obtained with the other stationary phases tested. For example, ODS and HILIC columns induced peak tailing and non repetitive retention times, while for C18 stationary phase, prior derivatisation is needed, increasing sample handling. Although Hypercarb needs several blank matrix injections to prime the column, suitable results were obtained using this stationary phase. After that, the composition of the mobile phase was optimised. A mobile phase containing 1 % of acetic acid in the aqueous solution and organic phase (acetonitrile) was tested, and it was observed that the peak shape improved. Then, as the organic phase, methanol and acetonitrile were tested. Methanol was selected because the peak shape of the compounds improved when methanol was used instead of acetonitrile. After that, several modifiers were added to the aqueous solution as acetic acid (0.1 %, 0.5 % and 1 %, v/v), or ammonium formate (5 mM), using methanol as an organic solvent. Suitable elution of the target compounds was achieved using methanol (1 % acetic acid) as eluent A, and a mixture of water/methanol/acetic acid (94/5/1, v/v/v) as eluent B, and this was used for further experiments.

Optimisation of the extraction procedure

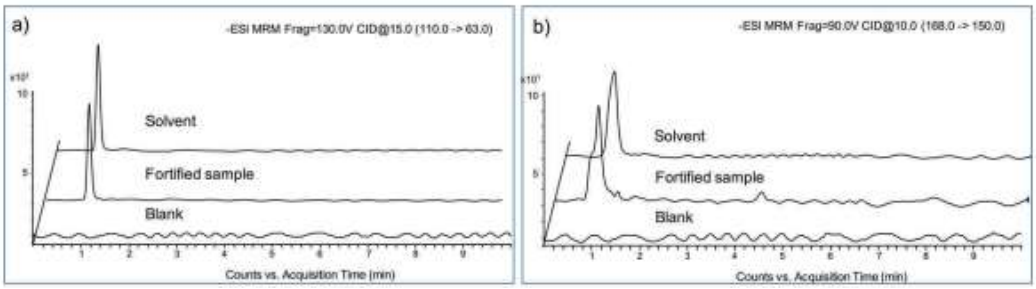
In this study, extraction procedures based on QuPPe approach were tested. Up to our knowledge, the QuPPe method has only been used in olive oil for the extraction of other pesticides. Thus, we evaluated it for the target compounds included in this study, and therefore, the composition of the extraction solvent was checked. Apart from the solvent used in the published QuPPe method, which is acidified methanol, acidified acetonitrile and acidified water were also tested using formic acid at 1 % (v/v). The extracts were prepared as described in Section Extraction of herbicide residues from oil samples, and for that, blank sample was spiked at 250 µg/kg with the target compounds and three replicates were performed. The extracts obtained when methanol and acetonitrile were used could not be analysed because when these solvents were added, a miscible mixture was obtained, and low recoveries (<60 %) and bad reproducibility, relative standard deviations (RSD) higher than 30 %, were obtained as can be observed in **Figure 1**. Nevertheless, suitable recovery and RSDs were achieved when acidified water was tested and it was used for further experiments. Then, acetic acid or lower concentrations of acid (0.1 % v/v) were tested but results were not improved.

Figure 1: Effect of the extraction solvent on the recovery of AMPA, glyphosate and glufosinate. H_2O/water; MeOH/Methanol; MeCN/Acetonitrile. Error bars obtained for n = 5.



Finally, a clean-up step using dispersive solid phase extraction (d-SPE) was tested in order to minimise the presence of interferents in the final extract. For that, the sorbents Zr-Sep+, C18 and PSA were tested. Thus, 1.5 mL of the final extract was mixed with 50 mg of each sorbent in an Eppendorf tube and after shaking during one min, the mixture was centrifuged for 5 min prior to chromatographic analysis. Similar results in terms of recovery and RSD that those obtained without clean-up step were achieved, and therefore, this step was not added to the final procedure in order to minimise sample handling. This can be explained considering that the use of water as extractant solvent avoids the co-extraction of non-polar compounds, achieving a "cleaner"extract. **Figure 2** shows the extracted ion chromatograms of the target compounds applying the proposed analytical methodology to blank and fortified samples at 10 µg/kg.

Figure 2: Extracted ion chromatograms for AMPA (a) and glyphosate (b) corresponding to a blank olive oil, a blank olive oil spiked at 10 µg/kg of the target compounds and a solvent standard at the same concentration (Chromatograms are offset from the timeline)



Analytical performance

Validation was performed according to SANTE guidelines (SANTE 2017). First, specificity was evaluated checking the absence of any signal at the same retention time of the analytes when the characteristic transitions of the target compounds were monitored. Moreover, identification was tested monitoring both MS/MS transitions for glyphosate and AMPA, and the ion ratio was compared with those values provided in **Table 1** according to SANTE guidelines. When ion ratios obtained in spiked samples were compared with those values, the difference was always within ±15 %. Matrix effect was evaluated, and six types of edible oils (extra virgin olive oil, virgin olive oil 1, virgin olive oil 2, olive pomace oil, soy oil and sunflower oil) were tested. **Table 2** shows the values of matrix effect obtained using equation 1. It was observed that matrix effect can be considered negligible (within ±20 %), ranging from −13.5 % to 5.0 %. Therefore, quantification can be performed using solvent standard calibration, allowing the determination of different types of oils with a common calibration curve, minimizing this step in routine analysis. It should be emphasized that no clean-up step is needed to minimize this matrix effect, and similar results were obtained during the determination of organophosphorus pesticides in vegetable oils. Linearity was evaluated by estimating R2, which were higher than 0.996 for the three compounds. In addition, it was checked that the deviation of the back-calculated concentrations of the calibration standards was lower than ±20 % (SANTE 2017).

Trueness was evaluated through recovery studies, and it was tested in six edible oils (extra virgin olive oil, virgin olive oil 1, virgin olive oil 2, olive pomace oil, soy oil and sunflower oil). Results are shown in **Table 2**, and it can be seen that recovery ranges from 81.4 % (AMPA in olive pomace oil) to 119.4 % (AMPA in virgin olive oil 1) for the three compounds in the six matrices at the three levels evaluated ensuring the effectiveness of the proposed extraction method for the quantitative determination of the targeted compounds in different types of edible oils. Intra and inter-day precision results are shown in **Table 2** and RSD values were lower than 19.0 % when intra and inter-day studies were performed, which are lower than the maximum value set by current SANTE guidelines (SANTE 2017). Considering the suitable recovery and precision values obtained the use of isotopically labelled standards was not

necessary, reducing the cost of the proposed method. The LOQ was set at 5 µg/kg for AMPA, whereas for glyphosate it was set at 10µg/kg. These values are lower than the MRLs established for the raw material, as well as in the edible oils if pre-concentration factor of 1 is applied and assuming 20 % oil yield.

Table 2: Validation parameters for the targeted herbicides and metabolites

Compound	Type of edible oil	Matrix Effect (%)[a]	Intra-day Recovery (%)[b]			Inter-day Recovery (%)[c]	
			10 µg kg^{-1}	*50 µg kg^{-1}*	*100 µg kg^{-1}*	*10 µg kg^{-1}*	*100 µg kg^{-1}*
Glyphosate	Extra virgin olive oil	−11.1	88.5 (9.7)	87.7 (5.1)	85.7 (3.1)	97.1 (7.9)	86.4 (7.3)
	Virgin olive oil 1	−7.6	110.3 (12.7)	105.9 (3.8)	101.9 (2.0)	101.2 (8.7)	98.6 (2.9)
	Virgin olive oil 2	−2.8	100.3 (9.5)	90.5 (5.9)	98.0 (1.9)	95.8 (7.1)	89.3(7.8)
	Olive pomace oil	−4.4	81.7 (16.6)	88.4 (5.6)	85.1 (0.6)	93.2 (7.9)	99.2 (13.9)
	Soy oil	4.4	94.4 (4.3)	94.3 (1.5)	87.5 (0.3)	96.8 (2.6)	91.1 (5.5)
	Sunflower oil	−13.5	100.8 (9.4)	113.9 (1.9)	92.8 (5.9)	96.4 (11.3)	95.9 (6.6)
AMPA	Extra virgin olive oil	−8.2	101.2 (4.3)	111.1 (0.9)	98.9 (6.4)	98.5 (11.2)	86.4 (9.5)
	Virgin olive oil 1	0.2	94.3 (6.8)	119.4 (3.8)	113.9 (3.2)	98.9 (5.2)	100.2 (12.1)
	Virgin olive oil 2	−1.4	106.2 (12.3)	93.4 (3.2)	92.1 (3.4)	94.5 (13.6)	98.5 (3.0)
	Olive pomace oil	−3.6	95.7 (12.3)	81.4 (5.2)	83.4 (7.8)	104.2 (7.3)	96.9 (5.1)
	Soy oil	−1.8	91.6 (6.9)	101.8 (3.0)	91.6 (2.7)	94.0 (4.9)	86.4 (9.5)
	Sunflower oil	−7.5	91.2 (6.4)	104.7 (3.7)	118.2 (2.7)	92.8 (13.1)	101.4 (3.1)
Glufosinate	Extra virgin olive oil	−4.3	100.0 (19.0)	93.5 (3.4)	83.9 (4.7)	99.2 (11.6)	87.4 (3.5)
	Virgin olive oil 1	−9.6	105.7 (12.3)	111.2 (2.8)	116.2 (1.0)	99.8 (6.5)	97.0 (17.2)
	Virgin olive oil 2	−7.2	93.1 (18.6)	87.2 (5.1)	91.2 (1.3)	90.6 (18.9)	93.2 (5.3)
	Olive pomace oil	−5.7	83.0 (11.3)	98.6 (5.3)	91.2 (6.6)	92.2 (15.5)	97.7 (6.4)
	Soy oil	5.0	85.5 (6.2)	99.1 (5.7)	98.7 (3.1)	93.1 (8.2)	91.2 (7.4)
	Sunflower oil	−0.4	92.2 (17.2)	112.7 (3.0)	101.6 (2.6)	93.4 (10.1)	100.9 (4.7)

[a] Estimated according to the following equation: *ME (%) = [(slope matrix/slope solvent) −1] X 100*
[b] Intra-day precision, expressed as RSD, is given in brackets (n = 5).
[c] Inter-day precision, expressed as RSD, is given in brackets (n = 5)

Sample analysis
The validated method was used for the determination of glyphosate and AMPA in 25 edible oils belonging to several classes (olive oil, 10; olive pomace, 5; soy oil, 5; and sunflower oil, 5) in order to evaluate the suitability of the developed method. During sample analysis, an internal control was carried out to ensure the reliability of the obtained results. Thus, a blank extract was injected to minimise the risk of false positives; a solvent calibration curve was also injected to check linearity and sensitivity as well as a blank sample spiked at 10 µg/kg to evaluate the extraction efficiency. When the method was applied to the samples, none of the target herbicides was detected at concentrations higher than the LOQ set by the proposed method.

Conclusion
A fast, sensitive and selective method was developed for the determination of glufosinate, glyphosate and AMPA residues in different edible oils. The developed method provides enough sensitivity for the detection of glyphosate, glufosinate and AMPA in edible oils, allowing quantification using solvent calibration. Neither internal standards nor matrix-matched calibration is needed, reducing the cost of the analysis and increasing sample throughput, so it is an interesting tool to be implemented in routine laboratories. In addition, this methodology can be considered as environmentally friendly since only acidified water is needed during the extraction step, avoiding the use of organic solvents and clean-up stated.

3. Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: The article describes the development of an analytical method for the determination of glufosinate, glyphosate and AMPA residues in samples of different edible oils (olive oil, 10; olive pomace, 5; soy oil, 5; and sunflower oil, 5). The method is described in detail and was successfully validated with limits of quantification ranging from 5 µg/kg (aminomethylphosphonic acid) to 10 µg/kg (glyphosate).
Olive oil is relevant to the uses considered for renewal in the EU. Only few real samples were analysed and all showed residues below the LOQ, which can be predicted from the physical-chemical properties of glyphosate and AMPA. Therefore the publication is classified as relevant but supplementary.

Assessment and conclusion by RMS:
The article describes primarily the development of an analytical method for the determination of glyphosate, glufosinate and AMPA in edible oil. Olive oil is relevant with regard to the defended uses within the current renewal

of glyphosate. However, no further impact expected on the existing risk assessment parameters. The study shows that no residues >LOQ were found in edible oil.

B.7.8.3.6. Study 6

1. Information on the study

Data point:	CA 6.9
Report author	Ehling S. *et al.*
Report year	2015
Report title	Analysis of Glyphosate and Aminomethylphosphonic Acid in Nutritional Ingredients and Milk by Derivatization with Fluorenylmethyloxycarbonyl Chloride and Liquid Chromatography-Mass Spectrometry.
Document No	Journal of Agricultural Food Chemistry (2015), Vol. 63, pp. 10562-10568
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	None stated
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

A straightforward analytical method based on derivatization with fluorenylmethyloxycarbonyl chloride and liquid chromatography-mass spectrometry has been developed for the analysis of residues of glyphosate and aminomethylphosphonic acid (AMPA) in a suite of nutritional ingredients derived from soybean, corn, and sugar beet and also in cow's milk and human breast milk. Accuracy and intermediate precision were 91 – 116 % and <10 % RSD, respectively, in soy protein isolate. Limits of quantitation were 0.05 and 0.005 µg/g in powdered and liquid samples, respectively. Glyphosate and AMPA were quantified at 0.105 and 0.210 µg/g (soy protein isolate) and 0.850 and 2.71 µg/g (soy protein concentrate, both derived from genetically modified soybean), respectively. Residues were not detected in soy milk, soybean oil, corn oil, maltodextrin, sucrose, cow's milk, whole milk powder, or human breast milk. The method is proposed as a convenient tool for the survey of glyphosate and AMPA in the ingredient supply chain.

Materials and methods

Chemicals and Reagents

Glyphosate, 1000 µg/mL in water, was purchased from AccuStandard (New Haven, CT, USA). Glyphosate (2-^{13}C, 99%; ^{15}N, 98 %) (96 % chemical purity), 100 µg/mL in water, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Aminomethylphosphonic acid (99 %), disodium tetraborate decahydrate, fluorenylmethyloxycarbonyl chloride (FMOC-Cl), acetonitrile (pesticide residue analysis grade), methanol (LC-MS grade), ammonium acetate, formic acid (98 % wt), phosphoric acid (85 % wt), and sodium hydroxide (10 N in water) were purchased from SigmaAldrich (St. Louis, MO, USA). Aminomethylphosphonic acid (^{13}C, ^{15}N, D2), 100 µg/mL in water, was purchased from Cerilliant Corp. (Round Rock, TX, USA). Chloroform was purchased from Burdick & Jackson (Muskegon, MI, USA). Hydrochloric acid (HCl) (34 – 37 % wt) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Laboratory deionized water was purified through a Milli-Q (EMD Milipore, Billerica, MA, USA) water purification system. A stock solution of AMPA was prepared at a concentration of ca. 1000 µg/mL in Milli-Q water. A mixed intermediate stock solution containing 5 µg/mL of glyphosate and AMPA was prepared in Milli-Q water. A mixed intermediate internal standard stock solution containing 5 µg/mL of glyphosate (2-^{13}C, ^{15}N) and AMPA (^{13}C, ^{15}N, D_2) was prepared in Milli-Q water. Mixed calibration standard solutions containing 5 − 500 ng/mL of glyphosate and AMPA were prepared in Milli-Q water. Borate buffer was prepared by dissolving 4.75 ± 0.05 g of disodium tetraborate decahydrate in 250 mL of MilliQ water and adjusting the pH to 10.0 ± 0.1 with sodium hydroxide 10 N. FMOC-Cl was dissolved in acetonitrile at a concentration of 1.5 mg/mL, and the solution was used within 30 min.

Samples

Soy protein isolate (90 % protein) was sourced from Archer Daniels Midland Co. (Decatur, IL, USA), The Solae Co. (St. Louis, MO, USA), The Scoular Co. (Omaha, NE, USA), and Farbest Brands (Montvale, NJ, USA). Soy protein concentrate (66 % protein) was sourced from The Solae Co. Maltodextrin was sourced from Grain Processing Corp. (Muscatine, IA, USA). Sucrose, soybean oil, and corn oil were sourced from Cargill Inc. (Minnetonka, MN, USA). Whole milk powder (28.5 % fat) was purchased from The Great American Spice Co. (Fort Wayne, IN, USA). Human breast milk (pooled, lactation stage III) was purchased from BioreclamationIVT (Westbury, NY, USA). Bovine milk (2 % fat) and soy milk were purchased from a local supermarket in the Columbus, OH, USA, metropolitan area.

Sample Preparation

Powders (1.00 ± 0.01 g for soy protein isolate/concentrate, whole milk powder, and maltodextrin; 0.50 ± 0.01 g for sucrose) were dispersed in 9.00 ± 0.01 g of Milli-Q water (9.50 ± 0.01 g for sucrose) in 50 mL polypropylene centrifuge tubes. Aliquots of 1.00 ± 0.01 g of dispersed powder or liquid sample (soy milk, bovine milk, human breast milk) or mixed calibration standard solution (5−500 ng/mL of glyphosate and AMPA in Milli-Q water) were added to 15 mL polypropylene centrifuge tubes, followed by spiking solution (if applicable), 10 µL of mixed intermediate internal standard stock solution, and 50 µL of hydrochloric acid (34−37 % wt). After vortexing, 2 mL of acetonitrile (soy protein isolate/concentrate and sucrose) or methanol (soy milk, whole milk powder, bovine milk, human breast milk, and maltodextrin) was added. Tubes were capped, shaken by hand for 30 s, and centrifuged at 3000 rpm for 5 min at 5 °C. Aliquots of 0.3 mL of supernatant were transferred to clean 15 mL polypropylene centrifuge tubes. Borate buffer (0.6 mL) was added, followed by 0.5 mL of freshly prepared FMOC-Cl solution in acetonitrile (1.5 mg/mL). Tubes were vortexed, capped, and incubated for 30 min at ambient temperature. After derivatization, 60 µL of formic acid (98% wt.) was added to each tube. After vortexing, ca. 1 mL of each extract was filtered through 0.2 µm PTFE syringe filters into 2 mL amber autosampler vials. For oils (soybean and corn), 1.00 ± 0.01 g samples were added to 15 mL polypropylene centrifuge tubes followed by spiking solution (if applicable) and 10 µL of mixed intermediate internal standard stock solution. After vortexing, 2 mL of chloroform and 1 mL of Milli-Q water were added. Tubes were shaken by hand for 30 s and centrifuged at 3000 rpm for 5 min at 5 °C. Aliquots of 0.3 mL of supernatant were further processed as described above.

Instrumentation

Analysis was performed on a Waters ACQUITY Ultra Performance LC coupled to a Xevo TQ-S triple-quadrupole mass spectrometer. Chromatographic separation was carried out on an ACQUITY UPLC BEH C18 1.8 µm, 2.1 × 100 mm, column (Waters Corp., Milford, MA, USA), operated at 40 °C at a flow rate of 0.3 mL/ min with a mobile phase system consisting of 10 mM ammonium acetate in water (solvent A) and 10 mM ammonium acetate in 95/5 (vol) acetonitrile/water (solvent B). The following gradient program was used: 0 − 1 min, 10% B; 1 − 7 min, 10 − 55% B; 7 − 9 min, 55 − 100% B; 9 − 12 min, 10% B. Injection volume was 10 µL. The mass spectrometer was operated in the positive electrospray mode with both quadrupoles tuned for unit resolution. Selected operating parameters were capillary voltage, 3.0 kV; cone voltage, 20 V; desolvation temperature, 350 °C; desolvation gas (N2), 800 L/h; cone gas (N2), 250 L/h; and collision gas (Ar), 0.15 mL/min. Two multiple reaction monitoring (MRM) transitions were recorded for the FMOC derivatives of each compound and internal standard (Table 1).

Table 1: Multiple Reaction Monitoring (MRM) Transitions Used for FMOC Derivatives of Analytes and Internal Standards

compound	MRM transitions	cone (V)	CE[a] (eV)	dwell (s)
FMOC-glyphosate	392 → 214	20	10	0.025
	392 → 170	20	10	0.025
FMOC-glyphosate ($2\text{-}^{13}C$, ^{15}N)	394 → 216	20	10	0.025
	394 → 172	20	10	0.025
FMOC-AMPA	334 → 112	20	10	0.025
	334 → 156	20	10	0.025
FMOC-AMPA (^{13}C, ^{15}N, D_2)	338 → 116	20	10	0.025
	338 → 160	20	10	0.025

[a]Collision energy.

Results

Method Development

A limited number of reports exist in the scientific literature on the direct analysis of glyphosate and AMPA without derivatization. In virtually all cases the chromatographic stationary phases used suffered from poor ruggedness (e.g., ion exchange [16, 20, 21] liquid separation cell [18, 19]). In this work the authors have attempted the direct analysis

for glyphosate and AMPA on a number of polar stationary phases, such as hydrophilic interaction chromatography (HILIC), amino-functionalized C_{18}, porous graphite, and mixed-mode (reversed phase + ion exchange) without success. The authors have also failed to replicate the reported direct analysis of glyphosate in the void volume of a reversed phase column [17] or via flow injection into the mass spectrometer [22]. Besides poor retention and peak shape and lack of ruggedness, a further impediment was insufficient instrumental sensitivity to the underivatized analytes.

Even after derivatization with FMOC-Cl, extreme peak tailing was observed on the C_{18} column chosen for chromatographic separation. This was most likely due to the presence of the free phosphate group in the derivatized analytes. Phosphate groups are known to bind to active metal sites in the column and to also interact with free silanol groups in a pH-dependent manner, as described in Zhang et al. Hence, new columns were conditioned with 0.1% phosphoric acid at a flow rate of 0.3 mL/min for 30 min before use. This procedure, along with the use of a neutral mobile phase (pH 6.8) to ensure repulsion between the negatively charged silanol and phosphate groups, reduced the tailing problem. Glassware was avoided for the same reason. The entire procedure was carried out in plastic vessels, as suggested by Goscinny et al. The issue of complexation of glyphosate with various cations resulting in low recoveries has been well established in environmental water analysis [13]. Acidification with HCl to pH 1 has been used successfully to overcome this problem [12, 14, 31].

Surprisingly, this issue has not been described so far in the analysis of complex biological matrices, even though acidification with HCl has been reported in selected cases [7, 24, 26]. During method development we determined that acidification of soy and milk-based matrices with HCl is absolutely essential to achieve any meaningful recovery of glyphosate and AMPA.

In the present method protein precipitation with acetonitrile or methanol was employed for sample cleanup. Chlorinated solvents such as methylene chloride were not used for general sample extraction, unlike in Goscinny *et al.* [33]. However, chloroform was used in the particular case of oils. It was found that either acetonitrile or methanol works best in certain matrices in terms of overall process efficiency (apparent recovery), which is a combination of extraction recovery and matrix effect. For example, in certain batches of powdered soybased matrices (soy protein isolate, soy protein concentrate) and in sucrose, acetonitrile afforded slightly better process efficiency than methanol. Conversely, in milk-based matrices, soy milk and maltodextrin methanol gave clearly superior results (**Table 2**). Overall, it was remarkable that in certain matrices much higher extraction recoveries were obtained with methanol compared to acetonitrile. However, the matrix effect was reduced and extracts were visually cleaner and easier to filter with the use of acetonitrile compared to methanol. Besides this step, no further laborious cleanup steps using solid phase extraction cartridges were used. Aliquots of the extracts were directly subjected to derivatization with FMOC-Cl without the evaporation/concentration step used by Goscinny et al. [33]. The previously reported concentration of FMOC-Cl solution used for derivatization of glyphosate and AMPA varied in the range of 1 − 28 mg/mL [28−33]. In this work it was determined using a fluorescence detector that a concentration of 1.5 mg/mL of FMOC-Cl in acetonitrile provides sufficient excess reagent in both soy- and milk-based matrices, with 85 and 93 % of FMOC-Cl being hydrolyzed by water, respectively, in each matrix. Goscinny *et al.* [33] have used methylene chloride to remove the excess FMOC reagent. In the present method excess reagent was not removed from the reaction mixture, with no apparent adverse effect on the analysis or the instrumentation.

Table 2: Effect of Extraction with acetonitrile versus methanol on extraction recovery (ER), matrix effect (ME), and process efficiency (PE) in various matrices.

		acetonitrile extraction			methanol extraction		
compound	matrix	ER (%)	ME (%)	PE (%)	ER (%)	ME (%)	PE (%)
glyphosate	soy protein isolate	83.7	89.6	75.0	87.3	90.7	79.2
	soy protein concentrate	79.3	84.6	67.1	84.8	74.5	63.1
	soy milk	65.7	65.4	43.0	91.8	55.2	50.7
	whole milk powder	63.5	86.7	55.1	92.6	77.1	71.4
	bovine milk	44.4	92.0	40.9	81.5	70.3	57.2
	human breast milk	30.7	100	30.7	94.3	78.5	74.0
	maltodextrin	75.2	90.2	67.9	87.1	98.5	85.8
	sucrose	87.6	97.1	85.0	79.2	95.2	75.4
AMPA	soy protein isolate	79.2	69.9	55.4	89.6	71.3	63.9
	soy protein concentrate	79.9	63.5	50.8	87.7	64.2	56.3
	soy milk	64.7	63.5	41.1	84.4	66.2	55.9
	whole milk powder	58.9	73.0	43.0	89.1	81.2	72.4
	bovine milk	40.3	66.8	26.9	83.4	74.7	62.3
	human breast milk	26.3	60.7	16.0	88.9	56.0	49.8
	maltodextrin	67.9	80.1	54.4	89.7	76.8	68.9
	sucrose	86.0	98.9	85.1	78.9	103.2	81.4

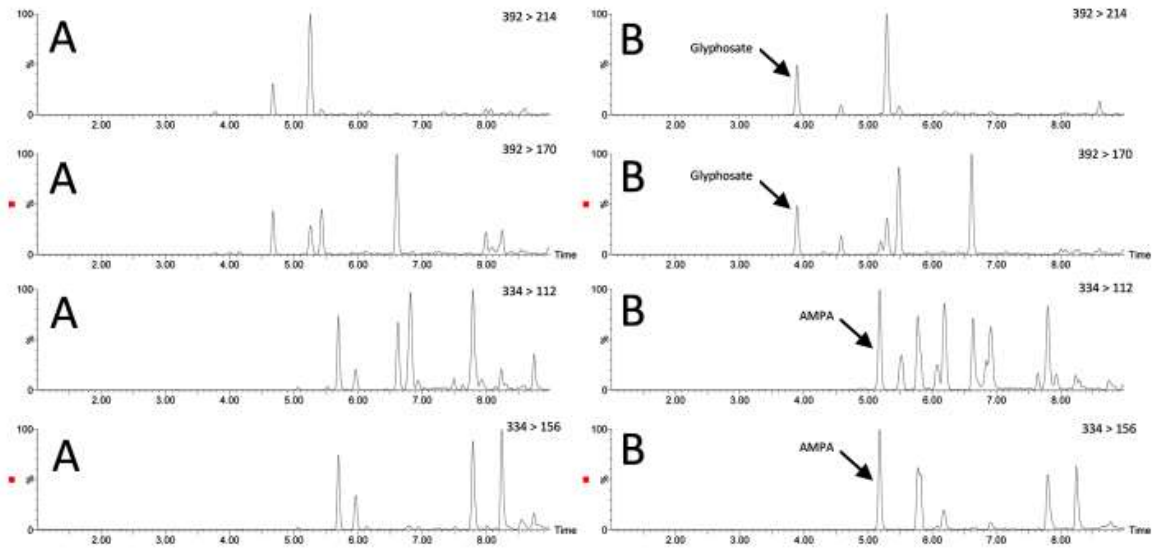
[a]ER, area internal standard in pre-extraction spiked matrix/area internal standard in post-extraction spiked matrix; ME, area internal standard in post-extraction spiked matrix/area internal standard spiked into solvent; PE, ER × ME, all expressed as percentages.

MRM transitions for derivatized analytes and internal standards were established by performing a daughter ion scan experiment at 15 eV collision energy on the expected protonated molecular ions of each FMOC derivative during a chromatographic run. After the identification of daughter (fragment) ions, collision energies were optimized for maximum sensitivity for each MRM transition by performing multiple chromatographic runs at collision energies ranging between 10 and 35 eV. Some of the MRM transitions selected (**Table 1**) are different from those used by Goscinny *et al.* [33]. For example, the fragment ion at m/z 88 resulting from the loss of the FMOC moiety and a phosphate group from FMOC glyphosate was not observed with the current experimental setup. Even though the fragment ion at *m/z* 179.1 corresponding to the loss of N-carboxyglyphosate and N-carboxy-AMPA from FMOC-glyphosate and FMOC-AMPA, respectively, was observed, the more intense fragment ions at *m/z* 170 and 214 resulting from FMOC-glyphosate and at *m/z* 112 and 156 resulting from FMOC-AMPA were preferred. These fragment ions result from the losses of 9-methylenefluorene and 9-formyloxymethylene-fluorene groups, respectively, from either FMOC-glyphosate or FMOC-AMPA. The other previous works using derivatization with FMOC-Cl and LC-MS have used single-stage mass spectrometers. Compared to the latter, triple-quadrupole mass spectrometry adds another dimension in terms of selectivity, in addition to enhanced sensitivity.

Method Validation

Soy protein isolate is the major ingredient used in nutritional products, which is derived from crops potentially treated with glyphosate. Hence, soy protein isolate from a batch derived from non-GM soybean was selected for core method validation. The selectivity of the method was established by analyzing seven batches of soy protein isolate (derived from non-GM soybean) from three different suppliers. There were no interfering peaks in the expected retention windows of the derivatized analytes, even though several matrix peaks were present at later eluting times (**Figure 1A**). Linear (1/x-weighted) six-point calibration curves (5 − 500 ng/mL) had coefficients of determination (R2) ≥0.99. Residuals were ≤20 % at the lowest calibration level and ≤15 % at all other levels. Three core validation runs consisting of four replicates each at four QC spiking levels were conducted on three different days. To ensure homogeneous distribution of the spiking solution, the powder was first dispersed in water (1/9 powder/water ratio), and spiking was carried out into the hydrated powder at 0.005, 0.025, 0.1, and 0.4 μg/g, which correspond to 0.05, 0.25, 1, and 4 μg/g in the dry powder.

Figure 1: MRM chromatograms of FMOC derivates of glyphosate and AMPA in extracts of soy protein isolate derived from conventional soybean (A) and GM soybean (B).



Overall, 48 data points each were collected for both derivatized glyphosate and AMPA, of which 2 were rejected for each compound as outliers (accuracy outside of the 75−125% range). The outliers were all at the lowest spiking level (0.050 μg/g). However, at least three valid data points were retained for each compound on each day. Accuracy, within-day precision, between-day precision, and overall (intermediate) precision are summarized in **Table 3**.

Table 3: Summary of Method Validation Data (Accuracy and Precision) for Glyphosate and AMPA in Soy Protein Isolate

			precision (% RSD)		
compound	spike concn (μg/g)	mean accuracy (%)	within day	between days	total
glyphosate	0.05	91.1	4.0	8.4	9.3
	0.25	104.7	2.3	4.7	5.3
	1	112.5	2.6	3.5	4.4
	4	103.2	2.7	1.9	3.3
AMPA	0.05	115.5	7.7	0.0	7.3
	0.25	108.0	2.1	3.8	4.3
	1	114.6	3.2	3.1	4.5
	4	115.2	3.1	2.0	3.7

Mean accuracy and overall (intermediate) precision were 91 – 116 % and <10 % RSD, respectively, for both analytes at all four spiking levels. The consistent performance of the method in terms of accuracy and precision over three core validation runs has demonstrated its ruggedness. The limit of quantitation (LOQ) for both derivatized analytes was 0.05 μg/g in soy protein isolate. This was the lowest practical concentration at which good accuracy and precision could be achieved. The limit of detection was estimated according to the procedure proposed by the Environmental Protection Agency35 as t·SD, where t is Student's variable (99% confidence level and n − 1 degrees of freedom), and SD is the standard deviation of n = 10 replicate measurements at the 0.05 μg/g spiking level (corrected for mean accuracy). Calculated limits of detection for glyphosate and AMPA were 0.012 and 0.01 μg/g, respectively, in soy protein isolate. Extract stability was determined by reanalyzing an entire validation run after the samples had been maintained on the autosampler tray for 48 h at ambient temperature. The run had acceptable mean accuracy and intermediate precision (80 – 116 % and <11 % RSD, respectively). The stability of the stock solution of AMPA was established over at least 100 days at 5 °C. The stock solution of glyphosate was used within the manufacturer's guaranteed shelf life period when stored at 5°C.

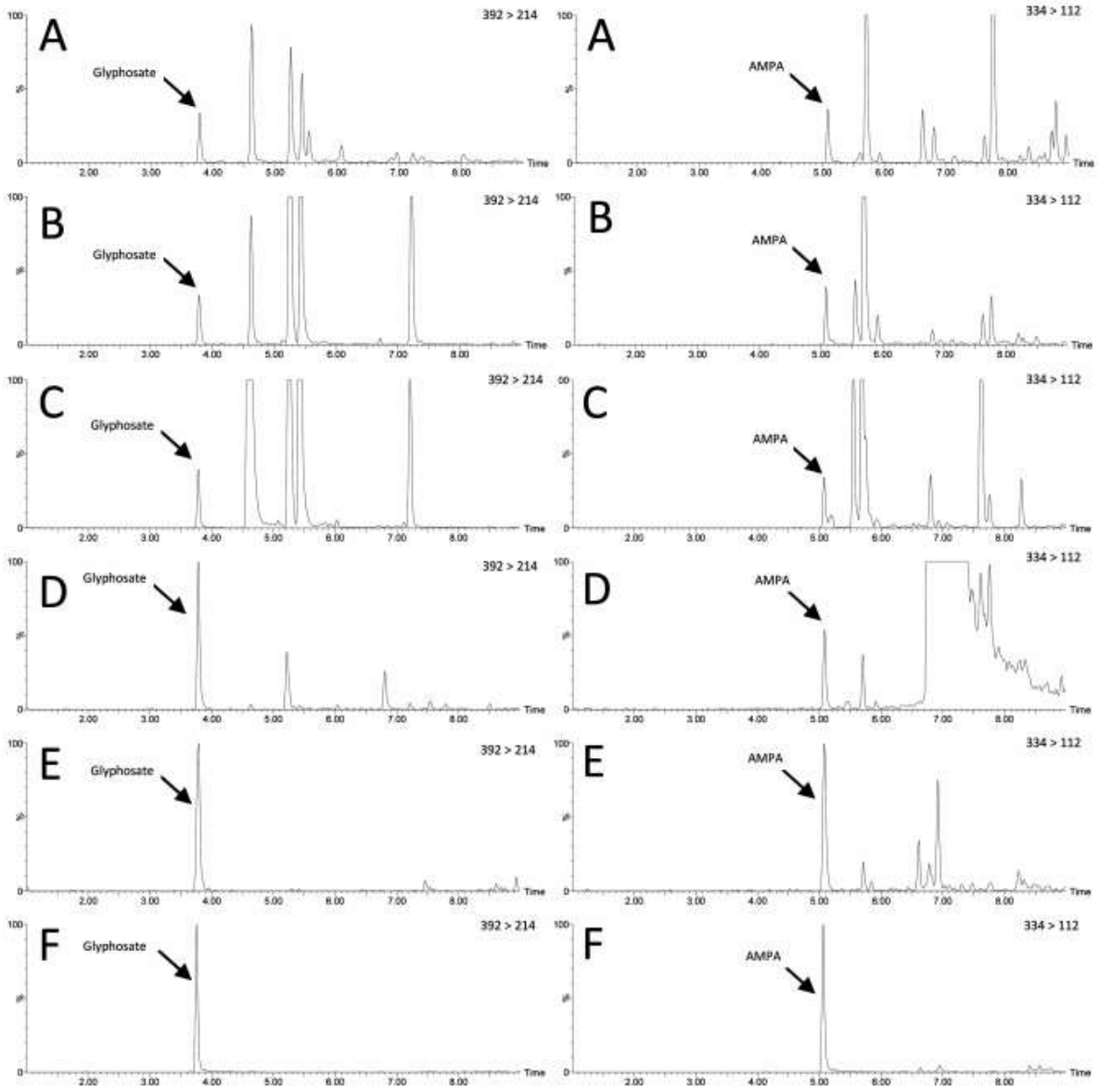
Method Applicability

The above method was applied to a variety of nutritional product ingredients derived from crops potentially treated with glyphosate, such as soy protein isolate, soy protein concentrate, soy milk, and soybean oil (derived from soybean), maltodextrin and corn oil (derived from corn), and sucrose (derived from sugar beets). Cow's milk and whole milk powder were also included because there was no previous study on the potential secretion of glyphosate and/or AMPA

into the milk of cows fed crops treated with glyphosate. Pooled human breast milk was also analyzed to address potential concerns regarding the exposure of breastfed newborns to this widely used herbicide. Because soy protein isolate is the major nutritional ingredient that is derived from crops potentially treated with glyphosate and is the main focus of the study, eight different batches were analyzed. In the case of maltodextrin four different batches were analyzed. For all other nutritional ingredients, one sample of each was included to demonstrate method applicability. Glyphosate and AMPA were detected in a batch of soy protein isolate sourced from GM soybean at concentrations of 0105 and 0.210 μg/g, respectively (**Figure 1B**), and in a batch of soy protein concentrate sourced from GM soybean at concentrations of 0.850 and 2.71 μg/g, respectively. The lower residue levels determined in soy protein isolate compared to soy protein concentrate reflect the higher degree of processing to which the former is subjected compared to the latter. Whereas MRLs for glyphosate have not been established in soy protein isolate/concentrate, the residue levels reported herein are at least 1 order of magnitude lower than the MRLs established in soybean. Glyphosate and AMPA were not detected in soy milk (sourced from non-GM soybean), as expected. Glyphosate and AMPA were not detected in soybean oil, corn oil, maltodextrin (four batches), and sucrose (all derived from GM crops), showing that the complex processing steps to which these ingredients are subjected are effective at removing any detectable levels of residues. Cow's milk, whole milk powder, and human breast milk did not show any trace of glyphosate and AMPA either, suggesting that secretion of these residues into either cow's or human milk does not take place. Given the practice of pooling of cow's milk by the dairy industry and also of breast milk by its supplier, a certain background concentration of glyphosate and/or AMPA in these mediums would be expected if secretion into milk occurred. To our knowledge this is the first confirmation of the absence of residues of glyphosate and AMPA in both cow's milk and human breast milk.

To demonstrate the method's applicability to the above matrices, each blank matrix was spiked with glyphosate and AMPA at 0.05 μg/g (powders) or 0.005 μg/g (liquids), and four replicate analyses were carried out (three replicates for oils). Mean accuracies were 86 – 118 % in all matrices, except for AMPA in sucrose (130 % accuracy). The latter is inconsequential given the absence of residues in this matrix. Precision was <10 % RSD in all matrices. Representative chromatograms are shown in **Figure 2**. The 0.005 μg/g spiking level in cow's milk is 10 times lower than the most stringent MRL established for glyphosate in the same matrix. Whereas MRLs have not been established in powdered nutritional ingredients, the 0.05 μg/g spiking level in powdered ingredients is 1 − 2 orders of magnitude lower than the MRLs established in soybean, corn, or sugar beet.

Figure 2: MRM chromatograms of FMOC derivatives of glyphosate and AMPA in extracts of whole milk powder (A), cow's milk (B), human breast milk (C), maltodextrin (D), sucrose (E), and soybean oil (F). Spiking concentrations were 0.05 μg/g in powders and 0.005 μg/g in liquids



Conclusion

In conclusion, a straightforward, rugged, quantitative, and confirmatory analytical method has been developed and validated for the monitoring of residues of glyphosate and AMPA in a variety of ingredients commonly used by the nutritional products industry and also in cow's milk, human breast milk, and soy milk. This study provides a first glimpse regarding the distribution of residues of glyphosate and AMPA in this suite of nutritional ingredients. Soy protein isolate and soy protein concentrate derived from GM soybean were the only ingredients that contained measurable residue levels of glyphosate and AMPA. This preliminary assessment helps reinforce the safety of ingredients used by the nutritional products industry and the dairy industry. The method is proposed as a convenient tool for the survey of glyphosate and AMPA in the ingredient supply chain.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: An analytical method for the analysis of glyphosate and AMPA based on derivatization with fluorenylmethoxyloxycarbonyl chloride (FMOC-Cl) and LC-MS/MS is presented. The method was applied to nutritional ingredients and products derived from genetically modified (GM) crops, as well as in cow's milk and human breast milk.

Glyphosate and AMPA were not detected in soy milk (sourced from non-GM soybean), in soybean oil, corn oil, maltodextrin (four batches), and sucrose (all derived from GM crops). Cow's milk, whole milk powder, and human breast milk did not show any trace of glyphosate and AMPA either. Glyphosate and AMPA were

detected in a batch of soy protein isolate sourced from GM soybean at concentrations of 0.105 and 0.210 µg/g, respectively, and in a batch of soy protein concentrate sourced from GM soybean at concentrations of 0.850 and 2.71 µg/g, respectively. MRLs for glyphosate have not been established in soy protein isolate/concentrate, but the residue levels reported in the study are at least one order of magnitude lower than the MRLs established in soybean.
The study presents an analytical method paper and includes selected analysis of samples, partially for GM crops. The information is relevant, but supplementary.

Assessment and conclusion by RMS:
The article primarily is an analytical method paper. In addition, glyphosate and AMPA have been analysed in several ingredients and commodities. No further impact expected on the existing risk assessment parameters.

B.7.8.3.7. Study 7

1. Information on the study

Data point	KCA 6.9
Report author	Jansons M. *et al.*
Report year	2018
Report title	Occurrence of glyphosate in beer from the Latvian market
Document No.	Food Additives & Contaminants: Part A, 2018, Vol. 35, No. 9, 1767-1775
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format
A sensitive LC-MS/MS method for the determination of glyphosate in beer has been developed, validated, and applied to analyse 100 beer samples from 24 different producers and distributors in Latvia. The selected samples represented most beer brands and varieties sold in local supermarkets. Different procedures for sample preparation and chromatographic separation were compared. The final version of the method consisted of solid phase extraction, chromatographic separation on aminopropyl stationary phase, and detection using tandem mass spectrometry. The concentration of glyphosate in beer varied from below the LOD of 0.2 µg/kg up to 150 µg/kg, higher than previously reported. Significantly higher ($p < 0.01$) content of glyphosate was observed in beers that did not have the country of production disclosed on the label and were sold in local supermarkets by distributors from Latvia (1.8 µg/kg median concentration in locally produced beer, 6.7 µg/kg in beer of undisclosed origin).

Materials and Methods
Materials and reagents
Glyphosate standard with 98.7% purity was obtained from Dr. Ehrenstorfer (Germany). $^{13}C_2{}^{15}N$-glyphosate internal standard as well as 36% hydrochloric acid and ammonium hydrogen carbonate (99% assay) were obtained from Sigma-Aldrich Chemie (Germany). The uncertainty of assay for the glyphosate and $^{13}C_2{}^{15}N$ glyphosate standard was 2%. Aqueous 25% ammonia solution was obtained from Merck (Germany). The solvents used in this study were ultra-pure water prepared by Millipore Milli-Q system, HPLC grade methanol and acetonitrile (Merck, Germany). Strata-X (500 mg, 6 mL) and Strata-XA (500 mg, 6 mL) solid phase extraction cartridges were purchased from Phenomenex (CA, USA). The following cartridges (500 mg, 6 mL) from Phenomenex (CA, USA) were used for comparison of different sample preparations: Strata-X polymeric polar reversed phase (RP), Strata-XC polymeric strong cation exchange (XC) resin, Strata-XA polymeric strong anion exchange (XA) resin, and Strata SAX silica-based strong anion exchange (SAX) medium.

Samples of beer and the sampling procedures
A total of 100 different bottled and canned beer samples were purchased from local supermarkets. The samples were stored refrigerated at 5°C in darkness until analysis. The selected samples represented the majority of available brands

and varieties of beer sold in local supermarkets by 24 different producers and distributors from Latvia. All available information about the samples was recorded: constituents, country of production, beer type by colour, type of packaging, presence of precipitate, and disclosed use of pasteurisation or filtering. There were 70 samples of light beer, 19 samples of dark beer, and 11 samples of specialty beer. A total of 71 samples were produced from barley malt only, 10 samples were produced from combined malt (wheat, rye, or rice), and 9 samples also contained other ingredients such as honey, fruit juices, or aromatisers.

Sample preparation
The samples were shaken, filled into polypropylene tubes and degassed by sonication in ultrasonic bath and centrifuged for 15 min at 4500 rpm. A 10.0 g portion of each sample was weighed and 10 µL of glyphosate internal standard solution was added to a concentration of 25 µg/kg. The glyphosate standard solutions to be added to the corresponding level of calibration and quality control (QC) samples were prepared separately for each calibration level and 10 µL of the respective solution were added to the weighed sample. The Strata-X SPE cartridges were conditioned with 3 mL of methanol and 6 mL of ultra-pure water. Each sample (1 mL) followed by 4 mL of ultra-pure water was slowly passed through the cartridge at approximately 1 mL/min and the eluent was collected. The remainder of the liquid was collected from the cartridges with suction. The Strata-XA cartridges were conditioned with 3 mL of methanol and 6 mL of ultra-pure water. The previously extracted extracts were quantitatively transferred to the conditioned Strata-XA cartridges and slowly passed through them. The cartridges were rinsed with 1 mL of ultra-pure water and 5 mL of methanol. Glyphosate was eluted from the Strata-XA cartridges with 5 mL of methanolic 10 mmol/L HCl solution and the eluted solutions were evaporated to dryness at 50°C under a purified air stream. The samples were reconstituted in 200 µL of ultra-pure water.

Instrumental analysis
Glyphosate was analysed using an Acquity UPLC system (Waters, USA) coupled to a QTRAP 5500 (AB SCIEX, USA) tandem mass spectrometer operated in multiple reaction monitoring (MRM) mode with Turbo V™ electrospray ionisation (ESI) source. Chromatographic separation was carried out on a Luna® NH_2 (Phenomenex, USA) 3 µm endcapped aminopropyl silica column (100 × 1 mm). A binary pump provided the gradient for separation at a flow rate of 0.110 mL/min by mixing acetonitrile with aqueous 10 mmol/L ammonium hydrogen carbonate solution adjusted to pH 10 with aqueous 25% ammonia solution. The following gradient programme was used (% of aqueous component indicated, linear transitions): 0 min – 20%, 15 min – 50%, 15.5 min – 90%, 16.5 min – 90%, 17 min – 20%, 25 min – 20%. The HPLC column was maintained at ambient temperature (20 ± 2°C); autosampler temperature was set at 8°C; the injection volume was 2 µL. The following MS ionisation parameters were applied: nebuliser gas pressure 30 psi; heater gas pressure 20 psi; curtain gas pressure 30 psi; source temperature 300°C; source voltage −4500 V; declustering potential 70 V. The MRM transitions were: *m/z* 168→63 (quantification); *m/z* 168→81 (confirmation); *m/z* 171→63 (internal standard).

Quality assurance and quality control
The samples where no glyphosate could be detected (below the LOD) were used as blanks for calibration and bias estimation. A maximum permissible deviation of ± 30% from the average quantitation and confirmation peak area ratio over all levels of the calibration set was selected as the confirmation criterion. The evaluation of LOD was based on the signal-to-noise ratio for the analyte ($S/N \geq 3$), the evaluation of LOQ was based on $S/N \geq 10$ and repeatability RSD $R \leq 20\%$. A six-point matrix-matched calibration with stable isotope labelled internal standard normalisation was used in the range from 0.2 µg/kg to 25 µg/kg with the internal standard at 25 µg/kg. Quantification was performed using a log-log quadratic model. Samples with glyphosate concentration above the calibration range were reanalysed with calibration at the levels of 25, 75, 125, and 175 µg/kg. Due to lack of a true blank material, blank subtraction was applied uniformly across the calibration of each batch by subtracting the blank (low level sample) peak area. The 100 samples were analysed in 5 batches of 20 samples. Different blank samples were used for calibrating each batch and a blank sample different from the calibration was used to prepare the QC samples. The criterion for acceptable QC recovery was the estimated method uncertainty. Validation was carried out by analysing 5 different blank samples (B1, B2, B3, B4, and B5) each spiked at the calibration levels of 0.2, 0.5, 1, 5, 10, and 25 µg/kg. The blank samples were light beers of different origin and malt type. Three of the blanks were of barley malt, two of the blanks were of wheat malt, and all three of the blanks had a precipitate. In this way, any bias due to the differences in matrix composition are included in the uncertainty estimation. On each validation day, one blank was used to prepare the calibration samples at each level. Selected other blanks spiked at each level were analysed by subjecting the samples to the whole analytical procedure. On the first validation day, B5 was used as calibration, with three repetitions of B5, one repetition of B1, B2, and B3 analysed. On the second validation day, B4 was used as calibration, with three repetitions of B1, one repetition of B2, B3, and B4 analysed. On the third validation day, B2 was used as calibration, with three repetitions of B2, one repetition of B3, B4, and B5 analysed. The uncertainty was estimated according to the Nordtest guidelines (Magnusson *et al.* 2012) modified to take the carry-over effect into account (Equation 3). The

carry-over effect was estimated by quantifying the glyphosate peak in solvent injection (deionised water) acquired after an injection of the 25 µg/kg calibration level. Pooled standard deviations were used to estimate the repeatability (RSD_R) and within-laboratory reproducibility (RSD_{WR}), expressed as relative standard deviation. For estimation of repeatability, *RSD* of measurements performed on the same sample on the same day were pooled with the pooled standard deviation, resulting in 9 repetitions for each calibration level. For estimation of within-laboratory reproducibility, measurements performed on the same sample on different days were pooled, resulting in 12 repetitions for each calibration level. Bias was estimated as the root-mean-square of a total of 11 bias determinations, expressed as relative differences from 100% recovery for 5 different blank samples at each level, after excluding the samples used as calibrants on the respective days.

Calculations
The matrix effect was estimated according to Equation 1:

$$ME = \frac{A_{sample} - A_{standard\ solution}}{A_{standard\ solution}} . 100\%$$

where A is the respective peak area at the same concentration. A situation free of matrix effect is defined as having the *ME* value equal to 0%.
Pooled standard deviations were calculated according to Equation 2:

$$s_{pooled} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + \ldots + (n_i - 1)s_i^2}{n_1 + n_2 + \ldots + n_i - i}}$$

where n_i is the number of measurements within the group, s_i is the standard deviation within the group to be pooled and *i* is the number of groups.
The measurement uncertainty at 95% CI was estimated according to Equation 3:

$$U = k.\sqrt{RSD_{WR}^2 + RMS_{bias}^2 + u_{C_{ref}}{}^2 + bias_{carry-over}{}^2}$$

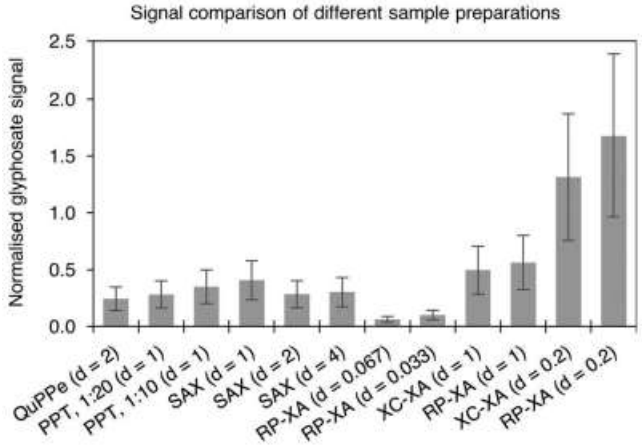
where k is the coverage factor, RSD_{WR} is the within-laboratory reproducibility and RMS_{bias} is the root-mean-squared bias, u_{Cref} is the relative uncertainty of the glyphosate standard certified value, $bias_{carry\text{-}over}$ is the absolute estimated carry-over after the acquisition of the 25 µg/kg calibration level divided by the nominal concentration of the level for which the uncertainty is estimated.
Chromatograms were processed by using the Analyst 1.6 software (AB SCIEX, USA) and all other calculations were performed in Microsoft Excel.

Statistical analysis
Mann-Whitney U tests were carried out with MYSTAT statistics software (Systat Software, USA) to evaluate the significance of the difference between the results of two groups. For statistical analysis, sample results below the LOD were assigned a value of zero, and the results above the LOD were unchanged. Regression analysis was carried out with the Minitab statistics software (Minitab, Inc., USA). Values of $p < 0.01$ were interpreted as a strong evidence against the null hypothesis.

Results
Optimisation of sample preparation
Different SPE stationary phases from Phenomenex® were tested to maximise the analytical method sensitivity to glyphosate: RP, XC, XA, SAX, and some combinations thereof, as well as protein precipitation with 10 mM HCl in methanol (PPT) at different ratios and the QuPPe method (instead of SPE extraction the spiked 10.0 g samples were diluted with 10 mL of 1% formic acid in methanol). The comparison was based on monitoring the normalised glyphosate signal, defined here as the ratio of response factors: peak area per µg/kg in blank spiked prior to the extraction to the peak area per µg/kg in standard solution (deionised water). The concentration of solutions for elution of glyphosate retained on SPE cartridges was optimised by preparing sequential ten-fold dilutions and selecting the weakest dilution that completely eluted glyphosate. For this purpose, fractions from the SPE extraction steps were monitored for the presence of glyphosate. The results of the signal comparison for different sample preparations are shown in **Figure 1**. The aim of this comparison is to identify the sample preparations that provided the highest sensitivity. In addition to the selectivity, also the selection of SPE cartridges or solvents used for the extraction, as

well as the dilution factor, here defined as the ratio of sample volume after reconstitution to the volume taken for the extraction, are important parameters to be optimised, because they strongly affect the sensitivity and matrix effects. Under the conditions of d = 1, the strongest signal was obtained by the RP-XA method, therefore preconcentration was attempted to further increase the sensitivity. Excessive pre-concentration (d = 0.067 and d = 0.033) resulted in the lowest observed signals due to signal suppression. The strongest signal was observed after purification with RP, followed by preconcentration with XA when d = 0.2 (**Figure 1**).
The estimated matrix effect of the final SPE procedure was (−65.8 ± 4.4%). The matrix effect was compensated with matrix-matched calibration and the use of a stable isotope labelled internal standard.

Figure 1: Signal comparison of different sample preparations, where 'd' represents the dilution factor, defined as the volume of sample after reconstitution to the volume of sample taken for extraction.



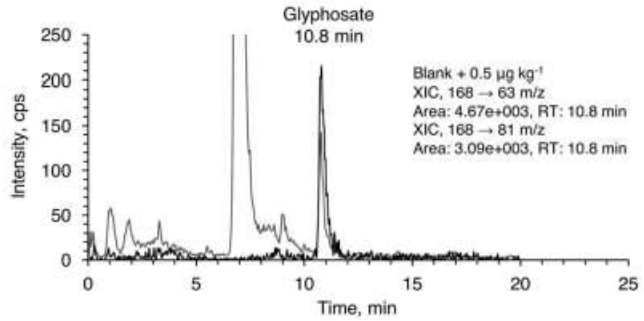
Optimisation of liquid chromatography
Five different columns and appropriate mobile phases according to **Table 1** were tested in order to optimise the chromatographic conditions at the same mass spectrometric parameters as in the final method. All of the tested columns retained glyphosate with the respective retention factors k = 6.5 (Column 1), 4.0 (Column 2), 0.7 (Column 3), 3.8 (Column 4), and 3.9 (Column 5). Under these conditions the peak shapes obtained from standard solutions on Obelisc® R and Luna® SCX columns were extremely broadened. Hypercarb™ and Luna® NH_2 columns produced tailing peaks. The selectivity obtained with spiked samples prepared using the final SPE procedure was tested on Hypercarb™ and Luna® NH_2 columns. The Hypercarb™ column produced distorted flat top peaks, possibly due to the insufficient retention factor. The Luna® NH_2 (100 × 1 mm) column was chosen for the final method based on the satisfactory peak shape and retention factor, as well as the low solvent consumption.

Table 1: Columns, mobile phases, and parameters tested for optimisation of the chromatographic conditions.

Columns	Mobile phases	Parameters
1 – Obelisc® R (SIELC, USA) 5 µm mixed phase (150 × 2.1 mm)	A – 20 mmol L^{-1} ammonium formate adjusted to pH 3,B – acetonitrile	isocratic, 80% A at 0.5 mL min^{-1}
2 – Luna® SCX (Phenomenex, USA) 5 µm cation exchange phase (50 × 4.6 mm)	A – 1% aqueous acetic acid,B – methanol	gradient, 0–5 min 10% to 90% A at 0.3 mL min^{-1}
3 – Hypercarb™ (Thermo Fischer Scientific, USA) 5 µm porous graphitic carbon	1% aqueous acetic acid phase (100 × 2.1 mm)	isocratic at 0.3 mL min^{-1}
4 – Luna® NH_2 (Phenomenex, USA) 3 µm aminopropyl phase (150 × 3 mm)	A – 10 mmol L^{-1} aqueous ammonium hydrogen carbonate adjusted to pH 10 with ammonia,B – acetonitrile	gradient, 0–8 min 20% to 50% A, 8–10.5 min 90% A,10.5–15 min 20% A at 0.75 mL min^{-1}
5 – Luna® NH_2 (Phenomenex, USA) 3 µm aminopropyl phase (100 × 1 mm)	A – 10 mmol L^{-1} aqueous ammonium hydrogen carbonate adjusted to pH 10 with ammonia,B – acetonitrile	gradient, 0–15 min 20% to 50% A, 15–17 min 90% A, 17–25 min 20% A at 0.11 mL min^{-1}

The gradient rate is an important parameter that can affect resolution. A beer sample spiked at 10 µg/kg, prepared using the final SPE procedure, was analysed at different gradient rates: 0.1% min^{-1}, 0.5% min^{-1}, 1% min^{-1}, 2% min^{-1}, 3% min^{-1}, 3.75% min^{-1}, and 7% min^{-1}. The S/N ratio of the quantitative ion peak reached maximum and levelled out at 2% min^{-1} gradient rate. The S/N ratio of the confirmatory peak (*m/z* 168→81) reached maximum at 2% min^{-1} and decreased markedly with increasing gradient rate. The optimised gradient rate was 2% min^{-1}. In the final method, glyphosate eluted at 10.8 min (**Figure 2**).

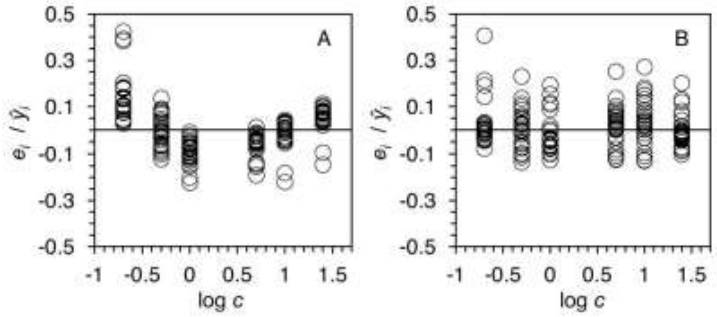
Figure 2: Chromatogram of a blank sample spiked with glyphosate at 0.5 µg/kg.



Method validation

The calibration levels are equidistant on a logarithmic scale, therefore a transformation must be applied, otherwise the highest levels would have a high degree of leverage leading to poor accuracy for the lowest levels and inflated R^2. Regression analysis using ANOVA was performed on data from each validation day consisting of 7 repetitions at each calibration level. The lack-of-fit F-tests suggested that a linear model did not fit the data ($p < 0.01$). Plots of residuals were constructed in the case of linear and quadratic calibration model (**Figure 3**). The residual plot was satisfactory only in the case of the quadratic calibration model where the residuals were distributed randomly around the horizontal axis. For the log-log linear calibration model, $R^2 > 0.99$ was observed. For the log-log quadratic calibration model, $R^2 > 0.999$ was observed. The peak area for internal standard deviated from the peak areas for calibrants by −53% to 30%, therefore calibration using stable isotope labelled internal standard should properly compensate for the matrix effects because the deviation does not exceed one order of magnitude. The peak area deviation for the internal standard appeared random and did not reveal any relationship with the type of beer or other known parameters of beer samples. One QC sample at the 0.5 µg/kg level was analysed after every 10 samples. The estimated concentrations of blank samples were 0.15, 0.08, 0.12, 0.11, and 0.08 µg/kg, and the standard deviation of the blank concentrations was 0.03 µg/kg. The recoveries of QC samples were: 119, 119, 113, 123, 90, 91, 87, 90, 99, and 93%. The QC sample recoveries were within the estimated method uncertainty. The estimated carry-over effect was 0.21% and was taken into account in the uncertainty estimation. The estimated LOD was 0.2 µg/kg and the LOQ was 0.5 µg/kg. The repeatability RSD_R ranged from 4.1 to 1.6%, decreasing with higher analyte concentration. The RSD_{WR} was 9.5%, with little variation and no trends observed over the concentration range. The estimated uncertainty due to the bias of measurements at 0.5 µg/kg and above was 13%. The estimated measurement uncertainty at and above the LOQ of 0.5 µg/kg was 32% (k = 2 at 95% CI), calculated as the average over the calibration levels. The analytical procedure developed and applied in this article has a superior sensitivity compared to the currently known methods for the determination of glyphosate in beer, however, the sample preparation procedure is not particularly cheap or simple.

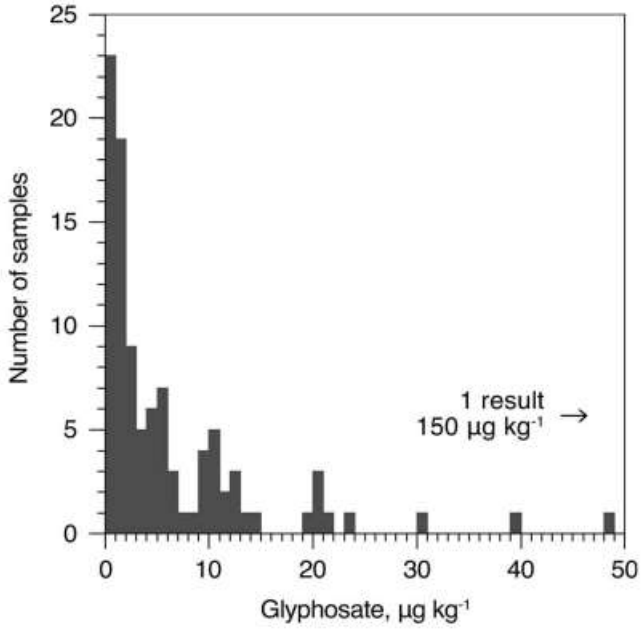
Figure 3: Scatter plots of residuals (e_i) divided by the respective fitted values ($\hat{y}_i$) versus the specified concentration for all validation and quality control samples with respect to the respective calibration curves – (A) in case of linear regression model, (B) in case of quadratic regression model.



The occurrence of glyphosate in 100 samples of beer from Latvia

The analysis of 100 beer samples revealed that the glyphosate content in beer ranged from below LOQ up to 150 µg/kg, with 2.9 µg/kg median value, 7.5 µg/kg average value, and the standard deviation of 16.6 µg/kg. The exponential distribution of glyphosate occurrence in beer is shown in **Figure 4**. Only in 8 samples was the concentration of glyphosate below LOD. Glyphosate was detected below LOQ in 9 samples. Glyphosate concentrations up to 30 µg/L have been reported (Pflaum *et al.* 2016). Our study shows that glyphosate content up to 150 µg/kg can occur.

Figure 4: Histogram of glyphosate content analysed in 100 beer samples from Latvia.



No correlation of glyphosate content with alcohol content was observed. No significant difference in glyphosate content between large and small producers was observed.

The results were categorised in following groups: by malt type (barley or combined/other), country of production (local or undisclosed origin), beer type by colour (light or dark), type of packaging (canned or bottled), presence of precipitate (precipitate or no precipitate), filtration (filtered or not filtered), pasteurisation (pasteurised or not pasteurised). The beer samples originated from a total of 24 different producers and distributors in Latvia. Of these, 25 beers originated from distributors, 3 of which were imported, and 22 of which had no country of production disclosed on the labelling. Some producers operate within consortia with several manufacturing or bottling plants across different countries and therefore choose not to disclose the country of production, which is legal.

No significant difference was observed between the groups by malt type, beer type by colour, presence of precipitate, filtration, and pasteurisation ($p > 0.1$). A significant difference in content of glyphosate was observed between locally produced and beer of undisclosed origin ($p < 0.01$) with 6.7 µg/kg median value in 25 beers of undisclosed origin and 1.8 µg/kg median value in 75 locally produced beers, and between canned and bottled beer ($p < 0.01$) with 6.8 µg kg median value in 16 canned beers and 2.2 µg/kg median value in 82 beers sold in glass bottles. Among beers of undisclosed origin 52% of samples were canned and 81% of canned beer were of undisclosed origin, therefore the beers of undisclosed origin were categorised in two groups by type of packaging and the significance of difference tested. No significant difference was observed between canned (6.9 µg/kg median value) and bottled (5.6 µg/kg median value) beer among the beers of undisclosed origin ($p = 0.586$). This suggests that beer sold by distributors from Latvia with no country of production disclosed on the labelling may contain significantly higher content of glyphosate than the locally produced beer. The reasons for this are beyond the scope of this article.

Taking into account the consumption of barley to produce a kilogramme of beer (processing factor of approx. 0.09) and assuming complete carry-over of glyphosate to beer, even the maximum glyphosate residue quantified does not lead to suspicion that the current MRL (Regulation (EC) No 396/2005) in barley or wheat used in the production could have been exceeded.

Conclusions

A sensitive LC-MS/MS method consisting of SPE extraction and detection using tandem mass spectrometry has been developed and validated to determine the content of glyphosate in 100 samples of beer sold in local supermarkets in Latvia. The analytical procedure developed and applied in this article has a superior sensitivity compared to the currently published methods for the determination of glyphosate in beer.

The content of glyphosate in beer varied from below the LOD of 0.2 µg/kg up to 150 µg/kg, with a median value of 2.9 µg/kg and an average value of 7.5 µg/kg. The selected samples represented most of the beer brands and varieties sold in local supermarkets by producers and distributors from Latvia. Our results show that glyphosate content up to 150 µg/kg can occur in beer, which is higher than previously reported, however, even the maximum glyphosate residue quantified does not lead to suspicion that the current MRL (Regulation (EC) No 396/2005) in barley or wheat used in the production could have been exceeded. Malt type, beer type by colour, presence of precipitate, type of packaging,

use of filtration and pasteurisation in manufacturing were not found to correlate with the content of glyphosate in beer. Our results show that the glyphosate content was significantly higher ($p < 0.01$) in those samples of beer that did not have the country of production disclosed on the label and were sold in local supermarkets by distributors from Latvia (1.8 µg/kg median in locally produced beer, 6.7 µg/kg median in beer of undisclosed origin).

3. Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: The article describes the development and validation of an LC-MS/MS based method for the analysis of glyphosate residues in beer. An LOQ of 0.5 µg/kg could be achieved and validated. The method was applied for the analysis of 100 beer samples from 24 different producers and distributors in Latvia. The concentration of glyphosate in beer varied from below the LOD of 0.2 µg/kg up to 150 µg/kg.
The report contains supportive information for the analytical section and provides glyphosate monitoring data for beers in Latvia. No regulatory relevant endpoints can be derived. Therefore the article is relevant but supplementary.

Assessment and conclusion by RMS:
The article primarily is an analytical method paper for the measurement of glyphosate in beer. In addition, glyphosate has been detected in several beers. No further impact expected on the existing risk assessment parameters.

B.7.8.3.8. Study 8

1. Information on the study

Data point:	CA 6.9
Report author	Liao Y. *et al.*
Report year	2018
Report title	Validation and application of analytical method for glyphosate and glufosinate in foods by liquid chromatography-tandem mass spectrometry.
Document No	Journal of chromatography. A (2018), Vol. 1549, pp. 31
Guidelines followed in study	SANTE/11813/2017 Commission Directive 2002/63/EC French Standard NF V03-110 SANTE/11945/2015
Deviations from current test guideline	None stated
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

3. Full summary of the study according to OECD format

A reliable and sensitive method was developed for simultaneous determination of glyphosate and glufosinate in various food products by liquid chromatography-tandem mass spectrometry. Based on extraction, derivatization with 9-fluorenylmethylchloroformate and purification on solid phase extraction column, quantification was done by using isotopic-labeled analytes as internal standard and calibration in matrix. Good selectivity and sensitivity were achieved with a limit of quantification of 5 µg/kg. The recoveries of these two pesticides ranged from 91 % to 114 % with inter-day and relative standard deviation of 3.8 – 6.1 % in five matrices of cereal group spiked at 5, 10, and 20 µg/kg. An accuracy profile was performed for method validation, demonstrating the accuracy and precision of the method for the studied food groups. The verification results in expanded food groups indicated extensive applicability for the analysis of glyphosate and glufosinate. Finally, the developed method was applied to analyze 136 food samples including milk-based baby foods from the French Agency for Food, Environmental and Occupational Health & Safety. Glyphosate residues were detected in two breakfast cereal samples (6.0 and 34 µg/kg). Glufosinate residues were found in a sample of boiled potatoes (9.8 µg/kg). No residues were detected in the other samples, including milk-based baby foods with limits of detection ranging from 1 to 2 µg/kg. The method has been applied for routine national monitoring of glyphosate and glufosinate in various foods.

Materials and methods

Chemicals and reagents

Glyphosate (purity 98 %) was provided by Cluzeau Info Labo (CIL, France) as certified analytical standard. Glyphosate (1,2-^{13}C, ^{15}N), used as internal standard, was also obtained from CIL. The solvents used were LC-grade acetonitrile (purchased from CARLO ERBA), formic acid, methanol (MeOH) and dichloromethane (from VWR). Other solvents and reagents were analytical grade such as disodium tetraborate decahydrate and ammonium acetate (NH_4Ac) (from SIGMA). SPE-C18 column was obtained from Waters and 9-fluorenylmethylchloroformate (FMOC) from SIGMA. Water was purified by the Milli-Q water purification system (Millipore). Standard stock solutions of glyphosate and glufosinate (1 mg/mL) were prepared in the Milli-Q water and stocked in the plastic bottles at -20 °C. The intermediate solutions (100 µg/mL and 10 µg/mL) were prepared from standard stock solutions. The composite working solutions (1 µg/mL and 0.1 µg/mL) were diluted with Milli-Q water in the plastic bottles at 4 °C. FMOC solution was 20 mg/mL in acetonitrile. Disodium tetraborate was used for the borate buffer (0.05 mmol/mL). The 100 mL eluent solvent was prepared as following: 10 mL NH_4OH (20 %), 40 mL the Milli-Q water and 50 mL methanol were added into a glass volumetric flask of 100 mL. A mixture of the two internal standards covered all the sample including calibration standard, matrix blank and samples at the concentration of 1 µg/mL.

Sample preparation

136 food samples came from ANSES. According to the European Union SANTE guideline, all the samples were divided into 3 groups for analysis (shown in **Table 1**). The samples with high water content (≥60 %) accounted for 21 % of the samples analyzed (mainly vegetables, fruits and drinks), and the ones with low water content such as biscuits or bread, for 13 % of the samples. The products from animal origin, including milk-based baby foods represented 66% of the samples. Some samples needed to be chopped and homogenized in a mixer (model Retsch-GM200) according to the documentation "Commission Directive 2002/63/EC". All samples were stored at -20 °C until analysis.

Table 1: Classification, group and food product of analyzed samples.

Classification	Groups	Food products (sample number)
Plant products with high water content (≥60%)	1. High water content (19 samples)	Bananas (1), kiwis (1), peeled pears (1), peeled apples (1), broccolis (1), carrots (1), zucchinis (1), cauliflowers (1), spinaches (1), beans (1), mixed vegetables (1), boiled potatoes (1*), fried potatoes (1), peas (1), leeks (1), pumpkins (1), mashed potatoes (1), vegetable soup (1), tomatoes (1)
	2. High acid content and highwater content (10 samples)	Oranges (1), clementines/mandarins (1), fruit juices (5), drinks with milk and fruits (1), diluted syrup extracted from fruits (1), sodas (1)
Plant products with low water content (<60%)	3. High sugar and low water content (2 samples)	Fruit compote except apples (1), applesauce (1)
	4. High starch and/or protein content and low water and fat content (6 samples)	Fruit biscuits (1), natural or fruit cereals (1*),brioche and brioche bread (1),chocolate bread (1*), chocolate mousse (1), chocolate cereals (1)
	5. "Difficult or unique commodities"(10 samples)	Cocoa powder (1), chocolate milk (1),chocolate spreads (1),Other products (7)
Products of animal origin	6. Meat (muscle) and seafood (1 sample)	Ravioli filled pasta (1)
	7. Milk-based baby foods and other milk products (80 samples)	Infant formulaefor infants below 6 months (27), infant formulae (6–12 months) (35), follow-on formulae (9), other milk products (6), yogurt (3)
	8. Fat from food of animal origin (8 samples)	Cheese (5), cream (2), butter (1)

Extraction procedure

An accurately weighed homogenized sample was put into a 50 mL plastic centrifuge tube. A mixture of solvents including MilliQ water, acidified water, methanol and dichloromethane (shown in **Table 2**) were added into the tube. The tube was rotated mechanically for uniform mixing for 20 min and then centrifuged at 3000 rpm for 20 min at 4 °C or -15 °C (Rotanta 460R, **Table 2**). The aqueous extract of sample was transferred into a 20 mL plastic tube for derivatization. The DCM organic phase was removed. For each analysis series, a calibration curve (5–10–25–50 µg/kg) in the same matrix group, a reagent blank, a matrix blank (free of analytes like glyphosate) and a spiked sample at 5 µg/kg were done.

Table 2: The extraction solvents for glyphosate and glufosinate.

Classification	Sample (g)	Acidified water (mL)	Methanol (mL)	Dichloromethane (mL)	Centrifugation temperature (°C)
High water content	5	20	–	20	4
Low water content	2.5	10	10	10	−15
Products of animal origin	5	10	10	10	−15

Derivatization and purification procedure

The derivatization steps were performed as follows: 4 mL of the aqueous extract of sample was pipetted into a glass tube of 20 mL together with 50 L of the labeled internal standard (1 µg/mL), 4 mL of the borate buffer (0.05 mmol/mL,

pH 9) and 4 mL of FMOC reagent (20 mg/mL in acetonitrile) and the tube was swirled and left overnight at room temperature. After that, 3 drops of hydrochloric acid (HCl, 37%) was added into the tube and pH value was adjusted to 1.5. A SPE-C18 cartridge (60 mg) was used to purify and concentrate the sample extract after derivatization. The cartridge was conditioned with methanol (3 mL) and then water solution (3 mL, with 0.1% formic acid). The total of the derivatized acidified extract was added into the cartridge and the cartridge was washed by acidified water (3 mL, with 0.1 % formic acid) and dichloromethane (3 mL). The eluent (1 mL) was added and collected to a vial of 2 mL. The eluate was evaporated to dryness at 50 °C under the protection of nitrogen. The residue was dissolved with 200 L of a solvent mixture (acetonitrile, the mobile phase A for LC–MS/MS and ammonium acetate at 0.05 mmol/L in water, the mobile phase B; 10/90; v/v). The final extract was then transferred into the conical vial for LC–MS/MS analysis after the ultrasonic shock.

Analysis by LC–MS/MS

Chromatographic conditions in liquid chromatography (LC). Dionex UltiMate 3000 LC system was used and equipped with a C18 column (Atlantic150 × 2.1 mm, 2.6 m, Waters). Acetonitrile was used as the mobile phase A and ammonium acetate at 0.05 mmol/L in water (pH = 5.0), as the mobile phase B. Correspondingly, the percentage of acetonitrile was changed linearly as follows: 0 min, 5 %; 2.5 min, 5 %; 8.0 min, 95 %; 10.0 min, 95 %, and 10.1 min, 5 % with a total flow rate of 200 µL/min and the injection volume of 5 µL. The retention times for glyphosate-FMOC/glyphosate (1,2-^{13}C, ^{15}N)-FMOC was 7.0 min. The total run time was 20.0 min.

Mass spectrometry conditions

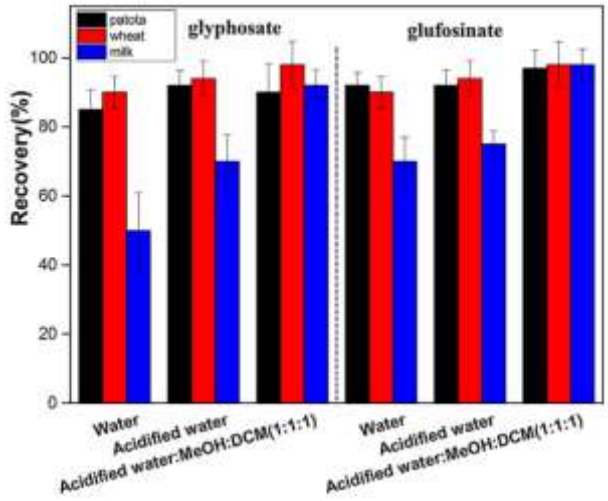
TSQ Quantiva mass spectrometer (Thermo Fisher Scientific) with an electrospray ionization (ESI) source was performed for MS analysis. The mass spectrometer analyses were carried out in positive ion mode and the ion source type was heated electrospray ionization (HESI). The selective reaction monitoring (SRM) mode was used. The MS instrumental settings parameters were shown as below: spray voltage was 4000 V, the sheath gas and auxiliary gas pressures were 40 (Arbitrary) and 15 (Arbitrary) respectively, ion transfer tube temperature was 300 °C, and the vaporizer temperature was 250 °C. The capillary temperature was 320 °C and the collision gas (Ar) pressure was 1.5 mTorr. The obtained data were handled by Xcalibur software. $[M+H]^+$ ions (*m/z*) were selected as the precursor ions and two transitions were selected for each derivative (392.2 → 88.2 for quantification, 392.2 → 214.1 for qualification) of glyphosate-FMOC. 395.2 → 91.1 transition of glyphosate (1,2-^{13}C, ^{15}N)-FMOC was used for internal standard quantification.

Results

Pre-treatment optimization

Extraction procedure. Based on the polarity and water solubility of glyphosate, water, acidified water (0.1 % formic acid), methanol (MeOH), and dichloromethane (DCM) were used as extraction solvent in the matrix samples at spiked level 100 µg/kg. When using just water as extraction solvent, the recoveries were not high, even only 50 % for glyphosate in the milk as **Figure 1** shown. The choice of an acidified water (0.1 % formic acid) as extraction solvent was dictated by the very polar character of glyphosate and its insolubility in most organic solvents. Although the recovery can be improved with acidified water (0.1 % formic acid), the recovery was not satisfied with milk sample and interferences persisted and affected the analyst. The main problem with this procedure is the presence of other water-soluble components in the extract that will hamper the analyte. Therefore, two other solvents were added to remove interferences: methanol for the removal of protein and starch precipitation (only for the matrix in medium and high protein content such as cereals) and dichloromethane (DCM) (for all the matrix) for the removal of hydrophobic matrix components or non-ionic low to medium water solubility matrix components such as lipids. A simultaneous process of extraction and purification was used to minimize the sample handing time. Adding dichloromethane and methanol for extraction improved the recovery of glyphosate from 50 % to 92 %. After a series of experiments, the proportion between acidified water (0.1 % formic acid), methanol and dichloromethane were chosen which is shown in **Table 2**.

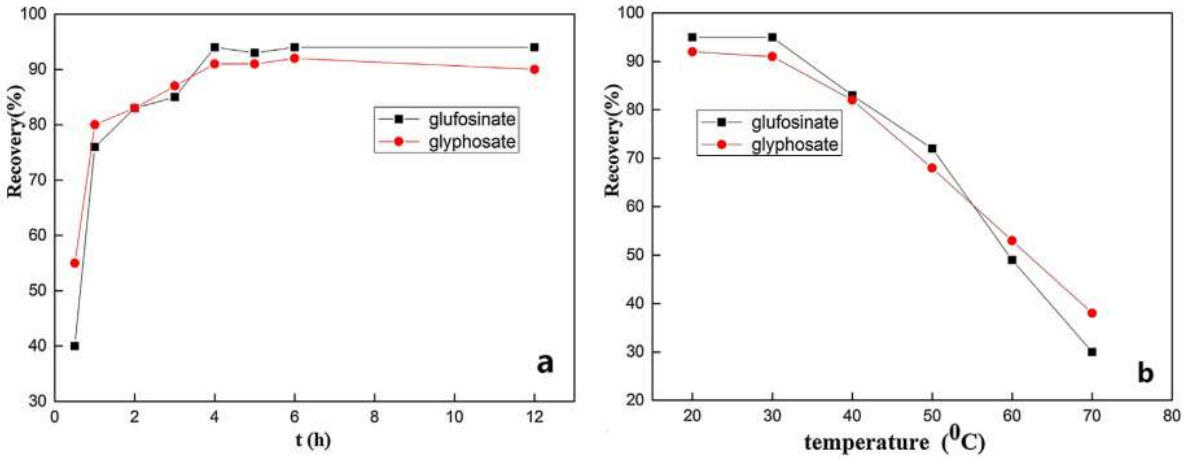
Figure 1: Influence of extraction solvent on the recoveries for glyphosate and glufosinate.



Derivatization conditions

Because of high polarity, retention in reversed phase columns and sensitivity in ESI/MS is either low in the case of underivatized glyphosate. Since the work of Moye and Boning, who were the first to use FMOC for glyphosate derivation, many procedures have been reported in the literatures. After derivatization, analytes' polarity is significantly reduced enabling better retention on the reversed phase and thereby better separation from matrix components. In addition, the ionization efficiency of analytes in ESI source was enhanced by its hydrophobic parts in molecules and limit of detection (LOD) was improved. Therefore, the FMOC was used as derivatizing reagent in this method. In terms of reaction time, date ranged from 5 min to overnight. The effect of different derivatization reaction times and temperatures was studied. With the increase of reaction time, the recovery rate increased significantly. The maximum reaction yield was observed after 4 h (shown in **Figure 2**). In practice, 12 h was chosen to be reaction time. An influence of the reaction temperature was observed as **Figure 2b**. Higher temperature reaction tended to decrease the recovery of product. It is the same as reported by Druart. Therefore, the derivatization was completed at room temperature. The effect of FMOC concentration was not investigated in our study but was fixed at 20 mg/mL. The use of FMOC in excess was to compensate for high reactivity with water leading to the formation of FMOC-OH products and matrix components-primary and secondary amines.

Figure 2: Influence of derivatization time and temperature on the recoveries for glyphosate and glufosinate.



SPE purification

To remove the interferences, excessive FMOC and concentrate the analytes to increase sensitivity, a SPE-C18 cartridge was chosen for purification and concentration. Firstly, methanol and acidified water were used to condition the cartridge. Once the derivatization reaction took place overnight, hydrochloric acid was added to stop the reaction, by lowering the pH. Then the derivatized acidified extract was added into the C18 cartridge. Acidified water and DCM were chosen for rinsing to remove the excess FMOC and some polar interference. In order to carefully check for

possible analyte losses during dichloromethane washing, the solution was also analyzed from washing step with water and dichloromethane and the result revealed no indication of analyte losses. The eluent at pH 9 was selected to elute the analytes from the cartridge. The eluate was then evaporated to dryness and the residue was dissolved with a solvent mixture of the mobile phases of LC–MS/MS to reduce solvent effect. Finally, the sample extract was concentrated 20 times (from 4 mL to 0.2 mL) before the analysis on LC–MS/MS. Following the above steps, the recovery of glyphosate could reach 92 % in the milk. A combination of several steps as indicated below makes our method more sensitive and selective: an efficient extraction with a solvent mixture, the FMOC derivatization, a SPE-C18 cartridge for purification and the concentration 20 times (from 4 mL to 0.2 mL) for sample extract before instrument analysis and finally, the determination by LC–MS/MS with its high sensibility and selectivity. Therefore, the combination of all these factors leads to the high sensitivity and selectivity of our method. In addition, a calibration curve in the same matrix group was used to quantify glyphosate in the presence of the labeled internal standard (glyphosate ^{13}C, ^{15}N) to reduce the matrix effect.

Method validation
Validation of the developed method was performed as described in the following documents: European Union SANTE guideline and French Standard NF V03-110. The calibration curves were obtained by the linear analysis (Y = aX + b and linear 1/X) at matrix-matched calibration, which was conducted at four levels ranging between 5, 10, 20 and 100 µg/kg with two replicates for five days. The accuracy was evaluated by the recoveries (%) and the precision was expressed by RSDR (%) of the spiked samples. The application fields, the limit of detection (LOD), the limit of quantification (LOQ), and the accuracy and precision of the method were discussed respectively as below.

Choice of the food groups and food categories
Glyphosate herbicides are used at pre- and post-emergence weed control, and their residues are theoretically mainly expected in cereals, which were divided into the commodity groups with high starch and/or protein content and low water and fat content shown in Annex A of SANTE guideline. Consequently, maize, rice, wheat and barley were selected as matrix blank to validate the developed method. Considering the diversity of food products to be tested, the verification of method in different matrices spiking samples was also done, as shown in method application. The linearity, the limit of detection (LOD), the limit of quantification (LOQ), and the accuracy and precision of the method were discussed respectively as below.

Linearity, LOQs and LODs
The linear range was determined by two replicate analyses of calibration standards (matrix-matched calibration), which was conducted at four levels ranging between 5, 10, 20 and 100 µg/kg with internal standard mixture for five days. The calibration curves were obtained by the linear analysis (Y = aX + b with 1/X). In five matrices (2 maize samples, 1 rice, 1 wheat and 1 barley) the coefficient of determination (R^2) for the standard curves of glyphosate were greater than 0.99. In theory, the LODs and LOQs are obtained by the ratio of the signal-to-noise (S/N) of 3 and 10 from the lowest concentration levels of the spiked samples. However, the results of reproducibility and repeatability were not satisfying in practical application using the value of 10 S/N as LOQ. In this study, although the target LOQ for glyphosate 10 µg/kg, the LOQs for the two pesticides were chosen at 5 µg/kg for French Infant Total Diet Study. The experiment was carried out for duplicate in the five matrices during five days. We used three parameters (accuracy, fidelity and homogeneity) to test LOQ. The accuracy, fidelity and homogeneity were calculated as following:

(1) Accuracy criterion (≤ 10 %):$\left| \frac{C_S - C_M}{SD \times \sqrt{10}} \right|$, where Cs was the chosen value; C_M, mean value and SD, standard deviation
(2) Fidelity criterion (≤ 20 %, relative standard deviation, RSD): $SD/C_M \times 100$ %
(3) Homogeneity: the homogeneity of data was tested by SHAPIRO-WILK statistic.

The values obtained of accuracy was 2.2% for glyphosate. Two values obtained of fidelity for glyphosate was 5.7% and reached the requirements of the precision. According to the test of SHAPIRO-WILK, the calculated results demonstrated the data followed a normal distribution. Consequently, it was satisfying with the homogeneity of data. Thus, the chosen LOQs of glyphosate at 5 µg/kg was suitable in the selected matrices. The LODs of glyphosate was 1.7 µg/kg correspondingly (LOD = 3/10 LOQ). In addition, it is observed that the values of the ratio S/N for glyphosate was higher than 1900 and 20000 respectively at 5 µg/kg (far more than 10 S/N).

Accuracy and precision
In the validation plan, we used an accuracy profile to validate a method. The details as follows:
The number (n) of series, repetition and levels for the validation plan were chosen (repeatability: n = 2, reproducibility: n = 5, level: n = 3, level of concentration: LOQ, 2 LOQ and 4 LOQ) according to the guidance document SANTE

(repeatability: n ≥ 1, reproducibility: n ≥ 5, level: n ≥ 2, level of concentration: LOQ and 2 LOQ or 10 LOQ) and NF V03-110 (repeatability: n ≥ 2, reproducibility: n ≥ 3, level: n ≥ 3, level of concentration: no request). First, the accuracy was evaluated by absolute mean bias, relative mean bias (%) and average recovery (%). The precision was expressed by standard deviations (SD) of repeatability, inter-series & intermediate fidelity and relative standard deviation (RSD_R %). The recoveries for glyphosate ranged from 93% to 114% in inter-day studies and the average recoveries (%) for three levels (5, 10 and 20 µg/kg) were 104, 104 and 100 respectively (**Table 3**) The RSD_R % values for glyphosate ranged from 4.5 to 6.4 in inter-day, which reached the evaluation requirements for the precision. The uncertainty (I) included relative mean bias (RMB) for accuracy and RSD for precision $\left(I = \sqrt{(RMB)^2 + RSD^2}\right)$, and it ranged from 9 % to 16 %.

Table 3. Accuracy and precision of the proposed method in studied matrices.

Matrix, day	[C*]	Recoveries (%)										Avg.R(%)	RSD_R (%)
		Maize, day1		Maize, day2		Rice, day3		Wheat, day4		Barley, day5			
glyphosate	5	100	94	106	100	114	105	109	100	108	103	104	5.60
	10	97	102	109	107	99	98	112	109	101	107	104	6.38
	20	99	103	100	95	101	108	100	93	106	99	100	4.53
Glufosinate	5	100	98	107	108	109	106	106	103	96	92	103	5.88
	10	107	98	95	94	99	103	108	108	102	105	102	5.12
	20	91	96	101	103	98	99	98	94	100	100	98	3.82

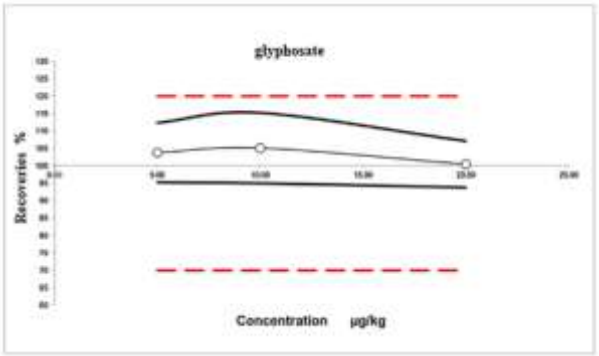
* Which is spiked level (µg/kg).

Second, a tolerance interval of expectation is constructed which contains a probability β % (80 % chosen). To use an accuracy profile to validate a method, the two decision criteria (limits of acceptability and probability β) was established as follows.

(1) Limits of acceptability. They serve to translate the practical objectives of the users and delimit an interval around the reference value. In most cases, these limits are regulated or derived from regulation. In this study, the recovery (between 70 and 120 %) for accuracy and RSD_R limits (≤ 20 %) were used according to the guidance document SANTE.
(2) Probability β. It represents the proportion of future average results within the tolerance intervals. The value chosen for β depends largely on the application field. It is obvious that the smaller β, eg. 70 %, the more likely the method is to produce results that do not correspond to the announced specifications. In NF V03-110 this probability β has been set at 80 %, at least.

Based on the accuracy, precision, uncertainty and a tolerance interval of expectation, the accuracy profile of the method was calculated and the results were shown in **Figure 3**. All different combinations complied with the acceptable method performance parameters suggested by SANTE guideline. The experiment results indicated that the developed method was in the satisfying range expected for analysis.

Figure 3: Accuracy profile of glyphosate for the proposed method.



Application
Application fields
This developed method has been certified by the French certification organization (COFRAC), and has been applied to the analysis of 136 food samples provided by ANSES in January 2013, and currently for routine national monitoring. The milk-based baby foods were mainly provided "as consumed" (liquid) by ANSES and analyzed as such by the laboratory. The food samples were divided into three groups as shown in **Table 1**.

Verification in expanded matrices

Considering the variety of food products to be tested (**Table 1**), the verification of the method in expanded new matrices spiking samples was also conducted according to SANTE guideline. The plant products with low water content (<60 %) have been validated. So according to three kinds of actual samples, the different matrices were selected for verification including fruit (banana) and vegetable (potato), milk, yogurt and cheese. The average recoveries (%) and RSD_R (%) were calculated by the spiked samples including five kinds of matrix in two replicates at two levels (5 and 10 µg/kg). The recoveries of glyphosate ranged from 82 % to 108 % (between 70 and 120 %) and the RSD_R, from 6.1 to 7.9 % (≤ 20 %). The result was displayed in **Table 4**.

Table 4: The recoveries of glyphosate and glufosinate in expanded matrices.

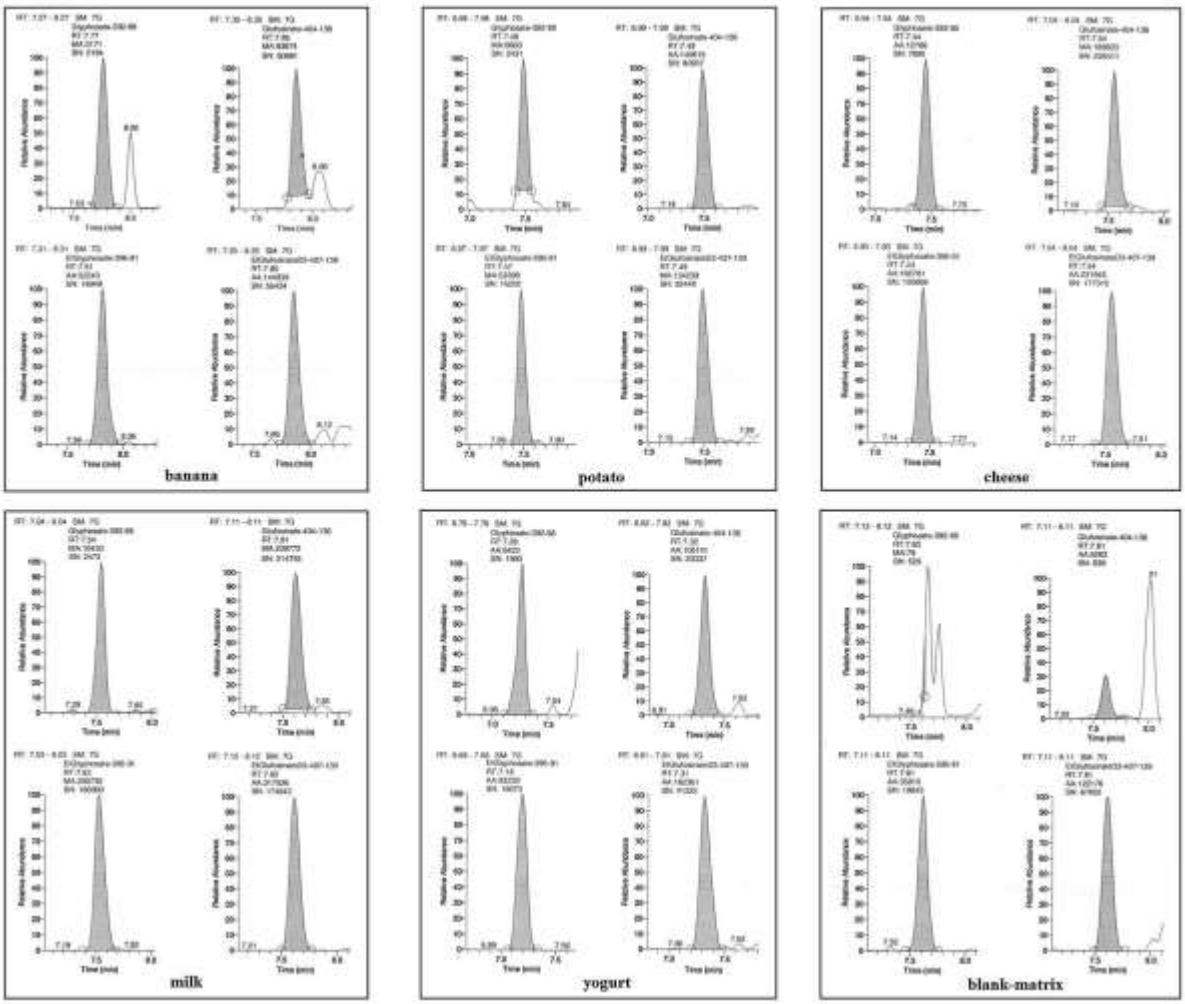
The recoveries of glyphosate and glufosinate in expanded matrices.

Matrices	[C*]	Recoveries (%)										Avg.R(%)	RSD_R (%)
		Banana		Potato		Milk		Cheese		Yogurt			
glyphosate	5	94	106	102	94	96	96	108	90	96	82	97	7.9
	10	103	96	98	103	97	88	100	105	102	89	98	6.1
Glufosinate	5	104	100	90	112	96	108	104	97	106	102	104	7.3
	10	97	99	99	95	83	88	116	92	96	101	95	6.0

* Which is spiked level (µg/kg).

So, the method was verified in these application fields with acceptable accuracy and precision. Meanwhile, the chromatograms at the level (5 µg/kg) in 5 different spiked matrices (banana, potato, cheese, milk and yogurt) and a blank matrix (free of analytes like glyphosate and glufosinate) were shown in **Figure 4**. The 4 peaks are respectively shown in the chromatogram for each matrix: the quantitative peak of glyphosate (top left) and one of its internal standard (bottom left), and one of glufosinate (top right) and one of its internal standard (bottom right). The last chromatogram (bottom right) was the blank matrix. From the figure, it displayed little interference in the matrices. Therefore, it indicated that the two pesticides in these different matrices can be determined, and that the developed method was sensitive enough to quantify glyphosate in foods.

Figure 4: Chromatograms (LC-MS/MS) in expanded matrices at the level (5 µg/kg)



Analytical results

To ensure the quality of the analytical results, a reagent blank, a matrix blank and two spiked samples at 5 µg/kg (QC) for two compounds were analyzed in each kind of matrix and each series experiment, for the 136 food samples analyzed by this method. Finally, among these samples, only 3 samples contained quantified levels of glyphosate or glufosinate residues. Glyphosate residues were quantified at 6.0 and 34 µg/kg in two breakfast cereal samples. No residues were detected in the other samples including milk-based baby foods, with LOD of 2 µg/kg for glyphosate as requested by ANSES. For these samples, in practice, better analytical limits were verified for each analytical batch and the results were satisfied. In order to better monitor the dietary exposure of infants and young children to this compound and its related residues (e.g. trimethyl-sulfonium, AMPA, acetyl-glyphosate and acetyl-AMPA), further analytical developments and most frequent analysis should be undertaken in the framework of monitoring programs, especially for cereals and potato products.

Conclusion

A reliable and sensitive method was developed to detect and quantify glyphosate and glufosinate residues in food by LC–MS/MS. High accuracy and precision could be achieved by off-line derivatization and purification. Good validation parameters for linearity, recovery, LOD and LOQ were acquired and the verification results demonstrated extensive applicability of the method for the analysis of glyphosate in various food products. The method can be used not only for high water content samples, but also for samples with low one, high protein and products of animal origin. In conclusion, the developed method can be a dependable and sensitive one for the routine monitoring of glyphosate in food. Analytical perspectives should include the development of analytical methods to better monitor glyphosate related metabolites in food, e.g. trimethyl-sulfonium, AMPA, acetyl-glyphosate and acetyl-AMPA.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The article describes the development and validation of an LC-MS/MS based method for the analysis of glyphosate residues. No metabolites were included. An LOQ of 0.5 µg/kg could be achieved and validated. The method was applied for the analysis of 136 food samples, representing different commodity groups. Residues of glyphosate were found in two food samples (breakfast cereal) at 6.0 and 34 µg/kg.
This is primarily an analytical method paper and, therefore, contains supportive information for the analytical section. It provides limited glyphosate monitoring data for various food products. In most cases only one sample per product type was analysed and residues above the LOQ were found only in two samples. No regulatory relevant endpoints can be derived. Therefore the article is relevant but supplementary.

Assessment and conclusion by RMS:
The article primarily is an analytical method paper for the measurement of glyphosate in various food products. In addition, glyphosate has been monitored in several of such products. No further impact expected on the existing risk assessment parameters.

B.7.8.3.9. Study 9

1. Information on the study

Data point	CA 6.9
Report author	McQueen H. *et al.*
Report year	2012
Report title	Estimating maternal and prenatal exposure to glyphosate in the community setting
Document No.	International Journal of Hygiene and Environmental Health, (2012), Vol. 215, No. 6, pp. 570-576
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

Glyphosate is a herbicide in common use, in both agricultural and residential settings. Controlled residue studies show that glyphosate persists in food crops, allowing for the potential of a large number of people to be exposed. Glyphosate is generally considered safe however there are a number of studies suggesting formulations or additives that may have adverse health effects. To assess the degree of exposure of pregnant women, this study measured glyphosate in composite food samples and estimated exposure based on food frequency questionnaire. 43 pregnant women were recruited and completed a self administered questionnaire with a food frequency component and provided a composite food sample. Twenty food samples were analysed with very low glyphosate concentrations (mean 0.08 mg/kg, range 0.002 – 0.5 mg/kg) with residues detected in more than 75% of the samples. Maternal dietary exposure was very low (0.001 mg/kg bw/day) and was considerably lower than the predicted National Estimated Daily Intake of glyphosate (0.02 mg/kg bw/day). The estimated exposure based on measured glyphosate in composite food samples corresponded to 0.4% of the acceptable daily intake for glyphosate, and the predicted concentration from dietary information was 4% which is comparable to the National Estimated Daily Intake of 5.5% of the Acceptable Daily Intake of glyphosate. Prenatal exposures were estimated to be significantly lower. While residues of glyphosate are present in food, this study demonstrates that exposure concentrations are low and confirms the current models used to estimate glyphosate exposure

Materials and Methods

The study was a descriptive cross-sectional study of glyphosate exposure via diet in non-occupationally exposed urban- dwelling pregnant women. Ethics approval was granted by Edith Cowan University's Human Research Ethics Committee (Reference# 06-45). All participants provided written informed consent prior to data collection.

Study area

The Perth Metropolitan Region was chosen as the study area to avoid confounding factors that might be introduced from the rural setting, particularly where agricultural spray drift of herbicides may be an issue.

Study population
The study recruited pregnant women from across the Perth Metropolitan Region. The selection criteria were pregnant women aged over 18 years, eating conventionally grown food, and with no reported occupational exposure to glyphosate in the household.

Recruitment
Forty three pregnant women were recruited through presentations at the antenatal clinics of several hospitals in the Perth metropolitan area and by ad hoc methods that included advertising in the community and through the local media and word of mouth. Thirteen women took part in the study during winter sampling in July/August 2006 and 30 during spring sampling in November 2006. Four participants took part in the study during both sampling periods.

Data collection
Each participant was asked to complete a self administered questionnaire that included a food frequency component, and to provide a composite sample of table ready food.

Self administered questionnaire
A self-administered questionnaire was developed to collect information on participants' demographic characteristics, their dietary intake and gardening activities in order to estimate exposure to glyphosate based herbicides. The questionnaire also included a semi-quantitative food frequency questionnaire (FFQ). The FFQ used was a modified form of The Cancer Council Victoria Dietary Questionnaire for Epidemiological Studies Version 2 (Giles & Ireland, 1996).

Composite table ready food samples
Participants were asked to provide a composite food sample by collecting a small serve (approximately one dessertspoon) of every food item they consumed during one twenty four hour period. Participants were asked to prepare food to table ready state as usual and place a sample of each food item in a labelled freezer bag/container at each eating occasion. Participants were asked to freeze their food samples in a domestic freezer as soon as possible after collection. Samples were collected by the research team and stored at − 20°C until analysis. As food was anticipated to be the major contributor to dietary intake, beverage samples were excluded from this study. All food samples were weighed and individual samples from each participant combined to create one composite sample per participant.

Recruitment
Forty-three pregnant women were recruited through presentations at the antenatal clinics of several hospitals in the Perth metropolitan area and by ad hoc methods that included advertising in the community and through the local media and word of mouth. Thirteen women took part in the study during winter sampling in July/August 2006 and 30 during spring sampling in November 2006. Four participants took part in the study during both sampling periods.
Food samples were shipped frozen to Agrisearch Analytical (NSW, Australia) for chemical analysis. Due to budgetary constraints, 20 food samples were analysed for glyphosate. Samples were randomly selected for analysis from the two sampling periods using Random Number Generator Pro (accessed June 2006 from: http://www.segobit.com/rng htm).

Chemical analysis of food samples
Food samples were analysed for residues of glyphosate and aminomethylphosphonic acid (AMPA), the principle degradation product of glyphosate, by electrospray ionisation–liquid chromatography tandem mass spectrometry at a laboratory that was ISO/IED 17025:2005 National Association of Testing Authorities accredited to test for glyphosate residues in food commodities by this method. The reported glyphosate concentrations were expressed as the sum of glyphosate and AMPA as per the Australian Pesticides & Veterinary Medicines Authority (APVMA) residue definition (APVMA, 2008b). The limit of quantitation was 0.01 mg/kg and the limit of detection (LOD) was 0.005 mg/kg for this method of determining glyphosate in assorted food products.

Data management and analysis
The food frequency questionnaire (FFQ) section of the questionnaire was analysed by The Cancer Council Victoria. The remaining questionnaire data was analysed using SPSS v14.0 (student version). Descriptive analysis of the entire study population was compared with the subset of 20 participants whose composite food sample had been analysed for glyphosate. There were no significant differences between the subset and the entire study population.

The general equation for calculating the average daily potential exposure for the ingestion of chemical residues was given by the equation:

Exposure (mg/kg bw/day) = Food consumption (kg/day) × concentration of residue in food (mg/kg) body weight (kg) (U.S. EPA, 1992)

For dietary intake, where food was consumed intermittently throughout the day, the average intake rate of the medium multiplied by the sum of the exposure durations for all events divided by the time period over which the exposure is averaged (IR × ED/AT) becomes the total amount of food consumed per day (U.S. EPA, 1992). Thus, the equation used by FSANZ (2003) for chronic dietary exposure to pesticides estimates average daily potential exposure in milligrams of chemical residue per kilogram of body weight per day for each individual and is given by:

Estimate of Maternal Dietary Intake Equation (U.S. EPA, 1992)

ADDpot = [C × IR × ED] / [BW × AT]

where, ADDpot, average daily potential exposure; C, average concentration of the chemical in the medium (chemical specific: 0.08 mg/kg); IR × ED, average intake rate of the medium x the sum of the exposure durations for all events (site specific: 1083 g/day); BW, body weight (kg) (site specific: 72 kg); AT, time period over which the exposure is averaged (converted to days) (site specific: per day).

Results

Socio-demographic characteristics

The study population comprised 43 individuals with a mean age of 32 years (**Table 1**). Twenty participants had their food samples analysed for glyphosate and their characteristics are also noted in **Table 1**. The study population was well educated with the majority having completed tertiary education (**Table 1**). On average, participants had resided at their current address for almost three years (**Table 1**).

Table 1: Participant reported socio-demographic characteristics of all participants and the subset having glyphosate measurements in food.

Number of participants	Total study population (n = 43)	Subset with composite food analysis (n = 20)
Age (years)		
Mean	32.1	32
Median	32.1	33.5
Range	22–41	22–41
Highest level of education (%)		
Tertiary	72.1	75
Year 12/TAFE equivalent	18.6	15
Year 10	7.0	10
Did not complete high school	2.3	0
Current or last occupation[a] (%)		
Managers	7.0	15.0
Professionals	53.5	45
Technicians and trades workers	2.3	0
Community/personal service	4.7	5
Clerical and administrative	23.3	25
Sales workers	2.3	10
Duration Lived at current address (years)		
Mean ± SD	2.8	2.8
Range	0.1–8.9	0.1–8.9
Median	2.7	2.7

[a] Occupations coded to 2006 Australian and New Zealand Standard Classification of Occupations (ANZSCO, 2006).

The median self-reported pregnancy body weight at the time of sampling was 67 kg, with the maximum body weight reported as 121 kg (**Table 2**). The subset of pregnant women were slightly heavier but the difference was not significant (**Table 2**).

Table 2: Participant reported body weight and derived gestational age of foetus.

Number of participants	Total study population ($n = 43$)	Subset with composite food analysis ($n = 20$)
Maternal pregnant body weight (kg)		
Mean ± SD	71.1	72.3
Range	51–121	51–121
Median	67	65.8
Gestational Age of foetus[a] at sampling (weeks)		
Mean ± SD	26.8	25.3
Range	9–38	9–36

[a] Gestational age of foetus calculated as 40 weeks minus the number of weeks between the sampling date and self-reported date of confinement.

Dietary consumption

Seventy-seven per cent of women reported eating 'no special type of diet' (data not shown). The types and amounts of food consumed by participants as well as the range of maximum residue limits of glyphosate for foods in selected major food groups is summarised in **Table 3**.

The study population ate a diverse diet, comprising of foods from all the major food groups as well as those from a wide range of sub-major food groups. At least 80% of participants reported eating from all major food groups (data not shown).

The food groups cereals, legumes and pulse products and fats and oil products represent the greatest potential for glyphosate exposure, due to high maximum residue limits (MRLs) for glyphosate associated with pre-harvest treatment during crop production (**Table 3**). The MRLs of glyphosate are highest for cereals and cereal products and cereal-based products/dishes, particularly those that contain wheat and wheat bran, for which the MRLs are 5 and 20 mg/kg respectively. High MRLs, ranging from 5 to 10 mg/kg, also apply to mature legumes and pulses. While MRLs are unlikely to reflect actual dietary intake, they nominate the foods in which residues of glyphosate are permitted and the variability of MRLs among food types indicates that consumers are exposed to different levels of glyphosate from eating different foods (**Table 3**).

Table 3: The range of maximum residue limits (MRL) of glyphosate for each food group.

Selected food groups	MRL[a] [range of MRLs for food items in food group] mg/kg
Cereals and cereal products	[T[b]0.1–20]
Cereal-based products and dishes	[T[b]0.1–20]
Fruit products and dishes	Most fruits [[b]0.05–0.5]
Vegetable products and dishes	Most vegetables [[b]0.01–[b]0.1]
Legume and pulse products and dishes	Most mature legumes/pulses [5–10]
Milk products and dishes	Milks [[b]0.1]; soya beans (dry) [10]
Meat, poultry and game products and dishes	Most meats [[b]0.1]
Fish and seafood products and dishes	No MRLs
Egg products and dishes	Eggs [[b]0.05]
Sugar products and dishes	[T0.3, T5]
Seed and nut products and dishes	Tree nuts and oilseeds [[b]0.05–0.2]
Fats and oils	[T[b]0.1, [b]0.1]

MRL, maximum residue limit. 'T', temporary use of the MRL or residue definition pending experimental work or during phase out of the MRL (APVMA, 2008a).

[a] Maximum residue limits apply either to individual foods or a food group, in which case the MRL applies to all members of the food group listed in the Codex Classification of Foods and Animal Feeds (FAO/WHO, 1993). Residues are also acceptable in processed foods that consist of, contain or are manufactured from primary food commodities for which MRLs have been set (e.g. fruit juices) provided that residues are lower than the respective MRLs of the raw ingredients (APVMA, 2008a). Maximum residue limits shown are current at October 2008 (APVMA, 2008b).

[b] MRL is set 'at or about' the analytical limit of quantitation and residues should not be detected.

The amount of food consumed by the study population from selected food groups and the percentage these food groups contribute to the total diet are summarised in **Table 4**. The mean food consumption was derived from the 74-item FFQ and represents an estimate of the amount of these foods and beverages consumed per day on average by study participants (**Table 4**).

The mean daily food intake of the study population was 1066 g/day and ranged from 525 to 2436 g/day (**Table 4**). The wide range of reported food intakes might indicate a degree of over- or under-reporting of food consumption by participants. However, the mean solid food intake reported in this study is very similar to average mean daily intake (1084 g) and range 564 – 2447 g (5^{th} – 99^{th} percentile) determined by dietary studies conducted on adults in 16 countries across Europe (EFSA, 2008). The combined cereals and cereal-based products food groups contributed a mean of 333 g to the participants' daily food intake (**Table 4**).

Table 4: Amount of food consumed by major food groups (n = 43).

Selected food groups	Mean (g/day)	Median (g/day)	Range (g/day)	% of diet
Cereals and cereal products	255.4	184.6	43.3–1386.2	23.9
Cereal based products and dishes	78.0	62.4	15.3–252.5	7.3
Fruit products and dishes	253.3	249.9	55.5–489.2	23.8
Vegetable products and dishes	166.3	147.2	27.6–392.0	15.6
Legume and pulse products, dishes	11.1	6.7	0–47.3	1.0
Total milk and milk products	103.1	111.8	0–291.6	9.7
Meat, poultry products and dishes	90.3	78.2	0–244	8.5
Fish and seafood product and dishes	27.8	22.0	0–108.1	2.6
Egg products and dishes	20.3	12.9	0–60.0	1.9
Snack foods	5.1	2.8	0–41.8	0.5
Sugar products and dishes	13.7	15.2	0–34.0	1.3
Confectionary	14.4	10.4	0.5–70.7	1.4
Seed and nut products and dishes	8.1	5.1	0–44.0	0.8
Fats and oils	15.3	14.0	0–42.0	1.4
Miscellaneous	4.2	2.6	0–20.6	0.4
Total food consumed	1066.4	1011.9	524.6–2436.2	100

Concentrations of glyphosate in food

Quantifiable residues of glyphosate were found in 75% of the composite food samples tested with a mean of 0.08 mg/kg and range of < 0.005 – 0.5 mg/kg (**Table 5**). The wide range of concentrations reported is due to one high value (0.5 mg/kg) and 2 values below the LOD. Wide-ranging concentrations of glyphosate are typically reported in supervised trials (FAO/WHO, 1986, 2006a).

Table 5: Concentration of glyphosate residuea concentrations determined in composite food samples.

By season	Number of samples	Mean ± SD (mg/kg)	Minimum (mg/kg)	Maximum (mg/kg)	% samples <LOD
Winter samples	5	0.03 ± 0.3	<0.005	0.07	40
Spring samples	15	0.09 ± 0.1	0.005	0.5	–
All samples	20	0.08 ± 0.1	<0.005	0.5	10

LOD = 0.005 mg/kg. Where glyphosate residue concentrations are <LOD, LOD/2 has been used to calculate mean concentrations (U.S. EPA, 1992).

[a] Glyphosate is expressed as the sum of glyphosate and aminomethylphosphonic as per Australian Pesticides & Veterinary Medicines Authority (APVMA) residue definition (APVMA, 2008b).

Estimated maternal exposure to glyphosate

The mean estimated maternal daily dietary exposure to glyphosate residue was calculated from the concentrations measured in composite food samples by the subset of twenty participants. The concentration calculated was 0.001 mg/kg bw/day with a range of 2 × 10−5 to 0.005 mg/kg bw/day (**Table 6**). The guideline value for the intake of pesticides via the oral route is the ADI which for glyphosate in food has been set at 0.3 mg/kg bw (DoHA, 2007). When the mean estimated maternal daily dietary exposure to glyphosate residues is expressed as a percentage of the ADI of glyphosate, estimated maternal exposure accounts for 0.4% of the ADI with a range of 0.005 – 2%. Thus, all estimated exposures were less than or equal to 2% of the ADI of glyphosate and well below the applicable health guideline.

It is expected that foetal exposures would be significantly lower due to the small percentage of glyphosate predicted to cross the placenta (15%) based on an in vitro placental perfusion study (Mose *et al*., 2008).

Table 6: Estimated maternal exposure to glyphosate (n = 20).

	Estimated dietary intake (mg/day)	Estimated dietary exposure (mg/kg bw/day)	% ADI
Mean	0.07	0.001	0.4
Median	0.06	9×10^{-4}	0.3
Range		2×10^{-5} to 5×10^{-3}	0.005–2

Estimated dietary intake, mean analytical results for concentrations of glyphosate in food multiplied by mean food consumption. Estimated dietary exposure, mean analytical results for concentrations of glyphosate in food multiplied by mean food consumption divided by mean maternal pregnancy body weight (U.S. EPA, 1992).

Dietary intake of glyphosate was also predicted based on MRLs (**Table 3**) allocated for food groups and estimated consumption (**Table 4**). The results of the prediction of concentrations and those estimates based on measurements of

intake and exposure to dietary glyphosate are shown in **Table 7**. These calculations show that the mean predicted estimate of glyphosate intake from the diet was 0.7 mg/day, with a predicted exposure estimate of 0.01 mg/kg bw/day (**Table 7**). When compared with the Acceptable Daily Intake for glyphosate, the predicted maternal dietary exposure estimate accounted for 4% of ADI for glyphosate. The predicted dietary intake of glyphosate is a factor of ten higher than the measured dietary exposure estimate (**Tables 6** and **7**).

Table 7: Predicted theoretical maximum daily intake of glyphosate for study population.

n = 20	Predicted dietary intake of glyphosate (mg/day)	Predicted dietary exposure to glyphosate (mg/kg bw/day)	% ADI
Mean ± SD	0.7	0.01	
Range	0.3–3	0.004–0.05	1–17
Median	0.5	0.008	3

[a] Theoretical maximum daily intake = $\Sigma MRLi \times Fi$, where MRL*i* is the maximum residue level for a given food commodity and *Fi* is the daily consumption value of that food commodity per person (WHO, 1997). Where MRLs vary among ingredients in mixed foods, AUSNUT recipes have been consulted to determine the percentage different ingredients contribute to a mixed food and proportional MRLs have been applied in calculations (FSANZ, 2009; FSANZ, n.d.-a, n.d.-b).

Conclusion

This study has confirmed the presence of residues of glyphosate in table-ready food. The estimated exposure of the pregnant women in this study to dietary glyphosate using measured concentrations in food represented a very small percentage of the ADI for glyphosate and that measured dietary intake of glyphosate in this group of women is approximately one order of magnitude lower than the nationally predicted dietary intake of glyphosate. This indicates the extent of conservatism inherent in the methodology for predicting the intake of residues of glyphosate from food. This study also found that the measured dietary intake of glyphosate in this group of women compared well with the most refined predicted exposure estimates available, supporting the current models for predicting dietary exposure to glyphosate.

It is suggested that a larger study be conducted to ascertain exposure to the formulation ingredients of glyphosate, as opposed to glyphosate alone, to confirm the low exposures in this study but importantly to assess the risks associated with formulation ingredients. It is also suggested this be combined with assessments of individual food items instead of composite food samples.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The article describes a dietary risk assessment approach for glyphosate for pregnant woman and prenatal children in Australia. The study was designed as cross-sectional study based on a volunteer questionnaire on individual food habits. Composite food samples were analysed for residues of glyphosate and AMPA as basis of the dietary risk assessment. The estimated exposure of the pregnant women in this study to dietary glyphosate using measured concentrations in food represented a very small percentage of the ADI for glyphosate.

The article does not provide relevant regulatory endpoints, however supports published dietary risk assessments and confirms the safety of glyphosate intake via food.

Assessment and conclusion by RMS:

The article investigated the dietary exposure to glyphosate of pregnant women in Australia. No further impact expected on the existing risk assessment parameters.

B.7.8.4. Category C studies, included in the renewal dossier as supplementary information (all studies relate to microbe-related effects)

B.7.8.4.1. Study 1

1. Information on the study

Data point	KCA 6.4
Report author	Ackermann W. *et al.*
Report year	2015
Report title	The Influence of Glyphosate on the Microbiota and Production of Botulinum Neurotoxin During Ruminal Fermentation.

Document No.	Curr. Microbiol. (2015), Vol. 70, pp. 374-382
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

The aim of the present study is to investigate the impact of glyphosate on the microbiota and on the botulinum neurotoxin (BoNT) expression during *in vitro* ruminal fermentation. This study was conducted using two DAISY^{II}-incubators with four ventilated incubation vessels filled with rumen fluid of a 4-year-old non-lactating Holstein-Friesian cow. Two hundred milliliter rumen fluid and 800 mL buffer solution were used with six filter bags containing 500 mg concentrated feed or crude fiber-enriched diet. Final concentrations of 0, 1, 10, and 100 µg/mL of glyphosate in the diluted rumen fluids were added and incubated under CO_2-aerated conditions for 48 h. The protozoal population was analyzed microscopically and the ruminal flora was characterized using the fluorescence *in situ* hybridization technique. *Clostridium botulinum* and BoNT were quantified using most probable number and ELISA, respectively. Results showed that glyphosate had an inhibitory effect on select groups of the ruminal microbiota, but increased the population of pathogenic species. The BoNT was produced during incubation when inoculum was treated with high doses of glyphosate. In conclusion, glyphosate causes dysbiosis which favors the production of BoNT in the rumen. The authors stated that the global regulations restrictions for the use of glyphosate should be re-evaluated.

Materials and Methods

Animals and Feeding

A 4-year-old non-lactating Holstein-Friesian cow was used as the donor animal for rumen fluids. The cow was fed 2 kg dry mass/100 kg body mass/day of a crude fiber-rich feed distributed over three feeding times per day and had *ad libitum* access to water. The composition of the feed corresponds to the experimental diet 1 (Table 1) used in this study. Two weeks prior to the initial collection of rumen fluids, the cow was adapted to the feed to ensure stable conditions in the rumen at the beginning of the experimental period.

Table 1: Ingredients of the experimental diets

	diet 1	diet 2
Hay (%)	84.17	59.17
Grass pellets (%)	5	0
Sugarbeet pellets (%)	10	0
Mineral feed[a] (%)	0.83	0.83
Concentrate feed for lactating cows[b] (%)	0	40

[a] Contains (%) Ca (4), P (6), Na (6.5), Mg (8); (IU/g) Vit A (1,000), Vit D3 (125); (mg/g) Vit E (5), biotin (0.15), β-Carotin (1.5), Cu (1.1); Mn (3.7), Zn (5), J (0.16), Se (0.04), Co (0.022)

[b] Contains (%) sugarbeet molasses pulp (20), rapeseed meal (14), rye bran (12), corn gluten (10), rye (10), wheat bran (10), wheat semolina bran (9), sunflower meal (7), $CaCO_3$ (3.5), soybean cups (3), sugar-beet molasses (1), NaCl (0,29); (IU/g) Vit A (7.5), Vit D3 (0.95)

In Vitro Fermentation of Diluted Rumen Fluids

The study was conducted using two DAISY^{II}-incubators (ANKOM Technology, Fairport, NY, USA), each of them containing four ventilated incubation vessels (volume 4 l).

Influence of Glyphosate on Rumen Microbiota

All chemicals and reagents were purchased from Sigma–Aldrich (Germany) unless otherwise stated. Before incubation, each vessel was filled with 800 mL buffer solution (10 g/l KH_2PO_4, 0.5 g/l $MgSO_4$ x 7 H_2O, 0.5 g/l NaCl, 0.1 g/l $CaCl_2$ x 2 H_2O, 0.5 g/l urea, 15 g/l Na_2CO_3 and 1 g/l Na_2S x 9 H_2O) and six filter bags (pore size 50 µm, R 510, ANKOM Technology, Fairport, NY, USA) which contained 500 mg each of either diet 1 or 2 (a maximum 3 mm particle size of the diet ingredients and crude nutrients (see Tables 1, 2). Glyphosate, Sigma-Aldrich, St. Louis, MO,

USA) was added from a 2% (w/v) base solution to reach final concentrations of 0, 1, 10, and 100 µg/mL in the diluted rumen fluids. Subsequently, the vessels were aerated for 1 min with CO_2 and pre-warmed for 2 h in the DAISY^{II}-incubators to ensure a temperature of 39°C at the beginning of the incubation.

Table 2: Composition of the crude nutrients of the experimental diets

	Diet 1	Diet 2
Dry matter (% of DM)	94.35	92.57
Crude ash (% of DM)	9.33	8.62
Crude protein (% of DM)	9.07	13.9
Crude fiber (% of DM)	27.07	21.27
Crude fat (% of DM)	0.88	1.47
Nitrogen free extract (% of DM)	53.65	54.74
Organic matter (% of DM)	90.67	91.38
Nitrogen (% of DM)	1.45	2.22

Rumen fluid was orally collected from the donor cow 3 h after the morning feeding and passed through four layers of cheese cloth. Each incubation vessel received 200 mL of the filtrate, mixed gently, aerated with CO_2 for another 2 min and incubated for 48 h at 39°C. After incubation, aliquots of the incubation medium were immediately fixed with a 1:10 methylgreen-formalin-saline-solution (contains 0.6 g/l methylgreen, 100 mL/L formalin, 8 g/l NaCl, 900 mL/L distilled water) in centrifuge tubes for protozoal analysis, 1:2 with 96% (v/v) ethanol or 1:4 with 4% (w/v) paraformaldehyde for bacterial analysis. The tubes for protozoal analysis were stored at 4°C and the tubes for bacterial analysis were stored 1 h at 4°C and then at -20°C (ethanol fixed vials) or 24 h at 4°C and then at -20°C (paraformaldehyde fixed vials) until further processing. Subsamples of the inoculum for the 0 h values were taken after the addition of rumen fluid and fixed as described above.

Influence of Glyphosate on C. botulinum Type B

C. botulinum type B (7273) was obtained from the National Collection of Type Cultures (NCTC, Salisbury, UK), cultured anaerobically in cooked meat medium (Oxoid, Wesel, Germany) at 37°C for 5 days and thereafter cultivated in Reinforced Clostridial Medium (RCM; Sifin, Berlin, Germany) anaerobically at 37°C for 3 days. The cultures were then heated at 80°C for 10 min, analyzed for sporulation using a Gram and Rakette stain and left aerobically at room temperature until further processing.

Preparation of the incubation vessels and buffer solution for experiment 2 was as described for experiment 1, except that smaller, rubber plugged gas-tight vessels were used (volume 125 mL) which were filled with 80 mL buffer solution that was added directly (without filterbags) with 1 g of either diet 1 or 2. Glyphosate was added to provide final concentrations of 0, 1, 10, 100, or 1000 µg/mL.

Twenty milliliter of orally collected, filtrated rumen fluid as described in experiment 1 was added to the vessels and preheated spores of *C. botulinum* type B were added to reach a final concentration of 10^5 cfu/mL in the inoculum. The vessels were aerated with CO_2 for 2 min, closed gas-tight and incubated for 48 h at 39°C. Gas produced during incubation was released with a gastight syringe after 3, 6 h and then every 6 h of incubation. After incubation, aliquots of the incubation medium were taken to estimate the concentration of *C. botulinum* and botulinum neurotoxin. Subsamples of inoculum for the 0 h values were taken after the addition of rumen fluid.

Analysis of the Protozoal Population

The protozoal population was analyzed using a modified McMaster slide and light microscope according to phenotypical criteria of Williams and Coleman. Each of the two counting chambers were filled with 1 mL of methylgreen-formalin-saline-fixed rumen fluid from experiment 1 and the population of *Entodinium* spp., *Diplodinium* spp. *Epidinium* spp., *Isotricha* spp., *Dasytricha* spp., *Ophryoscolex* spp. and total ciliates (as the sum of all previous species) were counted at 400x magnification and estimated as cells/mL.

Analysis of the Ruminal Flora

The ruminal flora was characterized using the fluorescence *in situ* hybridization technique using the following 16S rRNA/23S rRNA-targeted oligonucleotide probes (sequences of the probes are available at probeBase:

(1) an equimolar mixture of five bacteria-directed probes (EUB338, EUB785, EUB927, EUB1055, EUB1088) referred to as EUBmix to detect all bacteria;
(2) Bac303 for the detection of *Bacteroidaceae* and *Prevotellaceae*;

(3) Bfi826 for *Butyrivibrio fibrisolvens*-related clones;
(4) Rfla729 for *Ruminococcus albus* and *Ruminococcus flavefaciens*;
(5) Str for *Streptococcus* spp.;
(6) Eury498 for *Euryarchaeota*;
(7) Clit135 for the *Clostridium lituseburense* group;
(8) Chis150 for the *Clostridium histolyticum* group;
(9) Clept1240 for the *Clostridium leptum* subgroup;
(10) Erec482 for the *Clostridium coccoides*/*Eubacterium rectale* group;
(11) Lab158 for Lactobacilli and Enterococci;
(12) DSS658 for Desulfobacteraceae.

The oligonucleotides were purchased 5' labeled with the indocarbocyanine dye Cy3 (BioTeZ, Berlin, Germany). The ethanol and paraformaldehyde fixed aliquots from experiment 1 were hybridized on silanized slides, modified from Maddox and Jenkins, with eight wells. Hybridization, washing procedures, and enumeration were performed in the dark by transferring 10 µl of the fixed sample to the well, drying (37°C for 1 h) and dehydrating for 3 min in 50, 70, and 96% ethanol each. After air drying, 10 µl of lysozyme buffer (100 mM-Tris–HCl (pH 8,0), 50 mM-EDTA, 1 mg lysozyme (130'000 U/mg; Boehringer, Mannheim, Germany) was added to each well and incubated in humidity chambers at 37°C for 30 min to obtain improved permeability of the cell walls of Gram-positive bacteria during hybridization. After another drying process (45 min at 37°C), the samples were hybridized for 45 min at 46°C (50°C Lab158) in a humidity chamber after adding 10 µl hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl and 0.05% (w/v) sodium dodecyl sulfate, pH 7.2) containing 5 ng probe/mL. The hybridization buffer for the Clept1240 and DSS658 probes also contained 10 and 60% (v/v) formamide, respectively. After hybridization, the slides were gently rinsed with distilled water (DW) and washed for 10 min in washing buffer (0.9 M NaCl, 20 mM Tris-HCl and 0.05 % (w/v) sodium dodecyl sulfate, pH 7.2) at the same temperature as the hybridization process. The washing buffer for the Clept1240 and DSS658 probes contained only 0.45 M and 10 mM NaCl, respectively. EDTA (50 mM) also was added to the DSS658. Thereafter, the slides were again rinsed with DW and air dried.
To exclude false hybridization and counting of artifacts, the samples were counter-stained 5 min with 10 µl DAPI solution [0.9 M NaCl, 20 mM Tris–HCl, 1 lg/mL 4,6-diamidino-2-phenylindole [Sigma-Aldrich, St. Louis, MO, USA)], rinsed with DW, air dried and embedded in antifading solution (233 mg of 1,4-diazobicyclo(2,2,2) octane) (DABCO, Sigma-Aldrich, St. Louis, MO, USA), 10 mL 19 phosphate buffered saline [130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4 (PBS)]. Enumeration of bacteria was done with an epifluorescence microscope (eclipse Ni-U; Nikon, Düsseldorf, Germany) equipped with a mercury lamp (HBO 100 W/3, Osram, Munich, Germany) and a 12 bit CCD camera (ProGres CF, Jenoptik, Jena, Germany). The cells were counted at 1000x magnification (100x immersion oil objective) equipped with a DAPI (UV-2A), a Cy3 (G-2A) filter and a counting net (1 cm^2, Nikon, Düsseldorf, Germany) installed between the ocular and the objective. The concentration of bacteria was calculated as cells/mL.

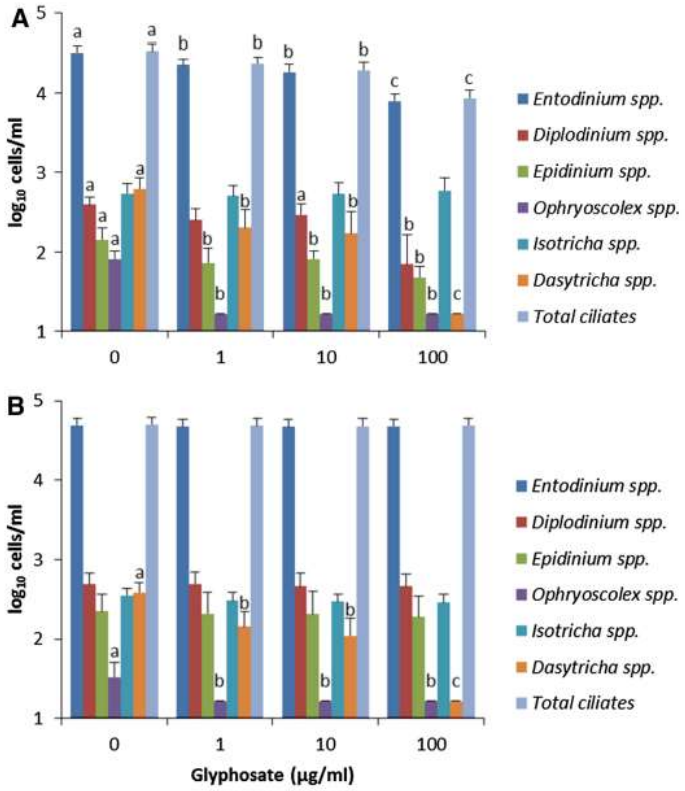
Determination of C. botulinum and Botulinum Neurotoxin
To estimate the population of *C. botulinum*, the samples from experiment 2 were heat treated (10 min at 80°C) and incubated anaerobically in Differential Reinforced Clostridial Medium (DRCM, Sifin, Berlin, Germany) at 37°C for 6 days followed by quantification of *C. botulinum* as colony forming units (cfu)/mL using the three-tube most probable number (MPN) method. Before the heat treatment, botulinum neurotoxin was detected using an ELISA as described by.

Statistical Analysis
To determine if variables differed among the treatment groups, the values before and after incubation were compared using the Statistical Package for Social Science (SPSS version 18.0; SPSS Inc., Chicago, IL, USA). The values of the flora and fauna were $\log_{10}$-transformed. For non-detectable parameters, a half detection limit was used which was 1.67 x 10^2/mL for protozoa and 2.82 x 10^4/mL for ruminal flora. Normality was tested using the Kolmogorov–Smirnov test. One-way ANOVA was performed for normally distributed variables. The Kruskal–Wallis H test and Mann–Whitney U test were used for non-normally distributed variables. Differences were considered to be significant at $p < 0.05$.

Results
Influence of Glyphosate on Rumen Microbiota
Glyphosate was toxic to all ciliates except *Isotricha* spp. and cell counts decreased after 48 h *in vitro* incubation with diet 1 (Fig. 1a). The population of all species except *Isotricha* spp. and *Diplodinium* spp. were significantly lower when treated with 1 and 10 µg/mL glyphosate, and of Diplodinium spp. treated with 100 µg/mL glyphosate. Only

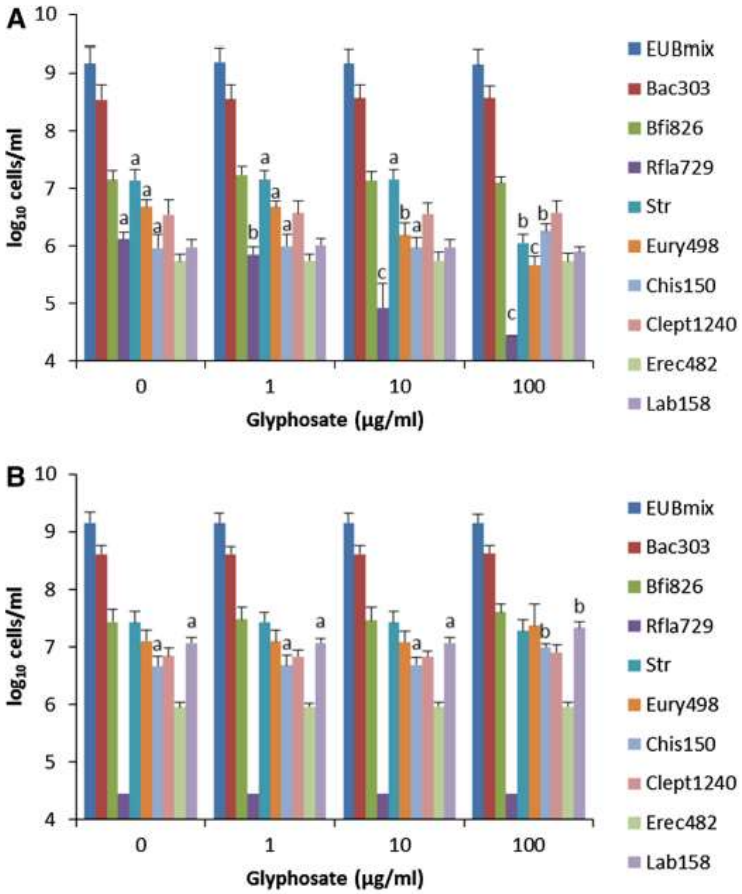
Ophryoscolex spp. and *Dasytricha* spp. were inhibited by glyphosate in diet 2 where glyphosate inhibited *Ophryoscolex* spp. at the lowest concentration and *Dasytricha* spp. at the highest (Fig. 1b).

Figure 1: Cell counts of ruminal protozoa with diet 1 (a) and diet 2 (b) after 48 h in vitro incubation with different concentrations of glyphosate. Values are mean ± SD (N = 6). Different letters within a species represent significant differences ($p < 0.05$).



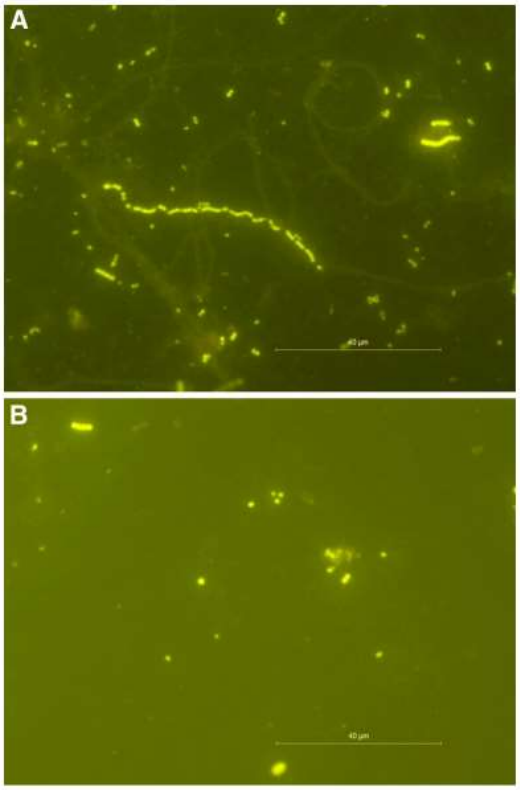
The ruminal bacterial flora was also affected by glyphosate (Fig. 2a, b).

Figure 2: Cell counts of ruminal flora incubated 48 h in vitro with diet 1 (a) and diet 2 (b) after 48 h in vitro incubation with different concentrations of glyphosate. Values are mean ± SD (N = 6). Different letters within a species represent significant differences ($p < 0.05$).



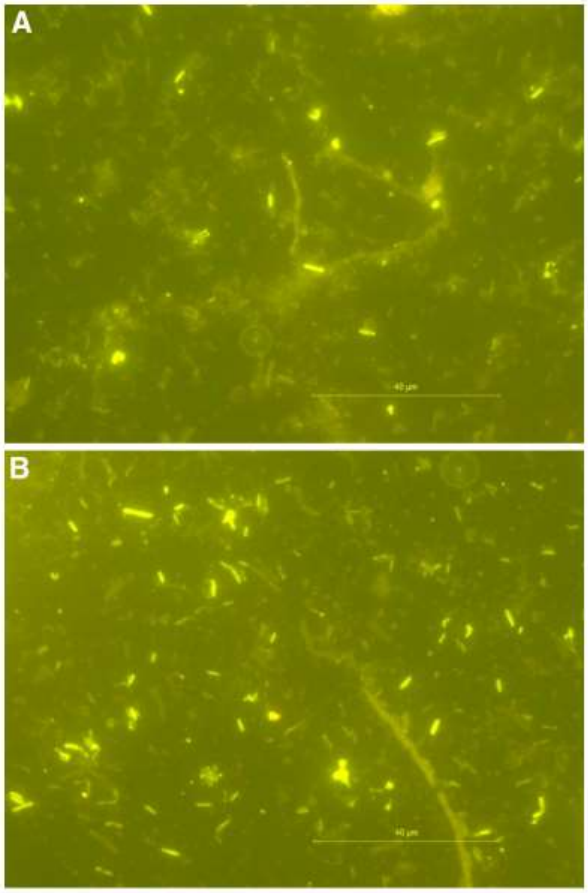
After incubation with diet 1, the cell counts of Rfla729 were significantly lower with 1 µg/mL glyphosate, for Str with 100 µg/mL and for Eury498 with 10 and 100 µg/mL (Fig. 3a, b).

Figure 3: Appearance of Rfla729 in diet 1 supplemented with 0 µg/mL (a) and 10 µg/mL (b) glyphosate incubated 48 h in vitro.



In contrast, cell counts of Chis150 were increased at the highest concentration of glyphosate (100 µg/mL) (Fig. 4a, b). Chis150 and Lab158 were significantly higher with 100 µg/mL glyphosate in diet 2. Before incubation, the population of all organisms was similar.

Figure 4: Appearance of Rfla729 in diet 1 supplemented with 0 µg/mL (a) and 10 µg/mL (b) glyphosate incubated 48 h in vitro.



Influence of Glyphosate on C. botulinum *Type B*

Botulinum neurotoxin type B could not be detected at 0, 1, 10, and 100 µg/mL glyphosate after 48 h *in vitro* incubation. However, it was detected at 1000 µg/mL glyphosate after 48 h *in vitro* incubation with both diets even though the population of *C. botulinum* type B did not differ significantly before or after incubation (Table 3). The toxin production was significantly higher with diet 1. It was 38–98 and 25–45 ng/mL for diet 1 and 2, respectively.

Table 3: The effect of glyphosate on the growth of *C. botulinum* type B (log 10 cfu/mL) at 0 and 48 h *in vitro* incubation with diet 1 and 2. Values are mean ± SD (n = 3).

Diet	Incubation time (h)	Glyphosate concentration (µg/ml)				
		0	1	10	100	1,000
1	0	5.14 ± 0.18	5.16 ± 0.15	5.06 ± 0.32	5.14 ± 0.18	5.11 ± 0.11
	48	6.25 ± 0.33	6.15 ± 0.26	6.11 ± 0.32	6.19 ± 0.28	6.29 ± 0.26
2	0	4.98 ± 0.03	5.14 ± 0.18	4.98 ± 0.23	5.14 ± 0.18	5.09 ± 0.18
	48	6.04 ± 0.23	5.93 ± 0.54	6.24 ± 0.34	6.30 ± 0.30	6.28 ± 0.28

Conclusion

This *in vitro* study shows that glyphosate causes a shift in the microbial population of rumen fluids to favor the production of BoNT within 48 h even though the population of *C. botulinum* spores was not significantly changed (Table 3). Several groups of bacteria and protozoa were inhibited by glyphosate. It was noted that more species were inhibited with the crude fiber-rich diet (Figs. 1, 2, 3, 4) than the lower fiber one to indicate there may be an inhibitory effect on the microbiota responsible for fiber degradation. This could be significant since 30–40% of microbial fiber degradation in the rumen is performed by entodiniomorphid protozoa and all entodiniomorphid protozoa were highly susceptible to glyphosate. *Ruminococcus* (R.) *albus* and *R. flavefaciens*, the most important ruminal bacteria for fiber degradation, also were strongly inhibited by glyphosate in trials with crude fiber-rich diets.

Among the carbohydrate-specific ruminal subpopulations, the cellulolytic bacteria are especially important. These bacteria convert the major plant polysaccharide (cellulose) and nitrogenous compounds to hydrolytic products of cellulose, volatile fatty acids, and microbial cell products which can be used by other microbes and the host animal. The cellulolytic ruminal bacteria are more sensitive than other ruminal bacteria to certain stress conditions such as low pH. These results are comparable to those of Zebeli and Metzler-Zebeli who reported lowered fiber digestion with metabolic stress of gastrointestinal microbiota. These results suggest that glyphosate leads to ruminal disorders influencing the whole body system of the animal. Inhibition of pH sensitive fibrolytic enzymes through pH changes or the binding of cations necessary as cofactors for microbial enzymes through the chelating properties of glyphosate could be possible mechanisms. Follow-up *in vivo* investigations are needed. Fiber fermentation provides important nutrients for many non-fibrolytic microbial species. These nutrients include salts of short-chained fatty acids such as acetate, propionate, butyrate, and lactate as well as glycerol and gases such as carbon dioxide, hydrogen, and methane, products of fiber degradation that are released in the rumen. Inhibition of fiber degrading microbes by glyphosate, as shown in our study, could also affect non-fibrolytic microbial species indirectly to explain the reduction of *Streptococcus* spp. during incubation with the crude fiber-rich diet.

Euryarchaeota were also reduced which could be due to direct effect of glyphosate or indirectly due to dysbiosis of protozoa, hence the rumen protozoa influence the population and composition of its microbiota. Also several symbiotic associations between anaerobic protozoa and methanogenic bacteria have been found, and the episymbiosis of bacteria and protozoa has been described.

While certain groups were inhibited, the population of members of the *C. histolyticum* group increased with both diets when treated with glyphosate (Fig. 2). The *C. histolyticum* group consists of cluster I and II of the *Clostridiaceae* including many species with pathogenic potential. Therefore, the glyphosate-induced dysbiosis of crude fiber-rich diets during the dry period of cows could induce a higher susceptibility for pathogens or support such microorganisms that normally occur in small numbers. Glyphosate stimulation of lactate producing bacteria with the concentrate rich diet (Fig. 2) could result in decreased pH to induce a sub-acute acidosis during the lactation period. Although BoNT/B was produced with both diets at the highest glyphosate concentration, the *C. botulinum* type B spore population did not change (Table 3). This indicates that glyphosate toxicity to the ruminal microbiota avoided degradation of BoNT/B by proteases of these microorganisms.

BoNT/B was detectable only with the highest dosage of 1000 µg/mL glyphosate in this study possibly because glyphosate concentrations of 1, 10, and 100 µg/mL could have been chelated cations inherent in the buffer solution that inactivate herbicide and nullify its activity on the microbiota. Freuze *et al.* reported that 1 µg/L of glyphosate (6 nM) in the presence of an equivalent molar concentration of free copper (0.4 µg/L Cu^{2+}) reduces free glyphosate to 10%. At 1000 µg/mL, glyphosate was inhibitory to neurotoxin degrading bacteria and their enzymes. The mechanistic effect could be explained due to dysbiosis induced by glyphosate, hence the proteolytic enzymes of the authochtonous microbiota of rumen are able to degrade BoNT. These enzymes are able to degrade BoNT. *Lactobacillus* suspensions are able to degrade BoNT after 48 h incubation at 37°C.

This could explain how glyphosate favors toxico-infection with *C. botulinum* in the rumen and/or the whole gastrointestinal tract of cows. Although BoNTB was only detectable at the highest concentrations of the herbicide, negative effects could accumulate in younger animals through the chronic ingestion of residual glyphosate in the milk replacer made with genetically modified (GM) soy, that is administered shortly after finishing the colostral milk period or in the milk of nursing cows. The authors believe that glyphosate will have a greater influence on cattle herd health in the near future due to increasing application rates of glyphosate to crops and much higher residual levels especially in glyphosate-resistant GM crops.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The article investigate the impact of glyphosate on the microbiota and on the botulinum neurotoxin expression during *in vitro* ruminal fermentation. Glyphosate was found to have an inhibitory effect on selected groups of ruminal microbiota, but increased the population of pathogenic species. Botulinum neurotoxin was produced during incubation when inoculum was treated with high doses of glyphosate.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. The system used in this study was not developed for microbiological research. Instead it was developed for comparing rates of digestion of feed. It is not a dynamic system like a rumen but a batch culture system. In 48 hrs the researchers showed that adding glyphosate resulted in greater drops in pH as a result of inadequate buffering. The endpoints are consistent with decreased pH. They are inconsistent with more sophisticated rumen simulation techniques that found no effects from glyphosate.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:
The article considers possible effects of glyphosate on the rumen microbiota *in vitro*. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. In addition, as described by the applicant, there are also some deficits regarding the study set up. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.2. Study 2

1. Information on the study

Data point:	CA 6.4
Report author	Bote K. *et al.*
Report year	2019
Report title	Effect of a Glyphosate-Containing Herbicide on Escherichia coli and Salmonella Ser. Typhimurium in an In Vitro Rumen Simulation System.
Document No	European journal of microbiology & immunology, (2019), Vol. 9, No. 3, pp. 94
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined relevance (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

Many studies in the past have shown that residues of the herbicide can be found in many cultivated plants, including those used as livestock feed. Sensitivity to glyphosate varies with bacteria, particularly those residing in the intestine, where microbiota is exposed to glyphosate residues. Therefore, less susceptible pathogenic isolates could have a distinct advantage compared to more sensitive commensal isolates, probably leading to dysbiosis. To determine whether the ruminal growth and survival of pathogenic *Escherichia coli* or *Salmonella* serovar Typhimurium are higher when glyphosate residues are present in the feed, an in vitro fermentation trial with a "Rumen Simulation System" (RUSITEC) and a glyphosate-containing commercial formulation was performed. Colony forming units of *E. coli* and *Salmonella* ser. Typhimurium decreased steadily in all fermenters, regardless of the herbicide application. Minimum inhibitory concentrations of the studied *Salmonella* and *E. coli* strains did not change, and antibiotic susceptibility varied only slightly but independent of the glyphosate application. Overall, application of the glyphosate-containing formulation in a worst-case concentration of 10 mg/L neither increased the abundance for the tested *E. coli* and *Salmonella* strain in the in vitro fermentation system, nor promoted resistance to glyphosate or antibiotics.

Materials and methods

The used in vitro fermentation system (RUSITEC) was run as described by Riede et al.

RUSITEC Set-up

For inoculation of the RUSITEC fermenter, ruminal content from 3 ruminally fistulated, nonlactating Holstein-friesian cows, fed with 25 % grass silage, 25 % maize silage, and 50 % concentrate, was obtained. The liquid and solid contents were separated by gauze filtration. Six fermentation vessels (V = 700 mL) were filled with the rumen liquid. Seventy grams of solid digesta were inserted into a nylon bag (11.5 m × 6.5 cm, pore size 150 µm). A second nylon bag was filled with 15 g of fresh substrate (49.5 % grass silage, 39.7 % maize silage, 5 % wheat meal, 5 % soy cake, and 0.8 % mineral feed). Both nylon bags were introduced into each fermentation vessel. On the next day, the bag with the original rumen solid content was replaced with another substrate bag, and the day after that, the former feeding bag was exchanged, leading to a retention time of 48 h for each bag.

The pH and redox potential (mV) were measured daily prior to feeding, as well as the effluent volume. Concentrations of NH_3 and short chain fatty acids (SCFA) were determined at the end of the equilibration period on day 6.

Infection of the Fermenters

After 7 days of equilibration, each fermentation vessel was inoculated with 1 mL of an *E. coli* and a *Salmonella* ser. Typhimurium strain, respectively. Therefore, overnight cultures of the isolates were subcultured in Mueller Hinton I (CM0405 Oxoid Ltd., Hampshire) and grown to a concentration of 109 colony forming units (cfu)/mL each to obtain 106 cfu/mL in the fermenter (Table 1). The *E. coli* strain was initially isolated from a lactating cow with acute mastitis and provided by the German Federal Office of Consumer Protection and Food Safety. It is classified as an ESBL-*E. coli* and, among others, resistant to enrofloxacin and cefotaxime. To recover this isolate from the rumen fluid, CHROMagar™ Orientation (Merck KgaA, Darmstadt) supplemented with 4 µg/mL enrofloxacin and 2 µg/mL cefotaxime was thus used. The MIC for Roundup® LB Plus (RU, registration number 024142–00) was 40 mg/mL isopropylamine glyphosate (IPA). The *Salmonella* Typhimurium DT104 strain used in this study was initially isolated from a pig and was provided by the German Federal Institute for Risk Assessment. Selective XLD media (Oxoid GmbH, Wesel, Germany) was used to reisolate the strain from the fermenter. The initial MIC for RU was 80 mg/mL IPA. After inoculation of the strains, 3 out of 6 fermenters (fermenter numbers 2, 4, and 6) were challenged with the common glyphosate-based herbicide RU containing 360 g/L glyphosate (RU), whereas the other fermenters (fermenter numbers 1, 3, and 5) served as controls (CTRL). Schnabel et al. determined a daily glyphosate intake of up to 84.5 mg per day for lactating dairy cows. Rounding this value to 100 mg per day and taking the rumen content volume (about 100 L) into account, we established a daily glyphosate exposure level of 1 mg/L rumen content. To create a worst-case scenario, RU was added to obtain 10 times of this concentration (10 mg/L) daily. Strains were enumerated from the rumen fluid by standard dilution plating on respective selective agar plates at different time points after inoculation (0, 0.5, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h). If the strains were no longer quantitatively detectable, rumen samples were enriched overnight in buffered peptone water (DM494D Mast Group Ltd., Merseyside) and streaked out for qualitative analysis on the respective selective agar, as described above.

Table 1. Overview of the strains used to infect the RUSITEC fermentation vessels with the inoculum quantity and the used resistances to detect the isolates on our agar plates. Minimum inhibitory concentration (MIC) was determined for isopropylamine glyphosate in the formulation Roundup LB Plus (RU, registration number 024142-00) with and without pH adjustment with NaOH.

Species	MIC RU	MIC RU pH7	Original host	Selectivity resistances	Inoculum
Salmonella ser. Typhimurium	80 mg/mL	80 mg/mL	Pig	Nalidixic acid	8.42E+08 cfu
E. coli	40 mg/mL	80 mg/mL	Cow	Enrofloxacin, cefotaxime	1.25E+09 cfu

Susceptibility Testing

Three isolates of each strain from each fermenter and the last sampling time point from which bacteria could be recovered were further assessed for changes in antimicrobial susceptibility relative to the original parent strains. Prior to the fermenter experiments, the initial MICs of RU and RU supplemented with NaOH (to achieve pH7) for these isolates were determined as described previously. In short, serial twofold dilutions of RU in Mueller Hinton broth ranging from 160 mg/mL to 2.5 mg/mL IPA were prepared in conical 96-well plates and stored at −80 °C until use. For one of the isolates each, antibiotic susceptibility testing via VITEK® system (bioMérieux Deutschland GmbH, Nürtingen, Germany) with the test card VITEK® 2 AST N-248 with common relevant antibiotics (piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, and trimethoprim/sulfomethoxazole) was further performed. *E. coli* isolates were further tested for the presence of beta-lactamase genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} , and the CIT-type pAmpC genes (blaCMY), following the protocol described by Roschanski et al..

Statistical Analysis

All statistical analyses were performed using IBM® SPSS® Statistics Version 24. All fermenters were compared at each time point individually with a t-test. To compare vessels with and without Roundup®, the median of the bacterial counts in each fermenter group was calculated and compared with either a non-parametric Wilcoxon test or a t-test. Further, to determine potential statistical differences in qualitative analysis, a chi-squared test was performed when possible (i.e., where not all results were the same).

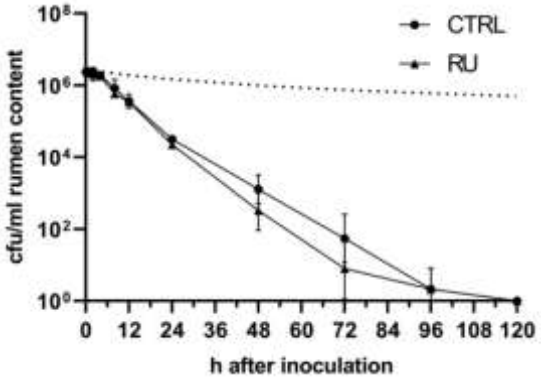
Results

To determine the effects of the glyphosate-containing formulation Roundup® LB Plus on growth and survival of *E. coli* and *Salmonella* ser. Typhimurium, we enumerated bacterial counts of the isolates after inoculation in vitro by means of the “Rumen Simulation Technique” (RUSITEC) and standard dilution plating.

E. coli in the Fermenters

After inoculation of 1.27E +09 cfu *E. coli*, the median starting concentrations in the fermenters were 2.73E+06 cfu/mL in the CTRL group and 3.12E+06 cfu/mL in the vessels, where RU equivalent to 10 mg/L glyphosate was added. In both groups, the concentration of *E. coli* did not vary significantly within the first 2 h. After 12 h, one logarithm step less was detectable, followed by a steady decline of about one to one and a half logarithm steps each day. At day 4 no more *E. coli* were quantitatively detectable in two out of three fermenters of each group (CTRL and RU). Qualitatively *E. coli* was still present in 5 out of 6 fermenter vessels on day 4 but not anymore on day 5. An overview of the cfu/ml rumen content can be found in Figure 1.

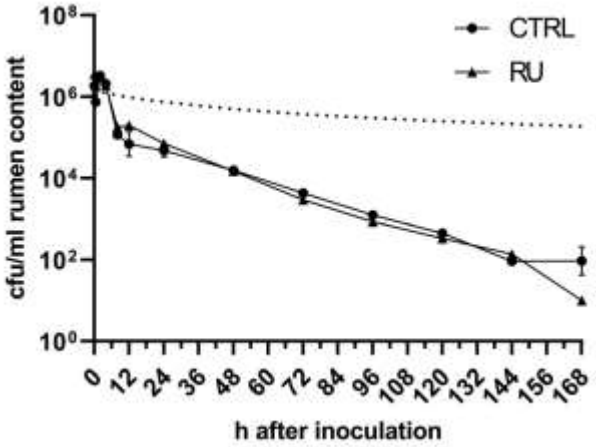
Figure 1. *E. coli* in the fermentation vessels measured by standard dilution plating on CHROMagar supplemented with 4 µg/mL enrofloxacin and 2 µg/mL cefotaxime. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup® LB Plus (10 mg/L, RU). The dotted line represents the theoretical loss of the *E. coli* due to the wash-out effect of the buffer if bacteria would be in a steady state.



Salmonella Ser. Typhimurium in the Fermenters

In addition to *E. coli*, vessels were simultaneously co-inoculated with 1.02E+09 cfu of the *Salmonella* ser. Typhimurium strain. Initial median starting concentrations were 1.50E+06 cfu/mL in the CTRL and 1.43E+06 cfu/mL in the RU group. After 30 min in both groups, the bacterial counts declined slightly followed by an increase after 2 and 4 h, where approximately the double amount of *Salmonella* compared to the starting concentrations could be detected (3.24E+06 cfu/mL after 2 h in the RU treated group and 3.22E+06 cfu/mL after 4 h in the CTRL group). This was followed by a steady decline in both groups (Figure 2). At the end of the experiment after 7 days, only 10 cfu/mL in the CTRL and 90 cfu/mL in the RU group were still present.

Figure 2. *Salmonella ser. Typhimurium* in the fermentation vessels measured by standard dilution plating XLD agar. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup LB Plus (10 mg/L, RU). The dotted line represents the theoretical loss of the *Salmonella* due to the wash-out effect of the buffer if bacteria would be in a steady state.



Comparison of the Treated and Non-treated Fermenters. Comparing the median from the control and the worst-case group, no statistically significant differences could be found in *Salmonella* ser. Typhimurium (P = 0.753) and *E. coli*

(P = 0.678) using Wilcoxon-test analysis or P = 0.967 and P = 0.825 using a t-test, respectively. More detailed statistical comparisons of all vessels at each sampling point are presented in Table 2.

Table 2. Statistical analysis of the differences between the control vessels and the vessels with 10 mg/L Roundup as a worst-case scenario for each sampling point quantitatively with the t-test. Further, a qualitative analysis with a chi-squared test for *E. coli* was performed (x: incalculable, because all fermenters are equal). No significant difference between the groups at any sampling point.

Time point		*t*-test		Chi-squared test
		E. coli	*Salmonella* ser. Typhimurium	*E. coli*
P0	Inoculation	$P = 0.244$	$P = 0.855$	x
P1	0,5 h	$P = 0.558$	$P = 0.503$	x
P2	2 h	$P = 0.456$	$P = 0.309$	x
P3	4 h	$P = 0.706$	$P = 0.970$	x
P4	8 h	$P = 0.275$	$P = 0.540$	x
P5	12 h	$P = 0.687$	$P = 0.539$	x
P6	24 h	$P = 0.151$	$P = 0.792$	x
P7	48 h	$P = 0.178$	$P = 0.339$	x
P8	72 h	$P = 0.257$	$P = 0.355$	$P = 0.273$
P9	96 h	$P = 1.000$	$P = 0.534$	$P = 0.273$
P10	120 h	–	$P = 1.000$	x
P11	144 h	–	$P = 0.729$	$P = 0.273$
P12	168 h	–	$P = 0.163$	x

Ruminal metabolism in the system was checked via pH and redox potential measurement (Table 3). Values were constant during the experiment in all fermentation vessels. SCFA and NH_3 have been checked after adaptation of the ruminal system and before the start of the experiment to ensure proper ruminal settings (data not shown).

Table 3. Control of ruminal metabolism. Means of the treated (RU) and non-treated (CTRL) vessels on each day of the experiment.

Days after inoculation	CTRL		RU	
	pH	Redox potential (mV)	pH	Redox potential (mV)
0	6.66	−273	6.65	−274
1	6.70	−281	6.64	−279
2	6.66	−261	6.68	−278
3	6.67	−277	6.69	−282
4	6.69	−279	6.74	−281
5	6.71	−281	6.70	−272
6	6.66	−264	6.67	−282
7	6.67	−265	6.63	−264
Mean	6.68	−273	6.68	−276
	6.69 ± 0.025	271 ± 10	6.68 ± 0.055	273 ± 9

Susceptibility Testing

MIC measurements were carried out for 3 isolates of each strain and fermenter from the last sampling point, which displayed bacterial growth. For E. coli, isolates recovered at day 2 from fermenters 2, 4, and 5 and at day 3 from the fermenters 1, 3, and 6 were investigated.

Salmonella Typhimurium isolates were examined after 5 days for all fermenters. The MIC values for RU did not change compared to the ancestor (Table 4).

Table 4. Minimum inhibitory concentrations (MIC) of isolated bacteria at the time point of the experiment with still solid growth on agar plates in comparison to the ancestral strain. MIC for IPA was tested in Roundup (RU) and RU adjusted to pH 7 (RU pH 7) (F: fermentation vessel).

F	RU	*E. coli*				*Salmonella* ser. Typhimurium			
		Sample number	Day	MIC RU (mg/mL)	MIC RU pH 7 (mg/mL)	Sample number	Day	MIC RU (mg/mL)	MIC RU pH 7 (mg/mL)
1	−	P8	3	40	80	P10	5	80	80
2	+	P7	2	40	80	P10	5	80	80
3	−	P8	3	40	80	P10	5	80	80
4	+	P7	2	40	80	P10	5	80	80
5	−	P7	2	40	80	P10	5	80	80
6	+	P8	3	40	80	P10	5	80	80
Ancestor				40	80			80	80

Further, for one isolate of each strain and fermenter, antibiotic susceptibility testing by VITEK® was performed. Individual strains differed in MIC for single antibiotics compared to the ancestor (Table 5). Differences were, in general, in the dimension of 1 or 2 dilution steps except for *E. coli* in cefepime, where ancestor showed a MIC of ≥64 µg/mL, and the isolates from Fermenter 1, 4, and 5, a MIC of 4 µg/mL.

Table 5. Minimum inhibitory concentrations in µg/mL tested with the VITEK® system and the test card AST N-248 with common relevant antibiotics. Shown in bold are the differences compared to the ancestor strain (R: resistant; S = susceptible).

	Ceftazidime	Cefepime	Aztreonam
E. coli Ancestor	16 R	≥ 64	16 R
E. coli Fermenter 1	16 R	**4**	**≥ 64 R**
E. coli Fermenter 4[a]	**4 S**	**4**	**≥ 64 R**
E. coli Fermenter 5	16 R	**4**	16 R
	Piperacillin/Tazobactam	Moxifloxacin	
Salmonella ser. Typhimurium Ancestor	8 S	0.5 S	
Salmonella ser. Typhimurium Fermenter 1	**≤ 4 S**	**1 R**	
Salmonella ser. Typhimurium Fermenter 2[a]	**≤ 4 S**	0.5 S	
Salmonella ser. Typhimurium Fermenter 3	**≤ 4 S**	0.5 S	

[a]Fermenter belonging to the RU treated group.

In addition, the *E. coli* isolates were tested for ESBL genes using multiplex real-time polymerase chain reaction (PCR). Isolates from all fermenters as well as the ancestor were positive for CTX and negative for SHV, TEM, and AmpC (data not shown).

Conclusion

Overall, no benefits for growth and survival of the tested pathogenic *E. coli* and *Salmonella* ser. Typhimurium strains with a worst-case glyphosate concentration of 10 mg/L present in the formulation Roundup® LB Plus could be detected in the in vitro rumen simulation system. Bacterial counts decreased equally in all fermenters. The MIC against RU did not change and antibiotic susceptibility only changed slightly for some antibiotics and strains regardless of glyphosate exposure.

Considering that there are various glyphosate-containing formulations on the market available worldwide, our findings are restricted to our experimental setup, where complete formulation Roundup® LB Plus and specific *E. coli* and *Salmonella* ser. Typhimurium isolates were used. We demonstrated that the worst-case concentration of Roundup has no effect on the pathogenic Enterobacteriaceae under our experimental conditions within a RUSITEC system. It therefore remains to be shown whether other formulations or pure glyphosate would influence the bacterial community in a fermenter model or in monogastric animals in vivo.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The study investigated whether the presence of glyphosate residues in feed may give an advantage to pathogenic enteric bacteria in colonization and infection of livestock, particularly cattle, by increasing resistance. The in vitro effects of a glyphosate-containing formulation on growth, survival, and resistance of *E. coli* and *Salmonella* ser. Typhimurium at a worst-case glyphosate concentration were measured. No benefits for growth and survival of the tested pathogenic *E. coli* and *Salmonella ser.* Typhimurium

strains with a worst-case glyphosate concentration of 10 mg/L present in the formulation Roundup® LB Plus could be detected in the in vitro rumen simulation system.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. This study used a rumen simulation technique that reasonably replicated rumen conditions that allowed for dynamic effects of feeding and removal of waste products. In the absence of a suitable dossier datapoint it was allocated to point KCA 6.4 as it concerns livestock. However, it is important to note that it is not a residue study and does not provide any data on the transfer of residues from feed to food of animal origin.

Assessment and conclusion by RMS:
The article considers possible effects of glyphosate on the rumen microbiota *in vitro*. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.3. Study 3

1. Information on the study

Data point	CA 6.4
Report author	Gerlach H. *et al.*
Report year	2014
Report title	Oral Application of Charcoal and Humic acids to Dairy Cows Influences *Clostridium botulinum* Blood Serum Antibody Level and Glyphosate Excretion in Urine
Document No.	J. Clin. Toxicol. (2014), Vol. 4, No. 2
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

The present study was initiated to investigate the influence of oral application of charcoal, sauerkraut juice and humic acids on chronic botulism in dairy cows. A total of 380 Schleswig Holstein cows suffering from chronic botulism were fed daily with 400 g/animal charcoal for 4 weeks (1-4 weeks of study), 200 g/animal charcoal (5-10 weeks of study), 120 g/animal humic acid (11-14s week of study), 200 g charcoal and 500 mL Sauerkraut juice/animal (13-16 weeks of study), 200 g charcoal and 100 mL Aquahumin/animal (15-18s week of study), 100 g charcoal and 50 mL Aquahumin (19-22 weeks of study) followed by 4 weeks without any supplementation. Bacteriological and immunological parameters investigated included *C. botulinum* and botulinum neurotoxins (BoNT) in faeces, *C. botulinum* ABE and CD antibodies, positive acute phase proteins (APPs) haptoglobin and LPS-binding protein (LBP) using serum ELISA, negative APP paraoxanase by its enzymatic activity and glyphosate in urine by ELISA. Neither BoNT nor *C. botulinum* was detected in feacal samples. From week six until four weeks before the end of the study, there was a significant reduction in antibody levels. All supplementation, except low doses of charcoal (200 g/animal) alone, led to a significant reduction of *C. botulinum* ABE and CD antibody levels. There also was a significant reduction of glyphosate in urine following supplementation with a combination of 200 g charcoal plus either 500 mL sauerkraut juice or humic acid. Haptoglobin, paraoxanase and LBP were significantly increased by the 24th week of the study. The positive APPs and *C. botulinum* antibodies were significant negative correlations. In conclusion, a charcoal-sauerkraut juice combination and humic acids could be used to control chronic botulism and glyphosate damage in cattle.

Materials and Methods

Animals and supplementations
A Schleswig Holstein dairy cow farm of about 380 cows with average milk production of 9000 L per year showed clinical symptoms of chronic botulism (flock stiff stilted gait, paresis, apathy, engorged veins on tarsus, positive venous pulse, mucous saliva, reduced tail tonus, small wounds in the udder region) in 10-15% of the cows and 60% of the cows suffered from Dermatitis digitalis (mortellaro). The entire animal population was involved in the various charcoal (CC) (≤ 8 mm diameter) and powdery humic acid (WH67) or sauerkraut juice (SJ) and liquid humic acid (Aquahumin) supplement. Each treatment represented 10 identical cows of the 1st, 2nd, and 3rd lactation and the dry cows group for the full time of the investigation. Their last polyvalent clostridial vaccination (Covexin, Intervet) was on 01.11.2012. The treatment regime with CC, SJ and/or humic acids was changed every 4 weeks (supplementation periods) in Table 1 and given as part of the total mixed ratio (TMR).

Table 1. Overview at the various times of supplementation. SP = Supplementation period, CC = Charcoal (Carboligni, Schottdorf, Germany), SJ = Sauerkraut juice (KronprinzKonserven, Meldorf, Germany), HA = humic acids WH67 (PharmawerkWeinböhla, Germany), AH = Aquahumin (Pharmawerk Weinböhla, Germany).

Date	SP1*	SP2	SP3	SP4	SP5	SP6	SP7
	11.11.2012-08.12.2012	9.12.2012-20.01.2013	21.01.2013–17.02.2013	18.02.2013-17.03.2013	18.03.2013-31.03.2013	01.04.2013-14.04.2013	15.04.2013-14.05.2013
Supplements	400 g CC1	200 g CC	200 g CC+ 500 mL SJ2	120 g HA3	200 g CC+100mL AH4	100 g CC+50mL AH	Without supplements

The TMR was composed of grass and maize silage (glyphosate concentration not tested), concentrated mixed feed (1.93 mg/kg glyphosate), ground grains (0.51 mg/kg glyphosate), wheat straw (0.03 mg/kg glyphosate) and alfalfa hay (0.02 mg/kg glyphosate). After 31.03.2013, 10 kg draff/cow (0.01 mg/kg glyphosate) was fed. At each sampling point, each of the 40 treated cows was evaluated for clinical symptoms (Table 2).

Table 2. Clinical estimation of cows at the various sampling points.

	06.01.2013	20.01.2013	17.02.2013	03.02.2013	03.03.2013	17.03.2013	31.03.2013	01.04.2013
Paresis	2/40	2/40	3/40	3/40	2/40	2/40	2/40	2/40
Cystitis	2/40	2/40	2/40	1/40	4/40	1/40	3/40	3/40
Diahrea	5/40	0/40	3/40	7/40	0/40	5/40	0/40	2/40
Viscus saliva	0/40	0/40	0/40	0/40	1/40	2/40	1/40	4/40
endometritis	0/40	1/40	0/40	0/40	0/40	0/40	0/40	0/40
Ataxia	0/40	0/40	0/40	0/40	0/40	0/40	0/40	1/40

Collection of samples
Blood, faeces and urine were analyzed 7 times at 4 week intervals with one exception (200 g CC over 6 weeks). Blood specimens were taken from the Vena coccygenamediana, coagulated blood centrifuged at 3000 x g for 15 min and the serum samples were stored at -20°C. Faeces were taken from Ampulla recti and spontaneous urination was sampled and stored at -20°C. All specimens were quickly cooled and sent to the laboratory.

Glyphosate testing of urine
Urine samples were diluted 1:20 with distilled water (aqua distillated, Braun, Germany) and tested for glyphosate by ELISA (Abraxis, USA) according to the manufacturer's instructions. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinsches Labor Bremen (Germany). The correlation coefficient between the two tests was 0.96 (Data not shown).

Analysis of free BoNT/A-E and C. botulinum spores in faeces

Preparation of faeces for detection of BoNT/A-E
Faecal samples were diluted 1:3 in PBS (Dulbecco, pH 7.4) containing 0.1% Triton X-100, 0.1% Tween 20 and 10 mM EDTA. The samples were thoroughly mixed and frozen at -20°C. After thawing, the diluted samples were centrifuged at 7000 g for 15 min and the clarified supernatants were analyzed with BoNT-ELISA.

Indirect detection of C. botulinum spores
Rumen fluid and faecal samples were diluted 1:10 in RCM (0.5 g in 4.5 mL), vigorously mixed, and heated at 80°C for 10 min. Samples were incubated at 37°C for 7 d under anaerobic conditions and subsequently stored at -20°C until

tested. After thawing, the sample was centrifuged at 7000 g for 15 min and the clear supernatant was analyzed for the type-specific soluble antigens of *C. botulinum* types A-E by ELISA.

BoNT-ELISA
BoNT/A-E were determined by an ELISA developed in the Institute of Bacteriology and Mycology at the University of Leipzig in Germany. The standard volume was 100 µL per well and the standard incubation condition was 1 h at room temperature (1 h at RT) on a microtiter plate shaker (400 rpm). The coating buffer was 0.1 M $NaHCO_3$ and the wash solution (WS) was 0.9% NaCl with 0.05% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). All washing steps were done in a Nunc-Immuno-Washer 12 (Nunc, Wiesbaden, Germany). After coating the ELISA wells with capture antibodies (3 mg/mL, BoNT-immunoaffinity purified-IgG from rabbits against BoNT/A-E, Institute of Bacteriology and Mycology, University of Leipzig, Germany) overnight at 4-6°C, they were incubated with 150 mL per well of 1% gelatin from cold water fish skin (Sigma-Aldrich, Taufkirchen, Germany) in 0.9% NaCl solution for 1 h at RT. The wells were washed twice with WS and loaded with the prepared faecal samples diluted 1:2 in 20 mM Tris, pH 8.0, assay buffer [adjusted with 1 M HCl] containing 0.9% NaCl, 5 mM EDTA, 1% gelatin from cold water fish skin, 0.2% bovine serum albumin, 0.1 mg/mL rabbit IgG from normal serum and 0.2% Tween 20 (chemicals from Sigma-Aldrich or Fluka, Taufkirchen, Germany). After incubation, the wells were washed five times with WS and loaded with the detection antibodies conjugated with HRP and diluted in assay buffer. *C. botulinum* types A and B were detected with 2.5 mg/mL horse $[Fab]_2$ from IgG against *C. botulinum* A and B (Novartis Vaccines and Diagnostics Co, Marburg, Germany). Types C and D were detected with 0.1 mg/mL of IgG from rabbits developed against BoNT/C and D (Institute of Bacteriology and Mycology, University of Leipzig). Type E was detected with 2.5 mg/mL IgG from horses against *C. botulinum* type E (WDT, Garbsen, Germany).
After incubation at RT, the plates were washed four times with WS and the HRP activity was determined by adding 100 µL/well of 3 mM H_2O_2 and 1 mM 3, 30, 5, 50-TMB. The substrate reaction was stopped with 1 M H_2SO_4 (50 µL/well) and the optical density (OD) was measured with an ELISA-reader at 450 nm. The sensitivity, specificity, precision, limit of detection, and range of quantification were determined previously. Cross reactivity of antibodies with *C. tetani*, *C. perfringens*, *C. sporogenes*, *C. sordellii*, *C. novyi*, *C. butyricum*, *Bacillus cereus*, *Streptococcus agalactiae*, *Streptococcus zooepidemicus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida krusei* were all negative.

Evaluation of BoNT-ELISA
The relative units (RU) were calculated from the measured OD values as follow: (sample-OD minus twice the value of the control-OD [BoNT-negative sample of bovine faeces]) multiplied by 1000 and dilution factors per minute substrate incubation time.

Analysis of C. botulinum antibodies using ELISA

Solid phase antigen for ELISAs
C. botulinum types A (7272), B (7273), C (2300), D (2301), and E (2302) obtained from the National Collection of Type Cultures (NCTC) were used for preparation of ELISA antigens. Culture supernatant from *C. sporogenes* and *C. perfringens* (Isolated and identified by the Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University) served as a control antigen to study cross reactivity. All strains were cultured in reinforced Clostridial medium (RCM; Sifin, Berlin, Germany) and incubated anaerobically at 37°C for 7 days followed by freezing at 25°C. Supernatants were checked for BoNT-type with type specific ELISA. After thawing and mixing, the culture suspension was centrifuged at 10000 g for 15 min and the clear supernatant was separated. BoNT-proteins in the supernatants were detoxified with 20 mM formaldehyde (four additions weekly) and incubated at 37°C. Active formaldehyde groups were blocked by the addition of 100 mM lysine and 100 mM glycine in 100 mM Tris/HCl (pH 8.0) solution and incubated at RT for 24 h. Complete detoxification was verified with the mouse test by Dr. F. Gessler (Miprolab, Göttingen, Germany), data not shown. The antigen preparation was washed with PBS (pH 7.4) and concentrated by ultrafiltration at a molecular weight cut-off of 50 kDa (viva- vivaspin 20, Sartorius Stedim Biotech, Göttingen, Germany). The protein concentration was measured with a spectral photometer (MBA 2000) and its integrated software (PerkineElmer, Norwalk, Connecticut, USA) and adjusted with PBS to 1 mg/mL.

Detection of IgG anti C. botulinum antibodies by ELISA
ELISA plates were coated with 100 ml/well of detoxified antigen from *C. botulinum* (1 mg/mL in 0.1 M $NaHCO_3$) and incubated overnight at 4-6°C. Coated plates were washed twice with 0.9% NaCl containing 0.05% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany) followed by 135 µL of blocking solution (1% bovine case) mixed with 15 mL diluted serum sample (1:10 in 50 mMTris buffer, pH 8, containing 0.9% NaCl, 10 mM EDTA, 1% yeast extract, 1% BSA, 20% RCM and 1% Tween 20) and incubated for 1 h at RT on a microtiter plate shaker. After washing four

times, IgG from rabbits against bovine IgG (Fc) conjugated with horse radish peroxidase (HRP) (Dianova, Hamburg, Germany) diluted 1:20000 in assay buffer (50 mM Tris pH 7.4, 0.9% NaCl, 0.2% yeast extract, 0.1% BSA, 0.1% bovine Casein, 2% RCM and 0.1% Tween 20) was added to each well and incubated 1 h at RT.

Haptoglobin analysis
The Hp concentration in blood serum was determined by ELISA as described by Schroedl et al.. Briefly, the coating antibody was IgG from rabbit anti-Hp (DAKO, Hamburg, Germany), which was diluted 1:3000. The standard was bovine plasma in which the Hp concentration was determined with a standardized colorimetric assay for bovine Hp (Tridelta Development Ltd., Greystones, Co. Wicklow, Ireland) and further checked against purified bovine Hp. The standard concentration ranged from 3 to 200 ng/mL. The samples were diluted 1:1000 and 1:50000 in assay buffer (50 mM Tris-HCl with pH 8.0, 0.15 M NaCl, 10 mM EDTA, 0.1% Tween 20 and 0.2% bovine casein, all from Sigma-Aldrich, Taufkirchen, Germany). The detection antibody was polyclonal IgG (rabbit) anti-Hp (DAKO, Hamburg, Germany) conjugated with horseradish peroxidase. The detection antibody was diluted 1:10000 in assay buffer. The detection limit, including the dilution factor of 1000, was 1 µg/mL.

LBP analysis by ELISA
The LBP coating antibody was affinity purified monoclonal IgG2a (mouse) anti-LBP-human (mAb-Abi-202) at 1.2 µg/mL. The standard LBP-range in the ELISA was 0.3 to 20 ng/mL human LBP (LBP-standard serum). The samples were diluted 1:1000 and higher. The assay buffer for dilution of the standard and plasma samples was 50 mM Tris HCl (pH 8.0), 0.15 M NaCl, 10 mM EDTA, and 0.1% Tween 20 (v/v). The detection antibody was affinity purified monoclonal IgG1 (mouse) anti-LBP-human conjugated with horseradish peroxidase (mAb-Abi-204) diluted 1:6000 in assay buffer. The two mAbs and the standard serum were provided by Prof. Ch. Schuett, Institute of Immunology, and University of Greifswald, Germany

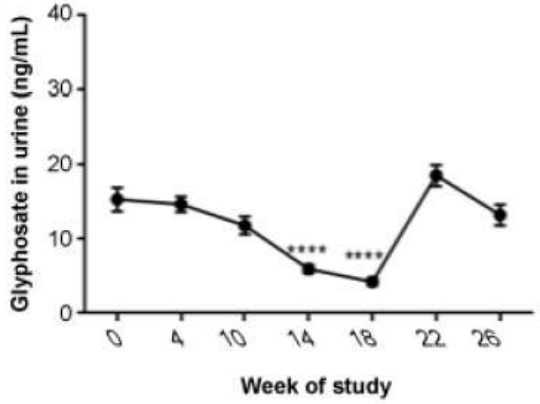
Paraoxanase analysis
Paraoxonase/arylesterase activity was measured spectrophotometrically using paranitrophenyl acetate (PNPA) as a substrate. A stock solution was prepared using 1M Hepes buffer (pH 7.5), 400 mM p-nitrophenyl acetate in DSMO and 100 mM $CaCl_2$. The working buffer contained 10 mM $CaCl_2$, 10 mM Hepes, and 2 mM p-nitrophenyl acetate in 50 mL distilled water. Blood serum specimens (25 µL) diluted 1:10 in distilled water were applied to microtiter plates and 200 µL of the working buffer were added. After 3 s shaking, the optical density was measured at 405 nm wave length (t0) and remeasured 10 min later (t1). The paraoxanase activity in U/mL is calculated with the following equation:

Paraoxonase activity = (t1-t0) x serum dilution x 1000 = (t1-t0) x 10 x 1000 = units/L

Statistical Analysis
The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). A two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results
Effect of supplementation on glyphosate in urine
A significant reduction in glyphosate excretion ($P < 0.0001$) was only seen at the 14^{th} and 18^{th} week of the study (Figure 1). The combination of CC (200 g) and SJ (500 mL) as well as HA (120 g) reduced glyphosate in urine significantly.

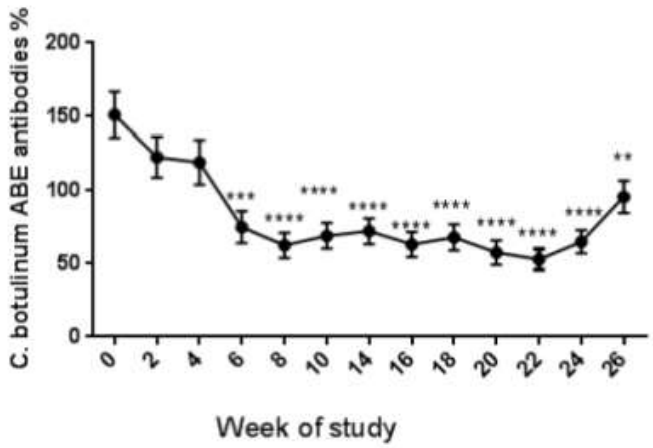
Figure 1. Dynamics of glyphosate excretion in urine with the application of 400 g charcoal daily (CC) the first four weeks (1-4 weeks) followed by 200 g CC daily for weeks 5-10, 200 g CC + 500 mL Sauerkraut juice (SJ) daily weeks 11-14, 120 g humic acid (HA) daily weeks 15-18, 200 g CC + 100 mL Aquahumin (AH) daily weeks 19-20, 100 g CC +50 mL AH weeks 21-22 and without supplementation weeks 23-26. A significant ($P < 0.0001$) reduction of glyphosate in urine was detected only in weeks 12 to 19 (4 weeks daily of 200 g CC + 500 mL SJ, 4 weeks daily of 120 g HA).



Botulinum neurotoxin (BoNT) and C. botulinum in faeces
No BoNT or *C. botulinum* was detected in feacal samples.

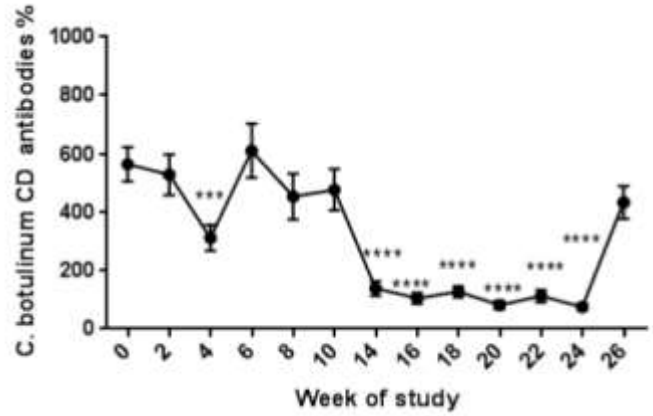
Detection of C. botulinum IgG antibodies in blood serum
The dynamic effects of different supplementations on *C. botulinum* ABE and CD blood serum antibody levels over 24 weeks are shown in Figure 2.

Figure 2. Dynamics of *C. botulinum* ABE antibodies in blood serum in relation to the daily application of 400 g charcoal (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC + 500 mL sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CC + 100 mL Aquahumin (AH) (weeks 19-20), 100 g CC + 50 mL Aquahumin (AH) (weeks 21-22), and without supplementation (weeks 23-26). There was a significant reduction of antibody levels with a daily supplementation of charcoal or humic acids beginning from week 6 ($P < 0.01$ for week 6, $P < 0.001$ for weeks 8-24, and $P < 0.05$ for week 26.



Daily supplementation with CC and/or humic acids initiated at week 6 significantly reduced antibody levels ($P < 0.01$ at week 6, $P < 0.001$ for weeks 8-24, and $P < 0.05$ for week 26). The effect of different supplements on *C. botulinum* CD blood serum antibody levels over the 26 weeks is shown in Figure 3.
Supplementation with daily 400 g CC significantly decreased CD antibody ($P < 0.01$) while a daily application of 200 g CC allowed the CD antibody level to increase. A highly significant ($P < 0.001$) reduction in CD antibody was detected only after two weeks supplementation with 200 g CC plus 500 mL SJ. Antibody reduction was constant from week 4 to 24; however, four weeks after finishing supplementation (week 26), CD antibodies increased.

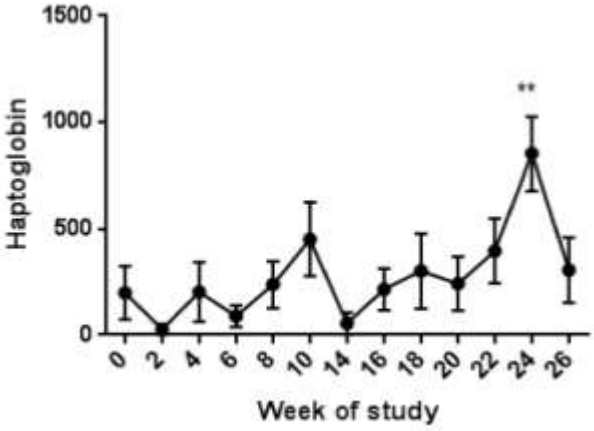
Figure 3. Effect of daily supplementation with 400 g CC (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC + 500 mL sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CC +100 mL Aquahumin (AH) (weeks 19-20), 100g CC +50 mL AH (weeks 21-22) and without supplementation (weeks 23-26) on the dynamic of *C. botulinum* CD antibodies in blood serum. There was a significant reduction in antibody levels from daily supplementation with charcoal and humicacids ($P < 0.01$ and $P < 0.001$) for weeks 14-24.



Detection of haptoglobin

Haptoglobin levels in blood serum were not significantly different with any of the supplements except for week 24 after they were taken off Aquahumin (Figure 4).

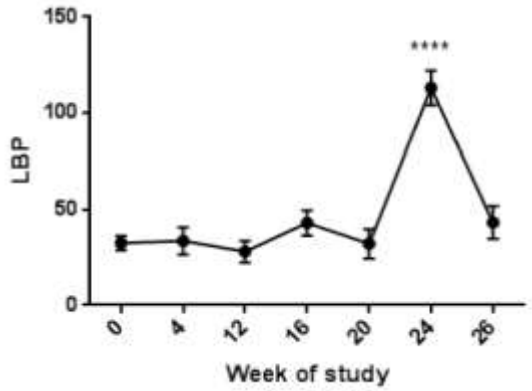
Figure 4. Haptoglobin in blood serum after the daily application of 440 g CC (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC + 500 mL sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CCl + 100 mL Aquahumin (AH) (weeks 19-20), 100 g CC + 50 mL AH (weeks 21-22) and without supplementation (weeks 23-26). A significant ($P < 0.05$) difference was only detected at week 24.



LBP results

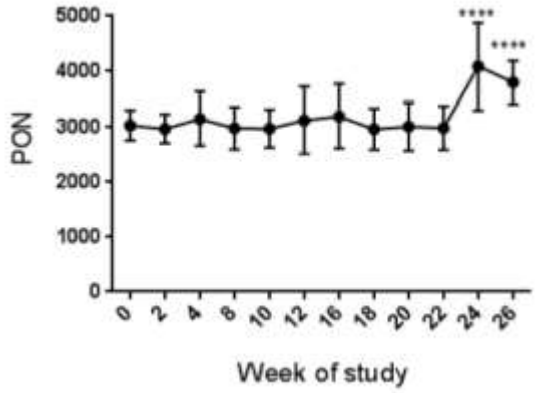
There was a significant increase in LBP in blood serum on week 20 ($P < 0.001$) (Figure 5).

Figure 5. LBP in blood serum in relation to daily oral application of 400 g charcoal (CC) (weeks 1-4), 200 g CC (weeks 5-10), 200 g CC + 500 mL sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CCl + 100 mL Aquahumin (AH) (weeks 19-20), 100 g CC + 50 mL (AH) (weeks 21-22) and without supplementation (weeks 23-26). A significant ($P < 0.001$) increase in LBP level was seen only at week 24.



Paraoxanase (PON) in blood serum
PON activity increased significantly only at 24-26 weeks ($P < 0.001$) (Figure 6).

Figure 6. Paraoxanaseactivity in blood serum in relation to daily supplementation with 400g charcoal (CC) (weeks 1-4), 200g CC (weeks 5-10), 200 g CC+500 mL sauerkraut juice (SJ) (weeks 11-14), 120 g humic acid (HA) (weeks 15-18), 200 g CC + 100 mL Aquahumin (AH) (weeks 19-20), 100 g CC + 50 mL AH (weeks 21-22) and without supplementation (weeks 23-26). Significant ($P < 0.05$) differences were only detected at weeks 24-26.



Conclusion
We investigated the effect of an oral application of CC and humic acids (HA) alone or in combination with SJ on blood serum *C. botulinum* ABE and CD antibody levels. Chronic botulism is characterized by the sub-lethal generation of *C. botulinum* progenitor toxins in the hind gut. The incorporation of the progenitor toxin and free BoNT from the gastrointestinal tract (GIT) into the body could happen via three different routes. Small concentrations of the progenitor toxin and BoNT bind with hemagglutinins (HA) or the HC part of the molecule can bind to receptors on the surface of epithelial cells and transcytosis can occur. Translocated HA disrupts the epithelial barrier. This is different with type A, B and C progenitor toxins. Type A and B HAs disrupt the epithelial cell line paracellular without causing cytotoxic effects in the epithelial cells of their susceptible hosts while type C HAs possibly evoke cytotoxic-barrier disrupting activity in the epithelial cells of susceptible animals. Damaged epithelial cells are not a barrier for progenitor toxins and BoNTs. The damaged epithelial barrier permits the toxins to be distributed throughout the body by blood and lymph vessels. Based on this knowledge, it is very important to bind these toxins with CC. The very strong reduction of CD antibodies after the daily application of 400 g of CC shows this effect. These very high CD antibody levels without the application of a CD vaccine have not been reported previously. Such high antibody levels have only been observed in conjunction with vaccination. Wang *et al.* showed good sorption of the hydrophobic herbicide terbuthylacin by CC. Maybe the hydrophobic surfactant of the commercial herbicide Roundup also could be absorbed by CC. Graber found that glyphosate can be absorbed by CC. Our results don`t support these results in animals. Four weeks daily application of 400 g CC reduced the CD antibody level dramatically (Figure 3) but did not

affect the excretion of glyphosate in urine (Figure 1). In our own investigation, we only found neutralization or absorption of a maximum of 300 µg glyphosate to 1 mg CC (data not shown). The daily application of 200 g CC in weeks 5-10 failed to reduce glyphosate excretion or *C. botulinum* type CD antibody levels. The mixed application of 200 g CC and 500 mL SJ significantly reduced the amount of glyphosate excreted and *C. botulinum* CD antibodies also significantly ($P < 0.001$) decreased (Figures 1 and 3). *C. botulinum* ABE antibodies were significantly reduced by all the treatments from week 4 on Figure 2. The application of HA (WH67) significantly ($P < 0.001$) reduced glyphosate excretion and *C. botulinum* ABE and CD antibody levels. Krüger et al. demonstrated that glyphosate reduced the *Enterococcus* spp. bacteria that are antagonistic to *C. botulinum*. Shehata et al. were able to neutralize the antibacterial activity of glyphosate with different humic acid preparations in vitro. Results from the application of 200 g CC and 100 mL Aquahumin (liquid preparation) for 2 weeks compared with 100 g CC with 50 mL Aquahumin for two weeks showed that a definite amount of these substrates is necessary to absorb or neutralize glyphosate and/or *C. botulinum* toxins. Mazzei and Piccolo found that glyphosate may spontaneously and significantly bind to soluble humic matter by non-covalent interactions at slightly acidic pH. Binding to matrices such as soluble fulvic and humic acids could be the reason. Glyphosate excretion was reduced with the soluble Aquahumin (Figure 1). It was not anticipated that the combination of 200 g CC and 500 mL SJ per day would be so very effective. Fermentation of cabbage to SJ is mostly done by *Lactobacillus plantarum* . Lactobacilli produce exopolysaccharides (EPS), homopolysaccharides and heteropolysaccharides. These biopolymers are widely distributed in nature and can be the polymers of neutral (pentoses and hexoses) or anionic sugars (hexoses). They are released into the extracellular medium by Archebacteria and Eubacteria (both Gram positive and negative). Approximately 30 species of Lactobacilli are described as EPS producers. Among them, the best known are *L. casei*, *L. acidophilus*, *L. brevis*, *L. curvatus*, *L. delbrueckii*, *L. bulgaricus*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. johnsonii*. *L. plantarum* generates heteropolymers of glucose, galactose and rhamnose. Galactose and lactose inhibit the absorption of *C. botulinum* progenitor toxins to the sugar bearing receptors on epithelial cells of the GIT. The sugar polymer concentrations in nutrient broth culture of Lactobacilli are in hundreds of mg per liter. EPS may also interact with proteins, mineral, ions and other compounds. Zhang et al. (2013) identified antioxidant effects of *L. plantarum* that may involve scavenging reactive oxygen species (ROS), up-regulation of enzymatic and non-enzymatic antioxidant activities, and reduction of lipid peroxidation. ROS and lipid peroxidation are induced by glyphosate. The neutralization of glyphosate with humic acids from WH67 was reported by Shehata et al.. The binding mechanism could be hydrogen bonding to phenolic groups of humic acid. The positive acute phase proteins (haptoglobine, LBP) only significantly increased at week 24 and by week 26, both acute phase proteins (APP) were reduced but *C. botulinum* ABE and CD antibodies increased. Inflammation indicated by the significant increase of haptoglobin ($P < 0.01$) and LBP ($P < 0.001$) may be induced by proliferation of *C. botulinum*. At week 26, when *C. botulinum* ABE and CD antibodies were high, the APPs were low. There is a negative correlation between LBP and *C. botulinum* ABE and CD antibodies ($R^2 = -0.41$ and -0.51, respectively). It is interesting that even though positive APPs increased, the negative APP paraoxanase also increased at week 24. This indicates that the anti-oxidative capacity of the cows increased, but the causes for this are unknown.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The article investigates the influence of oral administration of charcoal, humic acids and sauerkraut juice to dairy cows on *Clostridium botulinum* blood serum antibody levels and glyphosate excretion in urine. It was found that a charcoal-sauerkraut juice combination and humic acids could be used to control chronic botulism. The article might be relevant to veterinary pharmacology for control of chronic bovine botulism.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. Additionally there significant deficiencies (lack of control group, treatments). Glyphosate concentrations in urine would be highly impacted by urine volume which is affected by milk production and environmental temperature. Interestingly, aerobes from feces are tested and ruminants rely on strict anaerobes in the rumen and colon.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:

The study investigated the effect of oral administration of charcoal, humic acids and sauerkraut juice on chronic botulism in dairy cows. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse

effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.4. Study 4

1. Information on the study

Data point:	CA 6.4
Report author	Nielsen L. N. *et al.*
Report year	2018
Report title	Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels
Document No	Environmental Pollution (2018), Vol. 233, pp. 364-376
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

Recently, concerns have been raised that residues of glyphosate-based herbicides may interfere with the homeostasis of the intestinal bacterial community and thereby affect the health of humans or animals. The biochemical pathway for aromatic amino acid synthesis (Shikimate pathway), which is specifically inhibited by glyphosate, is shared by plants and numerous bacterial species. Several in vitro studies have shown that various groups of intestinal bacteria may be differently affected by glyphosate. Here, we present results from an animal exposure trial combining deep 16S rRNA gene sequencing of the bacterial community with liquid chromatography mass spectrometry (LC-MS) based metabolic profiling of aromatic amino acids and their downstream metabolites.

Materials and methods

Bacterial strains

Bacterial strains used in this study are listed in **Table 1**. All strains were grown in brain heart infusion broth (BHI) (Oxoid) or reinforced clostridial medium (RCM). *Escherichia coli* ATCC 25922 was additionally grown in AB minimal medium containing 2.5 mg thiamine/mL and 0.5% glucose (ABTG) to allow investigation of the effects of aromatic amino acids in the growth media.

Table 1. Minimal inhibitory concentration (MIC) towards glyphosate (Glyfonova® 450 PLUS) for selected bacterial strains grown in rich nutrient broth.

Species	Cell type	MIC (mg/mL) BHI	MIC (mg/mL) RCM
Bifidobacterium adolensis DSM 20083	Gram +	10	20
Bifidobacterium bifidum DSM 20456	Gram +	10	20
Bifidobacterium breve DSM 20091	Gram +	10	20
Bifidobacterium longum subsp. infantis DSM 20088	Gram +	10	20
Bifidobacterium animalis DSM 10140	Gram +	10	20
Bifidobacterium animalis lactis BL-04	Gram +	10	20
Clostridium perfringens CCUG 1795	Gram +	10	20
Clostridium leptum DSM 753	Gram +	10	20
Clostridium nexile DSM 1787	Gram +	10	20
Enterococcus faecalis ATCC 29212	Gram +	80	40
Enterococcus faecalis DSM 2570	Gram +	80	40
Lactobacillus johnsonii DSM 10533	Gram +	20	20
Lactobacillus planetarum DSM 20174	Gram +	40	40
Lactobacillus reuteri DSM 20016	Gram +	40	40
Lactobacillus rhamnosus ATCC 53103	Gram +	40	40
Bacteroides uniformis DSM 6597	Gram -	5	10
Bacteroides vulgatus DSM 1447	Gram -	5	20
Bacteroides ovatus DSM 1896	Gram -	10	20
Bacteroides fragiles DSM 2151	Gram -	5	40
Escherichia coli ATCC 25922	Gram -	80	20
Escherichia coli DSM 18039	Gram -	80	20
Akkermansia muciniphila DSM 22959	Gram -	20	20

BHI: Brain Heart Infusion broth, RCM: Reinforced clostridial medium.

Chemicals
Glyphosate was used in the formulations; Glyphosate (N-phosphonomethyl)glycine (Sigma-Aldrich 1071-83-6), Glyphosate salt N-(Phosphonomethyl)glycine with monoisopropylamine as counter-ion (Sigma-Aldrich 38641-94-0), Glyfonova® 450 Plus (450 g/L glyphosate acid equivalent) (kind gift from FMC Corporation, previously Cheminova A/S) and Roundup® Garden (120 g/L glyphosate equivalent) (Monsanto). Underlined names are used henceforth. For the aromatic amino acid analysis, LC-MS grade acetonitrile, methanol, ammonium hydroxide and formic acid were obtained from Merck (Darmstadt, Germany). All aqueous solutions for LC-MS analysis were prepared using ultrapure water obtained from a MilliQ Gradient A10 system (Millipore, Bedford, MA). Authentic aromatic amino acid compounds including L-Tyrosine, L-Tryptophan and L-Phenylalanine were obtained from Sigma-Aldrich. Aromatic amino acid internal standards (L-Phenylalanine (ring-d5, 98%), L-Tyrosine (ring-d4, 98%), L-Tryptophan (indole-d5, 98%) and indoleacetic acid (2,2-d2, 96%)) of the highest purity grade available were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). For the Glyphosate and AMPA analysis: Ammonium acetate, AMPA, ammonia solution 25%, and HPLC-MS grade water were obtained from Sigma-Aldrich (St. Louis, MO). Direct labelled internal standards, glyphosate-2-^{13}C and glyphosate (^{13}C, 99%; ^{15}N, 98%; methylene-D_2,98%), were obtained from Sigma Aldrich and Cambridge Isotope Laboratories Inc. (Andover, MA) respectively.

Minimal inhibitory concentration
The broth dilution method was used to determine the lowest concentration of glyphosate that inhibited growth under anaerobic conditions. Working solutions of the pesticides were prepared in broth and 100 mL of two-fold dilution series were distributed in 96-well flat bottom microtiter plates (Nunc, ThermoFisher). Subsequently, 100 mL aliquots of the CFU-adjusted bacterial suspensions were transferred to the wells. Positive and negative controls were included in each experiment. Plates were incubated under anaerobic conditions (80% N_2,10% CO_2 and 10% H_2) at 37°C and inspected after 24, 48 and 72 h. The MIC-value for each bacterial strain was defined as the lowest concentration of the challenge pesticide formulation giving rise to no visible growth. All experiments were performed in triplicates and repeated twice.

Bacterial growth
Growth experiments were performed with *E. coli* ATCC 25922 in minimal media (ABTG). Glyphosate, glyphosate salt, Glyfonova® or Roundup® were added to wells to a final concentrations of 0.04 mg/mL, 0.08 mg/mL and 0.16 mg/mL of the active compound. A mixture of the three aromatic amino acids phenylalanine, tyrosine and tryptophan was added to the ABTG minimal growth media to obtain final concentrations of 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL,10 mg/mL and 100 mg/mL for all three amino acids. Growth experiments were performed in triplicates and repeated twice.

Ethical statement
Animal experiments were carried out at the DTU National Food Institute (Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate with authorization number 2012-15-0201-00553 C2. Experiments were overseen by the National Food Institute in-house Animal Welfare Committee for animal care and use.

Animals, housing and experimental design
4-week old male Sprague Dawley rats (n = 80) were purchased from Taconic Biosciences (Lille Skensved, Denmark). Animals had access to *ad libitum* water and feed (Altromin 1324; Altromin Spezialfutter, Lage, Germany) throughout the experiment and were housed under controlled environmental conditions (12-h light/dark cycles, temperature 21.5 ± 0.3 C, relative humidity 51.3 ± 3.1%, 8-10 air changes per hour). Animal weight was recorded daily during the intervention period. Upon arrival animals were randomly caged in pairs and acclimatized for 7 days before initiation of the intervention, at which point cages were evenly allocated into four treatment groups based on weight. During the 2-week intervention period animals received water (CTR), glyphosate 2.5 mg/kg/day (GLY5), glyphosate 25 mg/kg/day (GLY50) or Glyfonova® 25 mg/kg/day glyphosate acid equivalent (NOVA) by oral gavage (**Figure 2A**). The pH of the glyphosate solutions were adjusted from pH ≈ 2 to pH = 5 using NaOH, as toxicity of glyphosate-based formulations has been found to be influenced by pH and to minimize any direct effects caused by low pH. Fecal pellets were collected directly from individual rats before the first treatment and immediately frozen at -80°C. Following the 2-week intervention period, all animals were euthanized by CO_2/O_2 sedation and decapitation, and blood was taken directly for serum preparation. One animal from each cage (n = 40) was dissected and inspected for abnormalities. The entire cecum was weighed, and multiple samples of intestinal content from the ileum, cecum and colon were taken and either snap-frozen in liquid N_2 or processed as described below for amino acid profiling.

Bacterial community composition

Bacterial Community DNA was extracted from 250 mg fecal samples collected on the initial day of intervention and lumen content of ileum, colon and cecum from the day of dissection using the MoBio PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's recommendations, including bead beating at 30 cycles/s for 10 min (Retsch MM 300 mixer mill). Total DNA concentrations were measured with the Qubit dsDNA HS kit (Life Technologies). The bacterial community composition was determined by sequencing of the hypervariable V3-region of the 16S rRNA gene in the extracted bacterial DNA. The DNA concentrations were measured using Qubit dsDNA HS assay (Invitrogen) and samples were pooled in equimolar concentrations to obtain two separate libraries containing samples originating from either ileum and cecum or colon and feces (before intervention). Prior to DNA extraction and library preparation, samples were randomized between treatment groups. Sequencing was performed by the DTU in-house facility (DTU Multi-Assay Core (DMAC). Taxonomy was assigned using the Ribosomal Database Project multiclassifier version 2.10.1 and the RDP database with confidence threshold set to 0.5 as recommended for sequences shorter than 250bp. Further downstream processing was performed in QIIME. A phylogenetic tree was generated (make_phylogeny.py) and rooted to an Archeae species following alignment of all OTUs. The OTU table was filtered to include only OTUs assigned as bacteria, excluding the Cyanobacteria/Chloroplast group and OTUs with average relative abundance below 0.005% of the total community, resulting in a total of 547 OTUs.

Short chain fatty acid (SCFA) content and pH of intestinal sample
Cecum content from each group of animals was analysed for acetic acid, propionic acid, butyric acid and valeric acid by GC-MS (MS-Omics, Denmark). Raw GC-MS data were processed with software based on the PARAFAC2 model and quantified values are calculated assuming that 1 mg feces corresponds to a volume of 1 mL.

Glyphosate and Aminomethylphosphonic acid (AMPA) in intestinal samples
Glyphosate and AMPA calibration standards were prepared in HPLC grade water at 1 mg/mL and dilutions hereof. Approximately 0.2 g of intestinal sample was transferred into a plastic vial, weighed and diluted with 10 mL of HPLC-grade water. The mixture was homogenized by vortexing, vigorously hand-mixing and sonication for 15 min. Subsequently vials were centrifuged for 20 min at 6000 rpm and 1 mL of supernatant was transferred to an HPLC vial. A total of 50 mL of stable isotope labelled internal standard solutions (10 mg/mL of each) were added to each sample and to calibration standards of glyphosate and AMPA. Intestinal samples were analysed in separated batches for ileum, cecum and colon samples. The mass spectrometer was an API 4000 (ABSciex, Framingham, MA, USA) and was operated with an ESI source in positive mode which was set at 400°C with a capillary voltage of 5500 V. Glyphosate and AMPA were detected and quantified in MRM mode (multi reaction monitoring). Labelled internal standards were used for quantification. The obtained data was treated with Analyst 1.6 Software. The calibration curves were established by plotting the peak area ratios between analytes and internal standards against the concentrations of the calibration standards. The calibration curves were fitted to a linear regression with weighting factor of 1/x. The correlation coefficient was above 0.99 for all regressions.

Aromatic amino acids and metabolite profiling
Stock solutions (1 mg/mL) of 23 aromatic amino acids and derivatives as well as four internal standards (IS) were individually prepared from their authentic compounds in water, methanol or 50% methanol. During dissection of animals (n = 40), intestinal content of ileum, cecum and colon (200-500 mg) was immediately diluted 1:2 with sterile milliQ water, vortexed for 10 s and centrifuged twice at 16,000 g, 4 °C for 5 and 10 min respectively with transfer of supernatant between steps. Finally, an aliquot of 300 mL was stored at -20 °C until analysis. For analysis, samples were thawed at 4 °C, centrifuged at 16,000 g, 4 °C for 5 min, and the supernatants were diluted in a total volume of 80 mL water corresponding to a 1:100 dilution of ileum content and a 1:5 dilution of cecum or colon content. Intestinal samples from each compartment were analysed separately in random order with a quality control (QC) sample of the given compartment injected before and after every ten samples throughout the analysis. The five standard mix solutions were also analysed once for every 10 samples. For each sample, a volume of 2 mL was injected into an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) system consisting of Dionex Ultimate 3000 RS liquid chromatograph (Thermo Scientific, CA, USA) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray interphase (Bruker Daltonics, Bremen, Germany) operating in positive mode. Data were processed using QuantAnalysis version 2.2 (Bruker Daltonics, Bremen, Germany) and bracket calibration curves for every 10 intestinal samples were obtained for each compound. The calibration curves were established by plotting the peak area ratios of all of the analytes with respect to the IS against the concentrations of the calibration standards. The calibration curves were fitted to quadratic regression with weighting factor of the reciprocal of the squared concentration (1/x2). The correlation coefficient was above 0.98 for all regressions. In ileum samples, only phenylalanine, tryptophan and tyrosine were quantified.

Analysis of host responses

The acute phase protein, haptoglobin, was measured in blood serum in random order using "PHASE"™ Haptoglobin Assay (Tridelta, Kildare, Ireland) according to the manufacturer's recommendations. The level of IL-6 in blood serum was quantified in random order using a specific sandwich ELISA kit (rat) (Cusabio Biotech) and a microplate reader (BioTek) at 450 nm, with a range of detection between 0.312 pg/mL and 20 pg/mL according to the manufacturer's recommendations.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.01 (GraphPad Software, Inc. CA) or R (version 3.1.0) (R Core Team, 2013). Differences between treatment groups were assessed by use of un-paired t-tests or non-parametric MannWhitney tests, if variances were found to be different. Differences between intestinal compartments were assessed by use of paired t-tests or Wilcoxon tests as appropriate. Correlation analysis was performed using Spearman's rank test. For all statistical analysis p-values less than 0.05 were considered significant. For multiple comparisons of bacterial groups at the genus level permutation based t-tests were applied. For assessing differences between aromatic amino acid metabolites in the treatment groups, KruskalWallis one-way ANOVA with Dunn's multiple comparison posttest was used. In both of these cases, correction for multiple testing was applied using the false discovery rate method with a threshold of $q < 0.05$ (Pike, 2011). The metabolite data were imported into LatentiX (version 2.11) (Latent5) for principle component analysis (PCA) to assess the quality of the data. QC samples clustered tightly in PCA score plots indicating a stable system.

Results

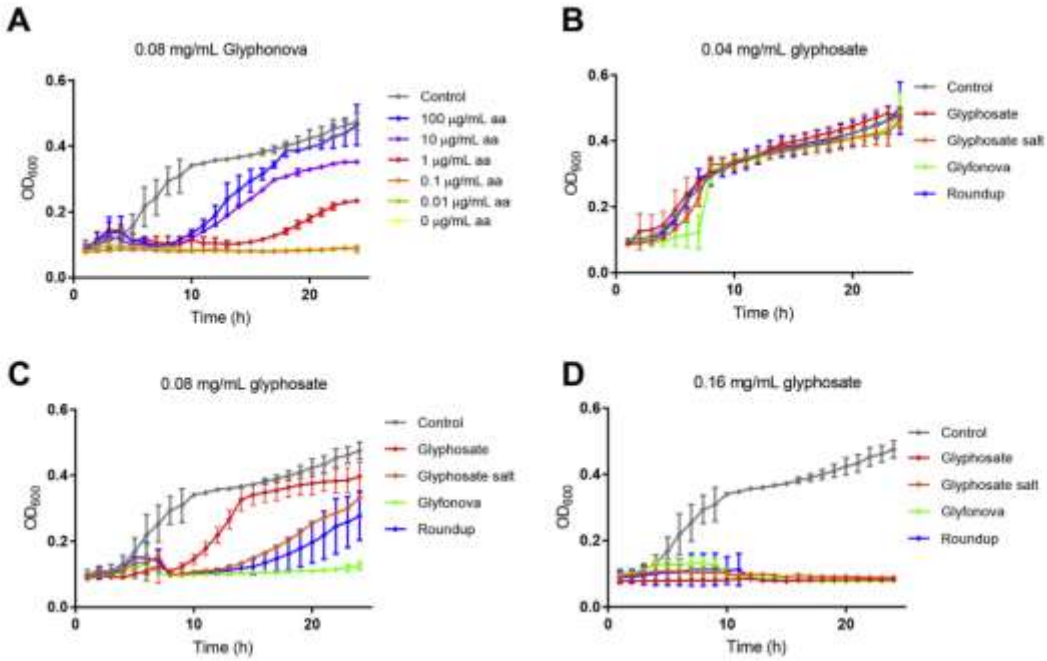
Minimal inhibitory concentrations

Minimal inhibitory concentrations (MIC) were determined for a selected group of bacteria representative of the gut microbiota in humans. Two different nutrient rich media, namely BHI and RCM, were chosen that both supported growth of all the included strains under identical growth conditions. The commercial formulation Glyfonova® was used as test compound because of its high solubility compared to glyphosate. Overall, all bacterial species tested showed very high MIC values in both growth media ranging between 5 mg/mL to 80 mg/mL, although some variability between bacteria and media was found (**Table 1**).

Importance of aromatic amino acids

Because glyphosate inhibits aromatic amino acid synthesis, we investigated the importance of bioavailable aromatic amino acids in the growth media by determining the MIC value for *E. coli* ATCC 25922 in ABTG minimal growth medium with or without supplementation of a mixture of phenylalanine, tyrosine and tryptophan. With no added aromatic amino acids a MIC of 0.08 mg/mL towards Glyfonova® was determined. Supplementation of the growth medium with either 50 mg/mL or 500 mg/mL of tryptophan, phenylalanine and tyrosine increased the MIC to 10 mg/mL and 20 mg/mL, respectively. To further investigate the importance of aromatic amino acids in the growth medium, 24-h growth experiments were set up with *E. coli* ATCC 25922 grown anaerobically in minimal medium containing 0.08 mg/mL Glyfonova® (equal to the MIC in ABTG medium without added amino acids) supplemented with 0 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL or 100 mg/mL aromatic amino acid mix. Bacterial growth was determined as OD600 every hour (Figure. 1A). The results revealed a clear dose-dependent alleviation of the inhibitory effect of Glyfonova® with aromatic amino acid supplementation, however even at the highest concentration a noticeable lag-phase before growth initiation was observed compared to the control group (no Glyfonova®) although the maximum growth rates (slope) were comparable. A concentration between 0.1 mg/mL and 1 mg/mL aromatic amino acid mix was required to allow growth of *E. coli* ATCC 25922 (Figure. 1A).

Figure. 1. (A) *Escherichia coli* ATCC 25922 grown in ABTG minimal medium containing 0.08 mg/mL Glyfonova®450 Plus supplemented with a mix of three aromatic amino acids (tyrosine, phenylalanine and tryptophan) at different concentrations. The control contains neither Glyfonova® nor amino acids. (BeD) Effect of glyphosate, glyphosateisopropylamine salt, Glyfonova®450 Plus and Roundup® on *E. coli* ATCC 25922 growth in ABTG minimal medium supplemented with (B) 0.04 mg/mL, (C) 0.08 mg/mL and (D) 0.16 mg/mL active compound. Data are presented as means with error bars showing SD.



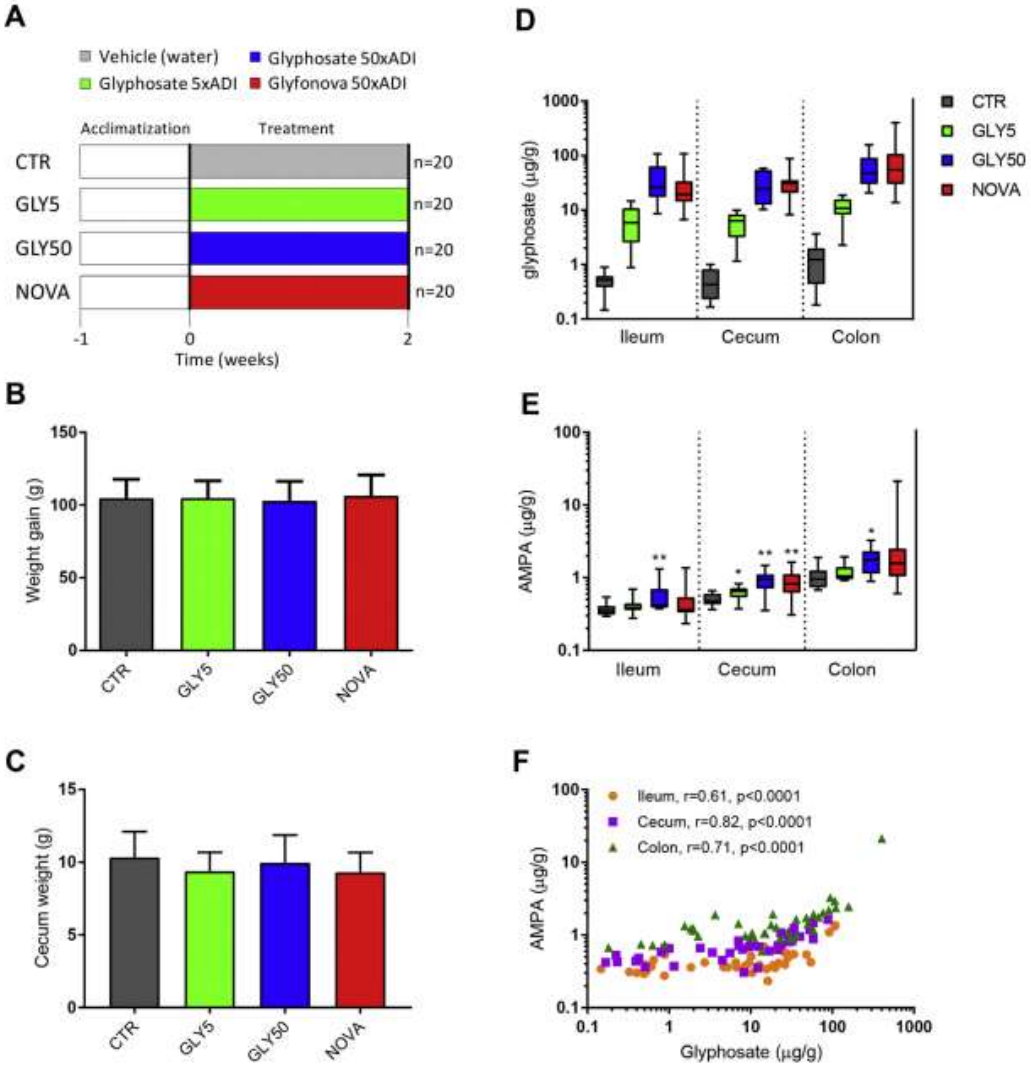
Impact of different glyphosate formulations on growth inhibition

The 24-h growth experiments of *E. coli* ATCC 25922 in minimal ABTG medium with different glyphosate formulations in the absence of aromatic amino acids showed, that Glyfonova® had the strongest effect on growth, with an apparent slight lag-phase observed in the presence of 0.04 mg/mL (Figure 1B) and a complete inhibition of growth during 24-h at the established MIC of 0.08 mg/mL (Figure 1C). At a concentration of 0.08 mg/mL all other formulations of glyphosate resulted in growth of the *E. coli* strain during the 24-h period after an initial lag-phase compared to the control (Figure 1C). The active compound in its pure form (Glyphosate) had a significantly lower impact on growth than the three other formulations as determined by the area under the curve (0.08 mg/mL glyphosate) during 24-h of growth ($p < 0.01$). All the tested formulations inhibited *E. coli* ATCC 25922 completely at a concentration of 0.16 mg/mL.

Effect of glyphosate on short-term weight gain in rats

After two weeks of exposure to Glyphosate or Glyfonova® (Figure 2A), no significant differences in rat body weight gain during the intervention period, or in cecum weight at termination, were observed between any of the treatment groups as compared to the control group (Figure 2B and C).

Figure 2. (A) Study design. Animals were caged in pairs and acclimatized for one week before receiving glyphosate, either in pure form or as a commercial formulation, by oral gavage daily for a period of two weeks. Fecal samples were taken before the first treatment and intestinal and blood samples were collected at termination. (B) Weight gain of rats during the treatment period. (C) Weight of cecum with content at termination. (D) Glyphosate and (E) AMPA concentrations (mg/g) in ileum, cecum and colon. (F) Spearman correlation analysis between glyphosate and AMPA concentrations in ileum, cecum and colon. For each compartment the Spearman r and p-value is shown. Data in (BeC) are presented as means with error bars showing SD. Data in (DeE) are presented as box-plots with whiskers showing the total range.



Levels of glyphosate and AMPA in ileum, cecum and colon

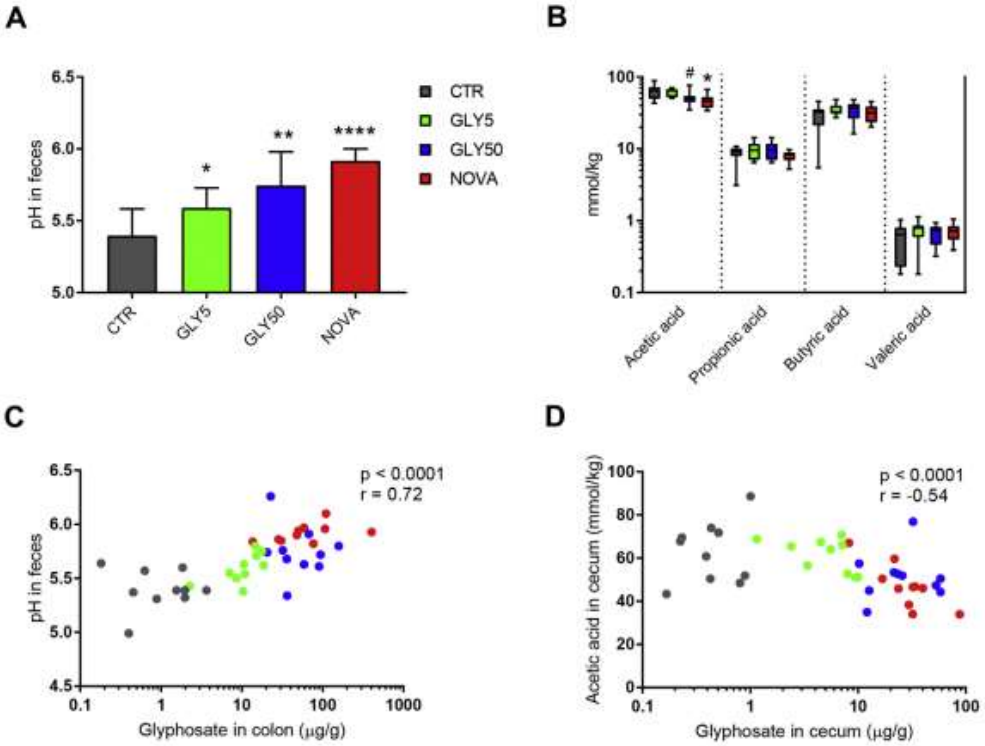
A clear dose-dependent relation between the concentration of orally administered glyphosate and detected levels of glyphosate in all three intestinal compartments was observed, with the colon compartment containing the highest concentration of glyphosate ($p < 0.0001$) compared to both cecum and ileum (Fig. 2D). The detection of both glyphosate and AMPA in intestinal samples from animals in the control group was explained by low residues being present in the standard animal feed (Glyphosate: 1.0 ± 0.03 mg/g and AMPA: 0.72 ± 0.12 mg/g). The primary degradation product of glyphosate, AMPA, was detected at levels above the background levels (CTR group) in all three compartments, but significant differences for the lowest treatment group (GLY5) were only detected in the cecum (Fig. 2E). Overall, the concentration of AMPA was highest in the colon, followed by cecum and then ileum ($p < 0.0001$ for all pair-wise comparisons). Significant correlations were found between the level of glyphosate and the concentration of AMPA in the same animal in all three compartments ($p < 0.0001$), and the AMPA to glyphosate ratio appeared to increase through the intestinal tract from the ileum through cecum to the colon (Figure 2F).

Fecal pH and levels of short chain fatty acids in the cecum

The pH in feces was measured in samples taken on the last day of the intervention period and showed a significantly

higher pH in all three treatment groups compared to the CTR group (Figure 3A). Concentrations of the SCFAs acetic acid, propionic acid, butyric acid and valeric acid were determined in cecum (Figure 3B). The level of acetic acid was significantly lower ($p = 0.01$) in animals in the NOVA group compared to CTR, and the same tendency was also noted for the GLY50 group ($p = 0.06$). A strong positive correlation was found between colonic levels of glyphosate and fecal pH (Figure 3C), whereas a weaker positive correlation was found between colonic levels of AMPA and fecal pH. Conversely, a negative correlation between cecal glyphosate and cecal acetic acid was found ($r = -0.54$, $P < 0.0001$) (Figure 3D), which was not found for the other SCFAs. Additionally, fecal pH and the concentration of acetic acid in cecum correlated negatively ($r = -0.58$, $p < 0.0001$).

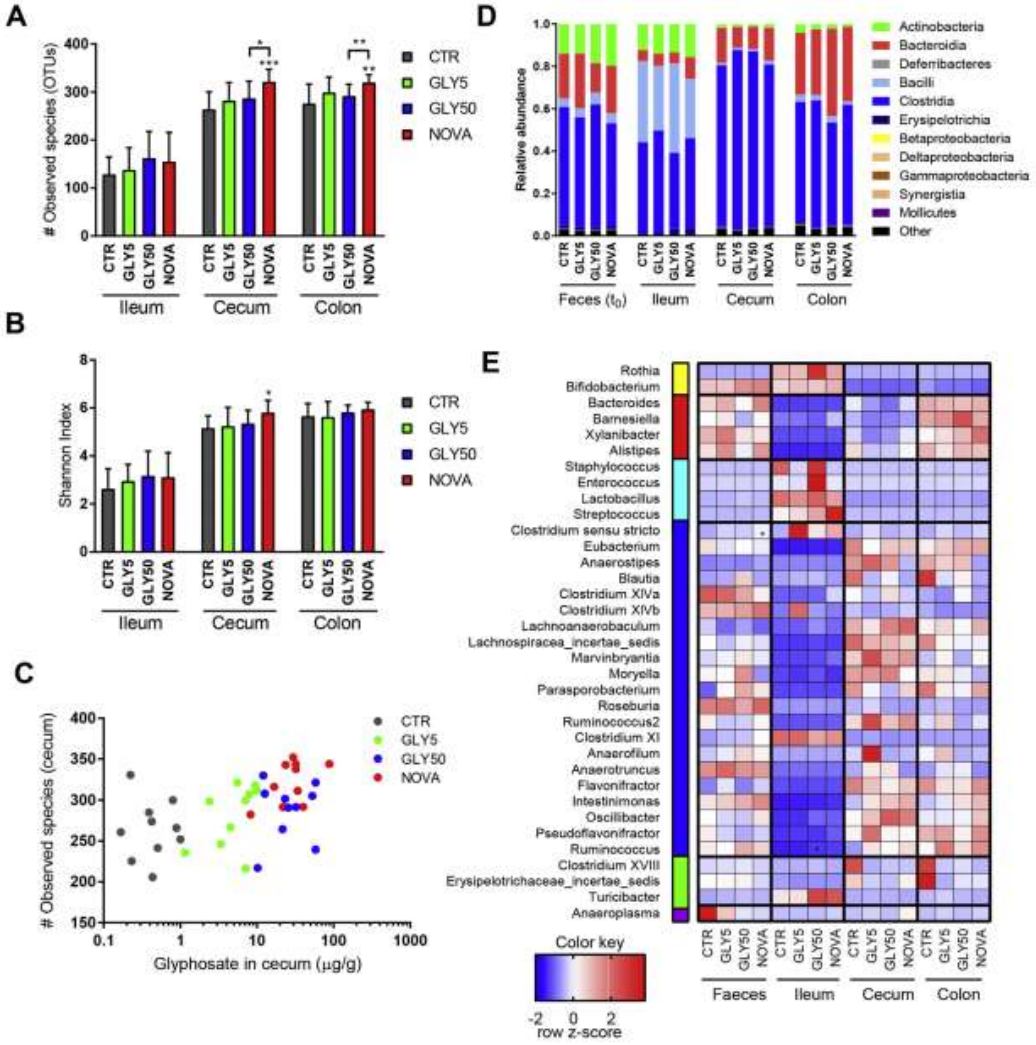
Figure 3. (A) Fecal pH after the intervention period presented as means with error bars showing SD. (B) Concentration in cecum of the SCFAs; acetic acid, propionic acid, butyric acid and valeric acid presented as box-plots with whiskers showing the total range. Spearman correlation analysis between (C) colonic glyphosate concentration and fecal pH and (D) glyphosate and acetic acid concentration in the cecum. In panels (AeB) *p < 0.05; **p < 0.01; ****p < 0.0001; #p < 0.1. For correlations (CeD) the p-value and Spearman r is shown.



Changes in bacterial diversity and community composition following glyphosate exposure

In all groups of animals, both the number of observed species (OTUs) and the Shannon diversity index were lower in the ileum as compared to the cecum and colon (Figure 4A and B). A significantly higher number of OTUs was found in the cecum and colon of the NOVA group as compared to the control group (Figure 4A). In the cecum, also a higher Shannon diversity index was found in the NOVA group (Figure 4B). A significant difference in numbers of observed species between the GLY50 and NOVA groups, both treated with the same concentration of active compound, was also noted in both the cecum and the colon (Fig. 4A) and also no effect of the active compound (GLY5 and GLY50 groups) on alpha diversity was observed based on *in situ* measurements of glyphosate (Figure 4C). Overall, the bacterial communities in all compartments were dominated by classes within the phyla Firmicutes, Bacteroidetes and Actinobacteria.

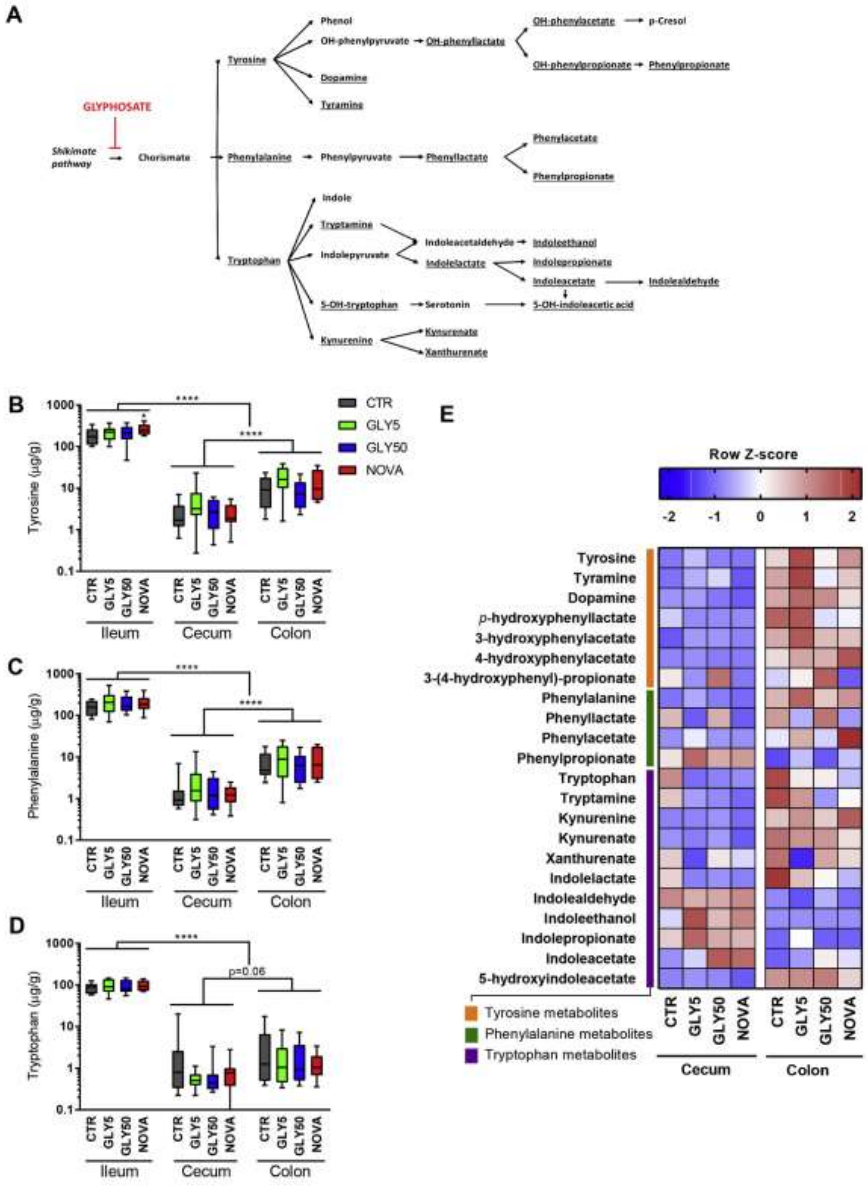
Figure 4. (A) Number of observed species (OTUs) and (B) Shannon diversity index for each group and compartment are shown as mean with error bars showing SD. In panels (AeB) *p < 0.05; **p < 0.01; ***p < 0.001. (C) Correlation analysis between glyphosate concentration and number of observed species in cecum. (D) The mean bacterial composition at the class level in feces (t0: Before intervention), ileum, cecum and colon. Bacterial classes with prevalence less than 25% across all samples and bacterial groups not classified to at least the class level were aggregated into the category: Other. (E) Heat-map showing average relative abundance of genera for each group of animals in feces (before intervention), ileum, cecum and colon, respectively. Colours indicate row z-scores. The bacterial genera shown represent above 1% of the community in any single sample and are present in at least 25% of all samples. Significant differences between the control group and the treated groups are shown as *q < 0.05 (FDR corrected permutation based t-test). The left-hand colour bar shows the classification of bacterial genera (Yellow: Actinobacteria, Red: Bacteroidetes, Cyan: Bacilli (Firmicutes), Blue: Clostridia (Firmicutes), green: Erysipelotrichia (Firmicutes) and purple: Tenericutes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Aromatic amino acids and metabolic profiling

Since glyphosate is known to inhibit the Shikimate pathway, the colonic levels of the three aromatic amino acids (tyrosine, phenylalanine and tryptophan) as well as their derivatives were measured (Figure 5A). The highest levels of all three aromatic amino acids were found in the ileum irrespective of treatment group (Figure 5B-D). Both tyrosine and phenylalanine concentrations were significantly lower in the cecum than in the colon, and a tendency for this was also seen for tryptophan (Figure 5B-D). A single significant difference in levels of aromatic amino acids was found for tyrosine in the ileum of the NOVA group (but not the GLY50 group) as compared to the CTR group. No differences between treatment groups and the control group were found for any of the downstream metabolites determined in cecum and colon after correcting for multiple testing (Figure 5E).

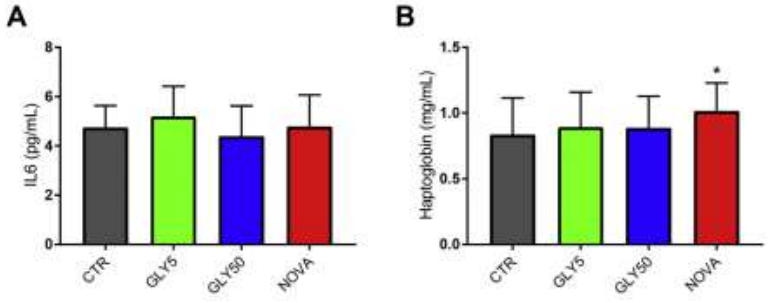
Figure 5. (A) Outline of how glyphosate potentially affects the catabolism of aromatic amino acids in the intestine. Underlined metabolites are those targeted in the present study.Concentrations of (B) tyrosine, (C) phenylalanine and (D) tryptophan in ileum, cecum and colon in treatment groups CTR, GLY5, GLY50 and NOVA are shown. Data are presented as box-plots with whiskers showing full range. In panels (BeD) *p < 0.05; ****p < 0.0001. (E) Heatmap showing mean concentration (z-score) of aromatic amino acids and derivatives in cecum and colon, collectively. No differences between the CTR group and the treatment groups were found for any of the metabolites after correcting for multiple testing.



Haptoglobin and IL-6 levels in blood serum

No differences were found in levels of serum IL-6 in any of the treatment groups as compared to the CTR group (Figure 6A). Serum levels of the acute phase protein haptoglobin were significantly higher in the NOVA group as compared to the CTR group (Figure 6B).

Figure 6. Concentration of (A) IL-6 and (B) haptoglobin in blood serum from rats in treatment groups CTR, GLY5, GLY50 and NOVA. Data are presented as means with error bars showing SD. *p < 0.05.



Conclusion

In conclusion, the authors have shown that pure glyphosate and the tested commercial formulation Glyfonova® had very limited effects on the gut microbial community composition in rats during a 2-week oral exposure trial at a concentration of 50x ADI for humans. This is likely to be explained by sufficient bioavailability of aromatic amino acids in the gut environment, alleviating the effect of glyphosate blocking the Shikimate pathway. However, in cases of human malnutrition or in subjects consuming special (e.g. low protein) diets that may cause lower levels of available amino acids in the gut, a detrimental effect of glyphosate cannot be excluded.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The study investigated the effect of glyphosate to intestinal bacteria of Sprague Dawley rats, which were orally dosed with glyphosate or formulation Glyfonova®. Effects found were discussed as limited, which was explained by sufficient bioavailability of aromatic amino acids in the gut.

Vertebrates are usually not orally exposed to formulated product. The effect of formulation components cannot be differentiated from the effect of the active substance.

However, after 2 weeks of excessive exposure of up to 50x ADI, effects on the gut microbial community were limited. Apparently, intestinal microbial composition tolerates exposure of glyphosate at 50x ADI.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:

In this public article, the effect of glyphosate on the gut microbiota of rats has been investigated. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.5. Study 5

1. Information on the study

Data point:	KCA 6.4
Report author	Riede S. *et al.*
Report year	2016
Report title	Investigations on the possible impact of a glyphosate-containing herbicide on ruminal metabolism and bacteria *in vitro* by means of the 'Rumen Simulation Technique'
Document No.	Journal of Applied Microbiology (2016), Vol. 121, pp. 644-656
Guidelines followed in study	None
Deviations from current test guideline	Not applicable

GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9):	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

This study was performed in a well-established *in vitro* model to investigate whether the application of a glyphosate-containing herbicide might affect the bacterial communities and some biochemical parameters in a cow's rumen.
The test item was applied in two concentrations for 5 days. In a second trial, fermentation vessels were inoculated with *Clostridium sporogenes* before the high dose was applied. Effluents were analysed by biochemical, microbiological and genetic methods. A marginal increase in short-chain fatty acid production and a reduction in NH_3-N were observed. There were minor and rather equivocal changes in the composition of ruminal bacteria but no indications of a shift towards a more frequent abundance of pathogenic Clostridia species. *Clostridium sporogenes* counts declined consistently.
No adverse effects of the herbicide on ruminal metabolism or composition of the bacterial communities could be detected. In particular, there was no evidence of a suspected stimulation of Clostridia growth.
Antibiotic activity of glyphosate resulting in microbial imbalances has been postulated. In this exploratory study, however, intraruminal application of concentrations reflecting potential exposure of dairy cows or beef cattle did not exhibit significant effects on bacterial communities in a complex *in vitro* system. The low number of replicates (n = 3/dose) may leave some uncertainty.

Materials and methods

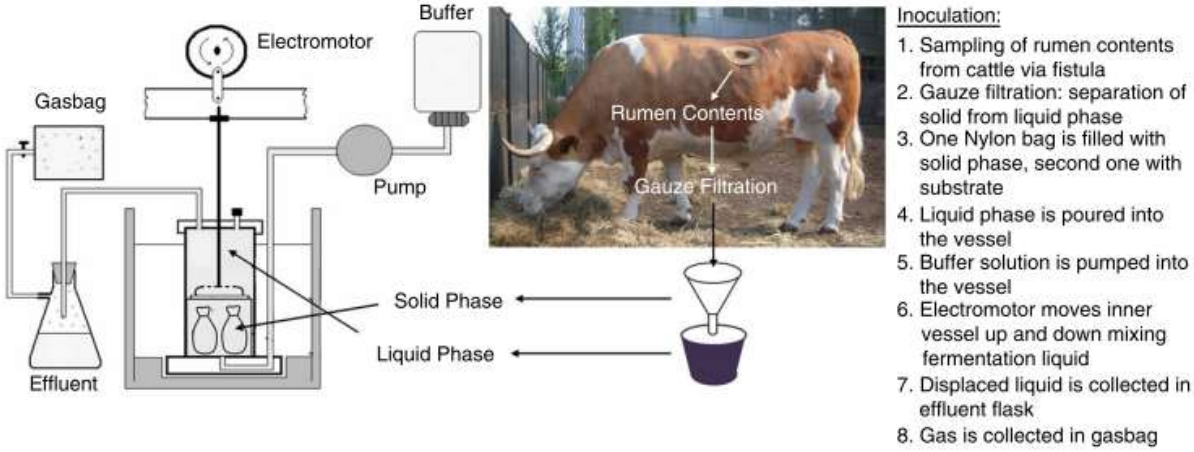
Test material

The glyphosate-based herbicide (GBH) Plantaclean® 360 (Plantan GmbH, Buchholz, Germany) served as test material. It is a commercially available, water-soluble concentrate containing 486 g glyphosate isopropylamine salt (equivalent to 360 g glyphosate acid) per litre.

In vitro experiments

The RUSITEC experiments were carried out similar as described by Czerkawski and Breckenridge (1977). Two trials (A, B) were conducted with six fermentation vessels (V = 700 mL) inoculated with rumen contents collected in the morning (approx. 3 h after feeding) from a ruminal-fistulated nonlactating cow (3 years of age, 450 kg body weight) that had been fed 9 kg hay and 200 g concentrate (provided by DEUKA Schaffutter, Erfurt, Germany) per day. Separation of the liquid from the solid phase of the rumen content was obtained by squeezing the fresh material through gauze. Rumen contents were kept at 39°C during this process. A schematic overview of a fermentation vessel is provided in Figure 1. Starting the experiments, one nylon bag (11.5 x 6.5 cm, pore size 150 µm) was filled with the solid phase of the rumen content (70 g), another one with a total of 10 g substrate (6 g hay, 4 g concentrate, as described above) and the pure liquid phase was poured into the vessels. The next day (24 h later), the nylon bag containing the solid phase was replaced by a substrate-filled one. From that day onwards, changing of nylon bags was carried out in an alternating way with a 24 h interval, leading to a retention time of 48 h for each nylon bag in the vessel.

Figure 1. One fermentation vessel of the RUSITEC system.



Crude nutrient content of the diet is given in Table 1. The rumen liquid was analysed prior to each run (means ± SEM) for pH (6.48 ± 0.05), redox potential (-348.5 mV ± 6.5) and concentrations of acetate (77.63 mmol/L ± 8.00), propionate (22.80 mmol/L ± 2.02), isobutyrate (0.70 mmol/L ± 0.00), butyrate (10.05 mmol/L ± 0.72), isovalerate (1.60 mmol/L ± 0.00) and valerate (1.08 mmol/L ± 0.00).

Table 1. Composition of the diet fed to the donor cow and the substrate in the RUSITEC system.

Hay		Concentrate	
Ingredient	Amount (% of DM)	Ingredient	Amount (% of DM or IU kg^{-1})
Ash	7·1	Ash	0·69
Crude protein	7·1	Crude protein	19·62
Crude fat	1·5	Crude fat	4·24
Crude fibre	31·9	Crude fibre	9·48
Acid detergent fibre	36·7	Acid detergent fibre	14·94
Neutral detergent fibre	60·4	Neutral detergent fibre	30·55
Acid detergent lignin	3·1	Acid detergent lignin	5·57
Nonfibre carbohydrates	23·9	Nonfibre carbohydrates	44·9
Organic matter	84·6	Organic matter	9·31
		Calcium	1·50
		Phosphorous	0·55
		Sodium	0·25
		Vitamin A	20 000 IU kg^{-1}
		Vitamin D_3	1600 IU kg^{-1}

DM, dry matter; IU, international units.

A buffer solution (for composition, see Table 2) similar to ruminant saliva was infused continuously into the vessels to reach a liquid turnover of once a day. Effluents of the vessels were collected in glass flasks on ice with the simultaneous collection of fermentation gases in gas-tight bags (Plastigas; Linde AG, München, Germany).

Table 2. Chemical composition of the buffer solution (in mmol/L)

Ingredient	Amount
NaCl	28·00
KCl	7·69
$CaCl_2 \cdot 2H_2O$	0·22
HCl (1N)	0·50
$MgCl_2 \cdot 6H_2O$	0·63
NH_4Cl	5·00
$Na_2HPO_4 \cdot 12H_2O$	10·00
$NaH_2PO_4 \cdot H_2O$	10·00
$NaHCO_3$	97·90

In both trials (A, B), an equilibration period of 6 days (day 0 to day 6) was followed by a control (day 7 to day 11) and then by an experimental period (day 12 to day 16) of 5 days each. That way, ruminal metabolism and microbiological parameters could be compared during control and experimental period for the same vessel.

In trial A, during the experimental period the GBH was added daily to fermentation vessels in triplicates at two different concentrations. The low glyphosate dose (LG) was chosen to reflect the estimated maximum dietary glyphosate intake of dairy cattle according to model assumptions (Germany 2013), that is, 43.4 mg/kg dry mass per day. Beef cattle might ingest higher residues since their diet might contain up to 103 mg/kg dry mass. The high

glyphosate dose (HG) did not directly correspond to that dose but was higher to ensure a sufficient margin between the two concentrations. The actual average amounts of glyphosate acid in the vessels (n = 3 per dose) were analytically determined to be 0.42 mg (LG) or 2.92 mg (HG).

In trial B, on the last day of the control period (day 11), each of the six fermentation vessels was inoculated with *Cl. sporogenes* in a concentration of 10^4 CFU/mL. Thereafter, three vessels received the HG daily throughout the experimental period, whereas the other three vessels served as controls and remained untreated. The concentration of 10^4 CFU/mL was chosen to detect either an increase or a decrease in CFU of Clostridia in response to the application of GBH. For safety reasons, *Cl. sporogenes* (provided by Ripac-Labor GmbH, Potsdam-Golm, Germany) was applied as a surrogate for *Cl. botulinum* to study its growth behaviour in the *in vitro* system. *Clostridium sporogenes* and *Cl. botulinum* exhibit morphological similarity and demonstrate a high degree of relatedness but *Cl. sporogenes* is not pathogenic. Therefore, this germ may be used as a nontoxigenic alternative (Bradbury *et al.* 2012) to facilitate research under normal laboratory conditions.

Sampling and analytical procedures

Glyphosate analysis
In trial A of the RUSITEC experiments, effluents from the control (day 11) and from the experimental period (pooled sample of day 12 to day 16) were analysed for concentrations of glyphosate in a state laboratory (LAVES, Oldenburg, Germany) by means of an LC-MS/MS method (Quick Method for the Analysis of Highly Polar Pesticides in Food of Plant Origin, QuPPe, Ver. 07) to exclude or verify its presence in the test system and to determine the total recovery. The limit of detection (LOD) for glyphosate by this method is 0.05 mg/kg and the limit of quantification (LOQ) is 0.1 mg/kg.

Ruminal metabolism
In trial A, the anaerobic status of the system, pH and redox potentials were monitored daily. Concentrations of short-chain fatty acids (SCFA) and NH_3-N in effluents were measured daily and degradation of organic matter (OM) was determined every 48 h in both, the control and experimental period. In trial B, in contrast, these examinations were performed only in the control period to avoid contamination of the laboratory with *Cl. sporogenes*. The analyses for SCFA concentrations, NH_3-N concentrations and for degradation of OM were carried out as described previously (Koch *et al.* 2006; Meibaum *et al.* 2012; Riede *et al.* 2013). Daily production of SCFA was calculated by multiplying their measured concentrations by the effluent volume.

Analysis of microbial communities
Single strand conformation polymorphism (SSCP) is a simple and powerful technique for identifying sequence changes in amplified DNA. In this study, it was used for the characterization of the composition and changes in the microbial community. For this purpose, the microbial communities of 'Total bacteria' and the 'Clostridium cluster I' (i.e. proteolytic Clostridia) according to Collins *et al.* (1994) were considered.
First, differential centrifugation was performed as described by Brandt and Rohr (1981). The SSCP procedure for 16S rRNA genes of bacterial sequences was carried out as published by Meibaum *et al.* (2012). Briefly, after isolation of genomic DNA, polymerase chain reaction (PCR) was used for amplification of 16S rRNA genes. The total reaction volume was 25 µL with a final concentration of 1x PCR buffer with 2.5 U/ µL HotStar HiFidelity DNA polymerase (Qiagen, Hilden, Germany). The F27 forward primer (AGA GTT TGA TC(A/C) TGG CTC AG; Lane 1991) and the R1492 reverse primer (TAC GG(C/T) TAC CTT GTT ACG ACT T; Weisburg *et al.* 1991) were also obtained from Qiagen and applied at a concentration of 50 µmol/L. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s and elongation at 72°C for 70 s. Final elongation was for 10 min at 72°C. For the subsequent nested PCR, the Com1 forward primer (CAG CAG CCG CGG TAA TAC) and the Com2-Ph reverse primer (CCG TCA ATT CCT TTG AGT TT; Schwieger and Tebbe 1998) were used.
For PCR amplification of Clostridium cluster I sequences, according to Dohrmann *et al.* (2011), the forward primer P930 (GTG AAA TGC GTA GAG ATT AGG AA) and reverse primer P932-Ph (GAT (C/T)(C/T) G CGA TTA CTA G(C/T)A ACT; Le Bourhis *et al.* 2005) were used (source as above). Reverse primers were phosphorylated at the 5' end for further single strand digestion. PCR reaction mixture and conditions were the same as for total bacteria with the exception that annealing took place at 58°C but for only 50 s. Gel electrophoresis of single strand DNA was carried out at 20°C and 300 V for 22.5 h (total bacteria) or 30 h (Clostridia). Polyacrylamide (0.625%) SSCP gels were air-dried and scanned (ScanMaker i800; Mikrotek, Willich, Germany).
Microbial profiles obtained by SSCP analysis of the total bacteria and Clostridium cluster I communities in pooled fermentation liquid of the control period (days 9, 11) were compared with those from the experimental period (days

13, 16) in trial A. In trial B, comparison to samples of days 13 and 16 was carried out separately to gain insight into possible temporal changes.

Bacteriological analysis
In samples taken on days 9 and 11 (control period, trials A and B), and on days 13 and 16 (experimental period, trial A) or 12 – 16 (experimental period, trial B), respectively, qualitative and quantitative determination of micro-organisms was carried out.
For the culturing and isolation of the aerobic and anaerobic bacteria, different protocols were used. The preparation of samples was performed according to ASU L 06.00-16, that is, an officially approved method according to the German food and feed legislation. For quantitative analysis, dilution series of samples were cultivated at 37°C on Columbia agar with 5% sheep blood, whereas moulds and yeasts were kept at 30°C on Sabouraud agar plates (Oxoid, Wesel, Germany). Cultivation time was 48 h.
For qualitative analysis of all present Clostridia, isolation was carried out additionally by enrichment using liver broth obtained also from Oxoid. After over-night cultivation of the dilution series at 37°C, 10 µL of the liver broth was transferred onto a Columbia-agar plate and Clostridia cultivated again in its presence for 48 h under anaerobic conditions at 37°C. This protocol was applied to quantifying *Cl. sporogenes* in trial B, too.
Once *Cl. perfringens* was detected, further differentiation was tried by genotyping its major toxins. For this purpose, a multiplex PCR as described by Meer and Songer (1997) was used. Quantification of the alpha toxin was done according to Beer and Al-Khatib (1968).
All colonies with different morphologies, including all cultivated bacteria, yeasts and moulds, were identified by means of the 'Matrix-assisted linear desorption/ionzation time-of-flight mass spectrometry' (MALDI-TOF MS) technique (Shimadzu/Kratos, Manchester, UK) as described by Kallow *et al.* (2010). The strains were analysed on a stainless steel target plate (MABRITEC AG, Basel, Switzerland), using a whole-cell protocol with 1 µL matrix solution of saturated α-cyano-4 hydroxy-cinnamic acid in a mixture of acetonitrile, ethanol and water (1/1/1) acidified with 3% (v/v) trifluoroacetic acid. For each strain, mass spectra were prepared in duplicate and analysed in the linear positive ion extraction mode. Mass spectra were accumulated from 100 profiles, each from five nitrogen laser pulse cycles, by scanning the entire sample spot. Ions were accelerated with pulsed extraction at a voltage of 20 kV. Raw mass spectra were processed automatically for baseline correction and peak recognition. Resulting mass fingerprints were exported to the SARAMIS (Spectral Archiving and Microbial Identification System; AnagnosTec GmbH, Potsdam, Germany) analysis program and compared to reference superspectra and spectra to identify the species. The available open database allows identification of more than 1500 different bacteria species and more than 300 yeasts and moulds.

Statistical analysis
Statistical analysis was carried out with GRAPHPAD PRISM 4.0 (GraphPad Software Inc., La Jolla, CA). For evaluation of ruminal metabolism parameters, mean values obtained from the control and experimental periods were calculated for each fermentation vessel. Control values were based on a 5-day observation period. In the experimental period, the data included were confined to the last 3 days in order to guarantee stable temporal conditions. One-way ANOVA was performed then to compare the means from the control period (six fermentation vessels) with the means obtained in three fermentation vessels receiving either the LG or HG during experimental period. Two-way ANOVA was applied for data on *Cl. sporogenes* counts. Post hoc, the Bonferroni test was applied. Differences were regarded as significant at $P < 0.05$, but trends at $P < 0.1$ already.
Automatically detected SSCP band patterns of digitalized images were compared using the software GELCOMPAR II (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analyses (clustering algorithm: UPGMA) with dendrograms were performed on the basis of similarity matrices using the Pearson product-moment correlation coefficient. A nonparametric multivariate analysis of variance that is suitable for statistical analysis of not normally distributed and discontinuous data was carried out using dissimilarity matrices as described previously by Anderson (2001). Calculations were conducted using the software PERMANOVA (ver. 1.6) leading to P-values obtained by a permutation procedure. Differences between treated ($n = 3$) and control vessels ($n = 6$) were regarded as significant at $P < 0.05$ (Anderson and Robinson 2003).

Results
Recovery of glyphosate
During the control period before treatment, glyphosate could not be detected in the effluents. Application of the glyphosate-based herbicide (GBH) into the fermentation vessels during the experimental period of trial A led to mean concentrations of 0.34 and 3.31 mg/L glyphosate in effluents for LG and HG respectively. The resulting mean recovery of 0.26 or 2.31 mg glyphosate per day accounted for 61.9 and 79.0%, respectively, of the actually applied low glyphosate dose (LG) or high glyphosate dose (HG).

Impact on rumen metabolism

The application of the GBH in the experimental period of trial A did not alter pH or redox potentials in fermentation vessels as compared to the control period (Table 3). However, the addition of HG led to a significant decrease in NH_3-N concentrations from 8.51 to 7.07 mmol/L ($P < 0.01$). While the production of propionate, butyrate and valerate remained unaffected in response to the addition of GBH, there was a trend of an increasing acetate and total SCFA production after the addition of GBH ($P < 0.1$). In contrast, isovalerate concentration was significantly higher after administration of HG ($P < 0.01$). For the isobutyrate concentration, a significant increase after addition of HG ($P < 0.05$) was only observed when compared with LG but not with the control. The addition of HG resulted in a significant decrease in molar proportion of propionate ($P < 0.05$) and in an increase for isovalerate compared with the control and LG-treated fermentation vessels ($P < 0.01$). For valerate, there was a trend of a decrease in molar proportion in response to the addition of GBH ($P < 0.1$). In addition, a trend of an increased degradation of OM was observed after the addition of GBH ($P < 0.1$).

Table 3. Impact of the GBH on ruminal metabolism in the RUSITEC system (Trial A, means with standard deviation; six vessels in the control and three in LG and HG groups each).

Parameter	Control	LG	HG	*P*-value
pH	6·75 ± 0·05	6·75 ± 0·03	6·73 ± 0·02	ns
Redox potential (mV)	−292 ± 36·2	−304 ± 12·3	−278 ± 36·6	ns
NH_3-N (mmol l^{-1})	$8·51^{a}$ ± 0·62	$7·49^{a,c}$ ± 0·28	$7·07^{b,c}$ ± 0·41	**
Total SCFA (mmol day^{-1})	28·3 ± 2·26	29·3 ± 2·31	32·2 ± 2·21	†
Acetate	15·9 ± 1·17	16·8 ± 1·32	17·0 ± 1·95	†
Propionate	5·97 ± 0·56	5·74 ± 0·36	6·42 ± 0·53	ns
Isobutyrate	$0·14^{a,b}$ ± 0·05	$0·11^{a}$ ± 0·01	$0·22^{b}$ ± 0·04	*
Butyrate	4·17 ± 0·45	4·52 ± 0·63	5·04 ± 0·49	ns
Isovalerate	$0·93^{a}$ ± 0·09	$0·93^{a}$ ± 0·12	$1·21^{b}$ ± 0·04	**
Valerate	1·42 ± 0·10	1·32 ± 0·04	1·42 ± 0·18	ns
Molar proportions (%)				
Acetate	56·1 ± 0·87	57·3 ± 1·18	55·8 ± 0·94	ns
Propionate	$20·7^{a}$ ± 0·67	$19·6^{a}$ ± 0·43	$19·9^{b}$ ± 0·29	*
Isobutyrate	$0·49^{a,b}$ ± 0·12	$0·4^{a}$ ± 0·03	$0·63^{b}$ ± 0·07	*
Butyrate	14·8 ± 0·94	15·4 ± 1·31	15·6 ± 0·54	ns
Isovalerate	$3·27^{a}$ ± 0·18	$3·17^{a}$ ± 0·19	$3·79^{b}$ ± 0·22	**
Valerate	5·04 ± 0·44	4·54 ± 0·27	4·39 ± 0·27	†
Degradation of OM (%)	47·4 ± 2·46	48·2 ± 0·23	51·2 ± 1·54	†

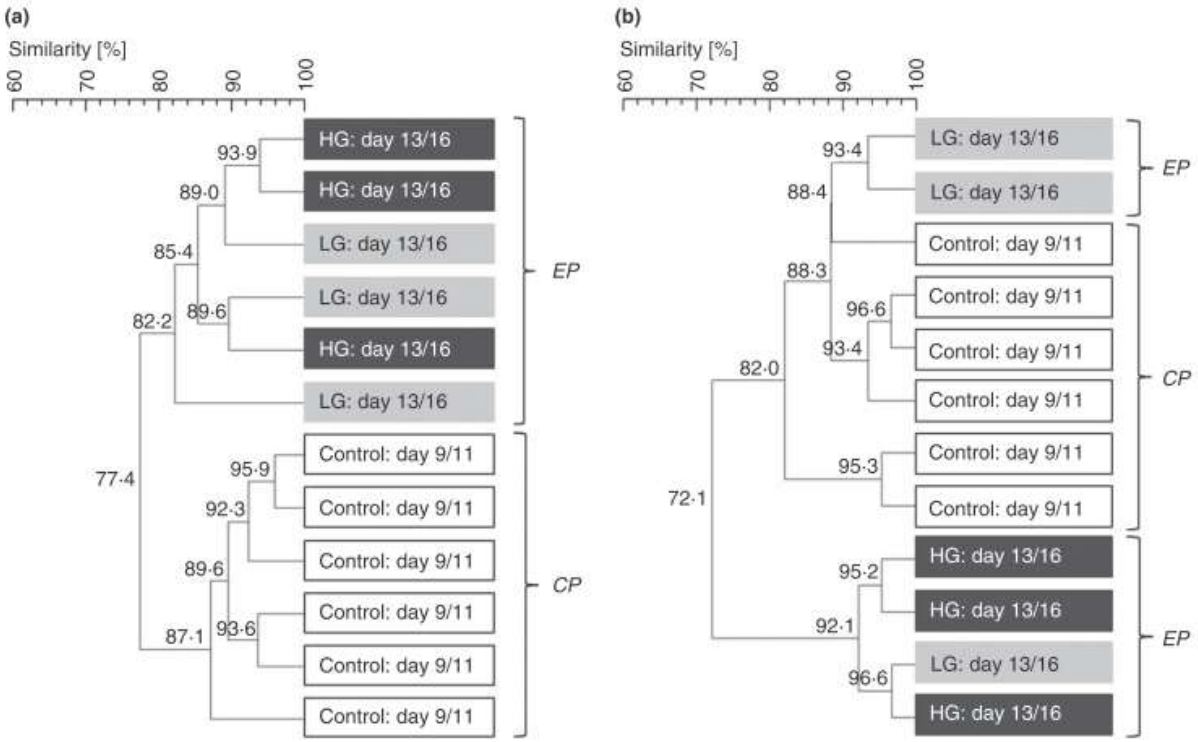
P-values obtained by One-way ANOVA; $*P < 0·05$, $**P < 0·01$, †trend: $P < 0·1$.
$^{a–c}$Means within a row with different superscripts differ significantly ($P < 0·05$).

Impact on microbial communities

Dendrograms with estimates of similarity for microbial communities of total bacteria and Clostridia, based on SSCP profiles, are presented in Figure 2 for trial A and in Figure 3 for trial B.

In trial A, SSCP profiles of the bacterial communities obtained from each fermentation vessel during the control period (pooled sample of day 9 and 11) resembled each other quite well with an average agreement of 87.1% (Figure 2a). SSCP profiles from the experimental period (pooled sample of day 13 and 16) formed a separate cluster. If directly compared, profiles from control period showed only a 77.4% agreement with profiles from the experimental period on average. This difference was statistically significant ($P < 0.01$) suggesting a treatment-related effect. However, a dose-dependent influence on SSCP profiles of total bacteria was not observed. For SSCP profiles of the Clostridia community, there were no defined clusters in response to the treatment in the experimental period (Figure 2b) and no statistical difference was obtained when compared to the control period.

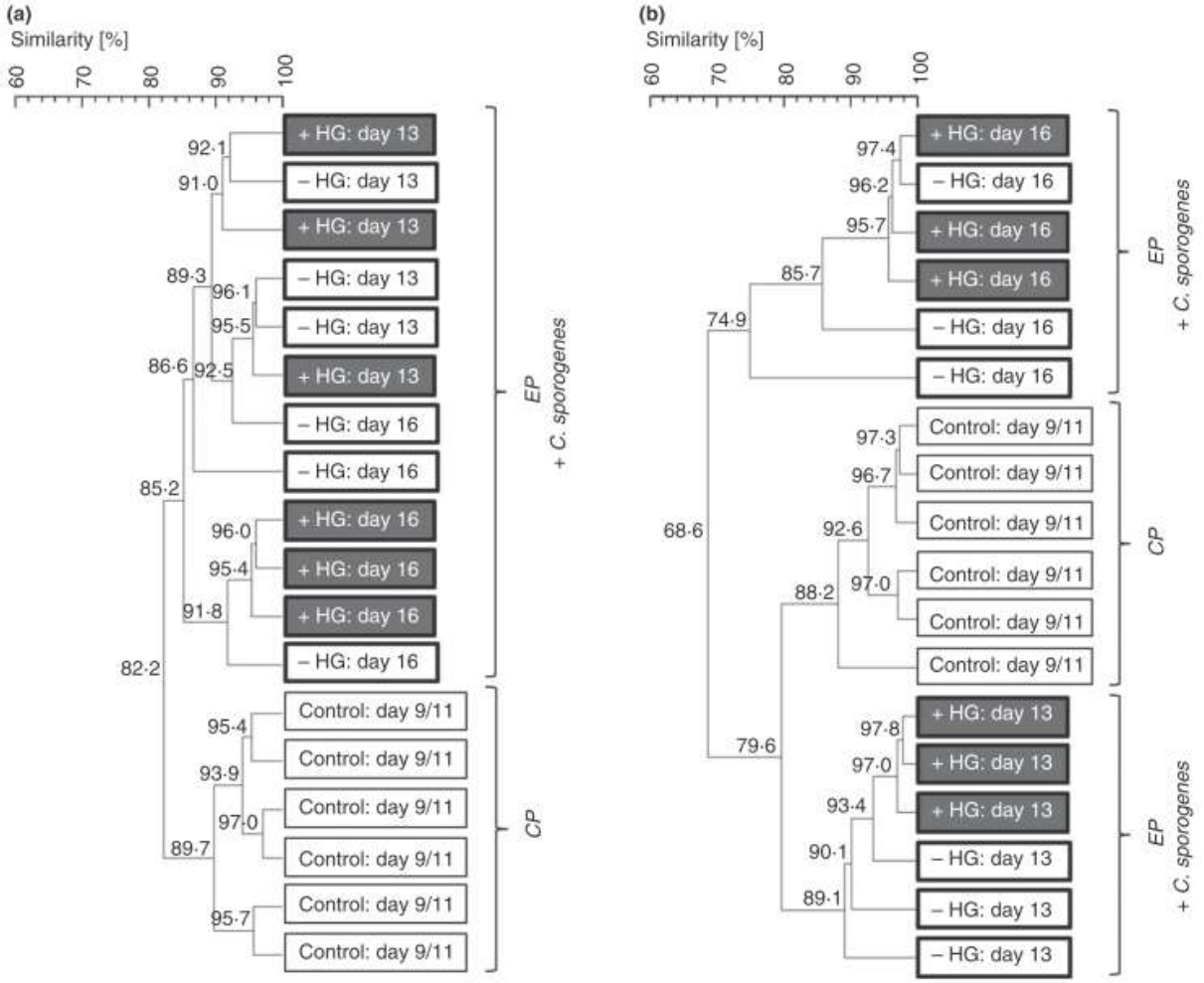
Figure 2. Trial A: Dendrograms of the 16S rRNA gene-based SSCP profiles for the microbial communities of total bacteria (a) and Clostridia (b) in the control period (CP, pooled sample of days 9/11) and the experimental period (EP: pooled sample of days 13/16) after the addition of a low (LG, 0.42 mg) or a high daily dose of glyphosate (HG, 2.92 mg). For the cluster analysis, Pearson's correlation and the unweighted pair group method with arithmetic mean (UPGMA) algorithm were applied.



In trial B, SSCP profiles of the bacterial communities from each fermentation vessel during the control period (pooled sample of day 9 and 11) were similar with an average agreement of 89.7% (Figure 3a). After the addition of *Cl. sporogenes* at the beginning of the experimental period, we found significant differences in SSCP profiles from the control period compared to those from all six vessels of day 13 ($P < 0.001$) and day 16 ($P < 0.01$) of the experimental period. In addition, profiles from day 13 compared with profiles from day 16 were significantly different ($P < 0.01$) suggesting a time-dependent influence. However, the application of the HG apparently had no impact on the SSCP profiles of the bacteria community.

As to be expected, the SSCP profiles of the Clostridia community were clearly altered by introduction of *Cl. sporogenes* (Figure 3b) at the beginning of the experimental period. SSCP profiles were significantly different on days 13 and 16 when compared to the previous control period ($P < 0.001$). A significant difference between SSCP profiles from day 13 and day 16 was also identified ($P < 0.01$). SSCP profiles of the Clostridia community were not affected by the addition of HG.

Figure 3. Trial B: Dendrograms of the 16S rRNA gene-based SSCP profiles for the microbial community of total bacteria (a) and Clostridia (b) in the control period (CP, pooled sample of days 9/11) and the experimental period (EP: pooled sample of days 13/16) after the addition of 10^4 CFU/g *Clostridium sporogenes* (– HG: = without high dose of glyphosate; + HG: = with high dose of glyphosate giving 2.92 mg glyphosate per day). For the cluster analysis, Pearson's correlation and the unweighted pair group method with arithmetic mean (UPGMA) algorithm were applied.



Bacteriological analysis

The following genera/species were identified by MALDITOF MS technology in trial A: *Aerococcus viridans*, *Bacillus licheniformis*, *Enterococcus faecalis*, *Escherichia hermannii*, *Lactobacillus* spp., *Leucobacter* spp., *Morganella* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Riemerella* spp., *Staphylococcus* spp. and *Streptococcus* spp. Interestingly, *Bifidobacterium* spp. was only detectable during the experimental period in response to the addition of both LG and HG but not during the control periods. Following treatment, concentrations of up to 2400 x 10^4 CFU/g were observed. There were no other differences in bacterial numbers in relation to treatment or time.

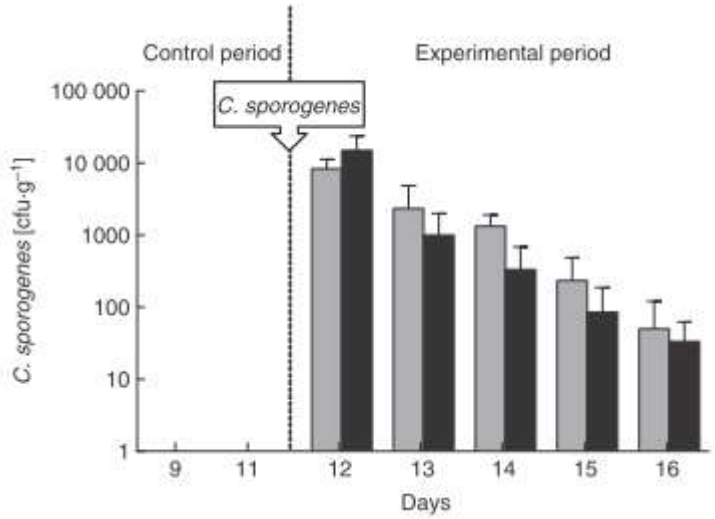
Because of artificial adding of *Cl. sporogenes* to the test system in trial B, spontaneous occurrence of Clostridia was of particular interest. In fact, in trial A, *Cl. perfringens* was identified on day 9 in the control period in one fermentation vessel in a low concentration of < 10 CFU/g but could not be typed. Following application of HG, *Cl. perfringens* type A was detected on day 13 in the same vessel at the same concentration. This bacterium produced small amounts of α-toxin (≤ 4 NE) but no β2-toxin. In a further fermentation vessel treated with HG, *Cl. perfringens* type A and *Clostridium bifermentans* were found in concentrations below 10 CFU/g on day 13. After application of LG, *Cl. perfringens* type A was found in one vessel and some production of α-toxin (≤ 4 NE) could be shown.

In trial B, *Clostridium sartagoforme* was identified in a concentration of 6 x 10^8 CFU/g in one fermentation vessel prior to HG addition. In the same fermentation vessel, after addition of *Cl. sporogenes* in a concentration of 10^4 CFU/mL at the beginning of the experimental period and HG, *Cl. sartagoforme* occurred on day 14 in a concentration of 3 x 10^7 CFU/g, on day 15 in a concentration of 1 x 10^6 CFU/g and on day 16 in a concentration of 2 x 10^7 CFU/g. In the remaining fermentation vessels, further Clostridia species were not detected.

Further identified genera/species in trial B were *Actinomyces viscosus*, species from the *Bacillus cereus* group, *Corynebacterium* spp., *Enterococcus* spp., *Escherichia coli*, *Gemella* spp., *Globicatella sulfidifaciens*, *Lactobacillus mucosae*, *Leucobacter* spp., *Paenibacillus odorifer*, *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Veillonella* spp. Again, there were no differences in bacterial numbers in relation to treatment or time.

Fate of Clostridium sporogenes in trial B
Following the addition of *Cl. sporogenes* (10^4 CFU/mL), a similar significant time-dependent reduction ($P < 0.001$) in the number of CFU in the fermentation vessels treated with HG and in the untreated vessels was observed (Figure 4). It became obvious that there was no evidence of growth stimulation of *Cl. sporogenes* by HG.

Figure 4. Trial B: Time-dependent decline of colony forming units of *Clostridium sporogenes* during experimental period after addition of 10^4 CFU/g *Cl. sporogenes* in fermentation vessels treated with the high glyphosate dose of 2.92 mg day (black bars) and without glyphosate (grey bars) (means with standard deviation; control n = 6; + HG n = 3; – HG n = 3; Two-way ANOVA, treatment: not significant, time: $P < 0.001$, treatment x time: not significant).



Conclusion
This study was performed in a well-established *in vitro* model to investigate whether the application of a glyphosate-containing herbicide might affect the bacterial communities and some biochemical parameters in a cow's rumen.
No adverse effects of the herbicide on ruminal metabolism or composition of the bacterial communities could be detected. In particular, there was no evidence of a suspected stimulation of Clostridia growth.
Antibiotic activity of glyphosate resulting in microbial imbalances has been postulated. In this exploratory study, however, intraruminal application of concentrations reflecting potential exposure of dairy cows or beef cattle did not exhibit significant effects on bacterial communities in a complex *in vitro* system. The low number of replicates (n = 3/dose) may leave some uncertainty.

3. Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case c) Relevance cannot be determined: This study used an *in vitro* model to investigate whether the application of a glyphosate-containing herbicide (Plantaclean®) might affect the bacterial communities and some biochemical parameters in a cow's rumen. No adverse effects on ruminal metabolism or composition of the bacterial communities were detected. In particular, there was no evidence of a suspected stimulation of Clostridia growth.
Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. In this study a system was developed for studying ruminal organisms that is dynamic, used mixed population of microbes, and is periodically fed with removal of waste products. There were no impacts of glyphosate formulation to this system.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:

In the current study, the effect of glyphosate on the gut microbiota in an in vitro cow rumen model was investigated. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.6. Study 6

1. Information on the study

Data point	CA 6.4
Report author	Schrödl W. *et al.*
Report year	2014
Report title	Possible Effects of Glyphosate on Mucorales Abundance in the Rumen of Dairy Cows in Germany
Document No.	Curr Microbiol (2014) Vol. 69, pp. 817-823
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

Glyphosate influences the soil mycobiota; however, the possible effect of glyphosate residues in animal feed (soybean, corn, etc.) on animal mycobiota is almost unknown. Accordingly, the present study was initiated to investigate the mycological characteristics of dairy cows in relationship to glyphosate concentrations in urine. A total of 258 dairy cows on 14 dairy farms in Germany were examined. Glyphosate was detected in urine using ELISA. The fungal profile was analyzed in rumen fluid samples using conventional microbiological culture techniques and differentiated by MALDI-TOF mass spectrometry. LPS-binding protein (LBP) and antibodies (IgG1, IgG2, IgA, and IgM) against fungi were determined in blood using ELISA. Different populations of *Lichtheimia corymbifera*, *Lichtheimia ramosa*, *Mucor*, and *Rhizopus* were detected. *L. corymbifera* and *L. ramosa* were significantly more abundant in animals containing high glyphosate (> 40 ng/mL) concentrations in urine. There were no significant changes in IgG1 and IgG2 antibodies toward isolated fungi that were related to glyphosate concentration in urine; however, IgA antibodies against *L. corymbifera* and *L. ramosa* were significantly lower in the higher glyphosate groups. Moreover, a negative correlation between IgM antibodies against *L. corymbifera*, *L. ramosa*, and *Rhizopus* relative to glyphosate concentration in urine was observed. LBP also was significantly decreased in animals with higher concentrations of glyphosate in their urine. In conclusion, glyphosate appears to modulate the fungal community. The reduction of IgM antibodies and LBP indicates an influence on the innate immune system of animals.

Materials and Methods

Animals

Blood (n = 258), urine (n = 243), and rumen fluid (n = 258) were collected from 14 dairies in Germany (Table 1). Blood was taken from the jugular vein, permitted to coagulate and centrifuged 30009 g for 15 min, and the serum samples were stored at -20°C. Rumen fluid samples (1000 mL per animal) were taken orally using a pumped stomach tube. Rumen fluid samples and urine were stored at -20°C until used.

Table 1. Animals and sampling.

Farm no.	Total no.	Urine samples	Blood samples	Rumen fluid samples
1	7	7	7	7
2	33	32	33	33
3	15	15	15	15
4	24	22	24	24
5	5	4	5	5
6	31	30	31	31
7	42	37	42	42
8	45	40	45	45
9	5	5	5	5
10	6	6	6	6
11	5	5	5	5
12	12	12	12	12
13	13	13	13	13
14	15	15	15	15

Glyphosate Detection
Urine samples were diluted 1:10 or higher with zero diluent (test kit, Abraxis, USA) and tested for glyphosate by competitive ELISA according to the manufacturer's instructions (Abraxis, USA). Test validation was done with Gas Chromatography–Mass Spectroscopy (GC–MS) by Medizinische Labor Bremen (Bremen, Germany). The linear correlation coefficient (Pearson) between the two test methods for glyphosate in bovine urine was 0.96 (data not shown).

Cultural Enumeration of Mucoraceae *in Rumen Fluid*
Following homogenization, a series of 10-fold dilutions (10^{-1}–10^{-4}) of the rumen fluid were made in sterile dilution-buffer (0.4% NaCl, 0.1% meat peptone, 3.72 mM NaH_2PO_4, 14.05 Na_2HPO_4, and 0.03% Tween 80). One-hundred microliter of each dilution was cultivated on Rose Bengal Agar with 0.01% chloramphenicol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 0.1% tergitol® NP 10 (Sigma-Aldrich, Taufenkirchen, Germany), and on Diclorane Glycerol Agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The plates were incubated aerobically at 25°C for three days and at least an additional four days at room temperature. Following incubation, the colonies were enumerated and the fungal species were determined by phenotypic and micromorphologic characteristics and by MALDI-TOF–MS-analyses. The fungal counts are expressed as $\log_{10}$ of colony-forming units (cfu)/mL.

Blood Analyses
LBP and antibodies (IgA, IgG1, IgG2, and IgM) against fungi were determined in blood serum with ELISA.

Common ELISA-Steps
All ELISAs were done with ELISA-plates (96 well, flat-bottomed, high binding, Corning, New York, USA) filled with 100 µL per well and incubated for coating overnight at 4–6°C and, in the other incubation steps, one hour at room temperature on a plate shaker (400 rpm). The coating buffer was 0.9% NaCl in ultra-pure water, and the wash buffer was 0.9% NaCl with 0.05% (v/v) Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). The plates were washed after the coating step two times and after the other incubation steps four times with wash buffer using a 12 canal-microtiter-plate-washer (NUNC, Wiesbaden, Germany). Horseradish peroxidase was determined with H_2O_2 (3 mM) and 3, 3', 5, 5'-tetramethylbenzidine (TMB, 1 mM) in 0.2 M citrate buffer (pH 4). The substrate reaction was stopped with 1 M H_2SO_4 (50 µl/well). The optical density (OD) was measured with an ELISA microplate reader at 450 nm.

Analysis of LPS-Binding Protein (LBP) in Blood Serum
The concentration of LBP was determined with ELISA as described by Ostermann et al.. Briefly, ELISA-plates were coated with 1 µg/mL monoclonal antibody-anti-LBP (mouse IgG_1, "big 48", biometec, Greifswald, Germany). After incubation the plates were washed. The standard was a bovine pooled serum in which the LBP-concentration was calibrated with affinity purified bovine LBP. Blood samples were diluted 1:1000 or higher and the standard range was between 3 and 200 ng/mL bovine LBP. After incubation with diluted samples and standards, the plates were washed four times before the monoclonal antibody-anti-LBP conjugated with horseradish peroxidase (mouse IgG_1, big 412 with POD, biometec, Greifswald, Germany) at 0.05 µg/mL was added to the wells. After incubation and washing, a

colorimetric substrate solution for horseradish peroxidase was added. The substrate reaction was stopped with 1 M H_2SO_4 (50 µL per well). The OD-value was measured with a microplate ELISA reader at 450 nm. The standard curve (LBP standard-concentrations vs. OD-values) was determined using Table Curve software (Systat Software, Erkrath, Germany), and the concentrations of LBP in the samples were then calculated by considering of the dilution factor.

Mucoraceae Antibody Analysis

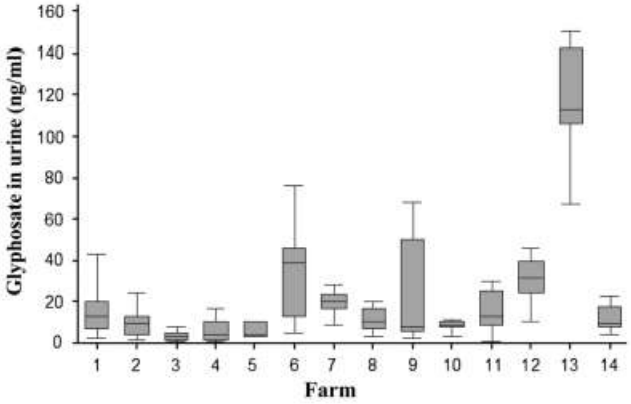
Antigen Preparation
Lichtheimia (*L.*) *corymbifera* (IBML-155-1), *L. ramosa* (IBML-155-2), *Mucor* (*M.*) *ramosissimus, M. circinelloides, Rhizopus* (*R.*) *oryzae*, and *R. microspores* (isolated from rumen fluid in this study) were cultured on 2% glucose-Sabouraud agar with chloroamphenicol (SIFIN, Berlin, Germany) for five days at 25°C. The mycelium was collected in PBS containing 0.03% Tween 80. The suspension was washed with PBS and the number of sporangiospores was determined. For isolation of the ELISA-coating antigen, 3 x 10^4 spores of each Mucoraceae isolate were inoculated in 200 mL synthetic broth (Difco Czapek-Dox-Broth and Difco-AOAC synthetic broth from Becton–Dickinson and Company, USA) with 2 mM H_2O_2. The *Lichtheimia* spp. and *Rhizopus* spp. were cultured seven days and the *Mucor* spp. ten days at 38°C. The cell free and sterile filtered (0.2 µm) culture supernatants were concentrated by ultrafiltration with a 5 kDa molecular weight cut-off concentrator (Vivaspin, Sartorius Stedim Biotech, Goettingen, Germany). The protein concentration was measured with a MBA 2000 spectrophotometer using the integrated software of Perkin-Elmer (Norwalk, Connecticut, USA).

Anti-Mucoraceae-Antibodies ELISA
The ELISA-plates were coated with 1 µg/mL fungal antigen (see above). Serum samples and an internal antibody-standard (serum pool from over 3000 cows, defined as 100 relative units [RU] mL^{-1}) were diluted in assay buffer (50 mM Tris adjusted with 1 M HCl at pH 7.35, 0.9% NaCl, 1 mM EDTA, 1% yeast extract, 2% fish gelatine, 0.1% Tween 20, all from Fluka or Sigma-Aldrich, Germany). The fungi-specific bound antibodies were detected with horseradish peroxidase-conjugated antibodies (Bethyl Lab. Inc., Montgomery, USA): sheep IgG-anti-bovine IgG1, IgG2 or IgM (diluted 1–10000) and sheep IgG-anti-bovine IgA (1–2500). After detection of bound peroxidase activity with H_2O_2 and TMB (see above), the enzyme reaction was stopped with 1 M H_2SO_4 (50 µl/well) and the OD-value was measured at 450 nm. The RU/mL for each serum sample was calculated relative to the internal antibody-standard (100 RU/mL) and dilution factors.

Statistical Analysis
The statistical analysis was carried out with the SigmaStat software (Systat Software Erkrath, Germany). The level of significance between two value groups was calculated with the non-parametric Mann–Whitney rank sum test. To compare the proportions of positive fungi detected in culture ($\geq$ 1 x 10^2 cfu/g), the z-test was used. The correlation coefficient and *P* value were determined with the Spearman rank order correlation.

Results
Glyphosate Detected in Urine
Glyphosate excreted in the urine of 243 dairy cows is shown in Fig. 1. The glyphosate concentrations ranged from 0.0 to 164.0 ng/mL. Cows on farm 13 excreted significantly ($P > 0.005$) more glyphosate than the other farms (Fig. 1). The samples were sorted into three groups according to glyphosate concentration in urine. Animals excreting < 10 ng/mL glyphosate (n = 95), animals excreting 10–40 ng/mL glyphosate (n = 113), and animals excreting > 40 ng/mL glyphosate (n = 35).

Figure 1. Glyphosate excretion from cows at 14 dairy farms. Cows in farm 13 had significantly ($P > 0.005$) higher glyphosate compared with the other farms.



Detection of Mucorales in Rumen Fluids

L. corymbifera, *L. ramosa*, *Mucor*, and *Rhizopus* could be isolated from the rumen fluid and could be differentiated based on the MALDI-TOF–MS profile. The frequency of isolation of these fungi depended on the glyphosate concentration in urine is shown in Table 2. Interestingly, cows with the highest glyphosate excretion in urine had lower frequencies of isolation of total *Mucorales*, *L. corymbifera* and *L. ramosa*.

Table 2. Frequency of *Mucorales* in rumen fluid of dairy cows in relation to the excretion of glyphosate in urine.

Glyphosate in urine (ng/ml)	Total *Mucorales* Pos. no. (%)	*L. corymbifera* Pos. no. (%)	*L. ramosa* Pos. no. (%)	*Mucor* Pos. no. (%)	*Rhizopus* Pos. no. (%)
<10 (n = 95)	59 (62,1)[a]	22 (23.2)	47 (49.5)[g]	2 (2.1)	0
10–40 (n = 113)	67 (59,3)[a]	34 (30.1)[c]	44 (38.9)[e]	6 (5.3)	0
>40 (n = 35)	9 (25,7)[b]	4 (11.4 %)[d]	6 (17.1 %)[fh]	0	2 (5.7 %)

a/b $P < 0.005$, *c/d* $P < 0.05$, *e/f* $P < 0.05$, *g/h* $P < 0.005$

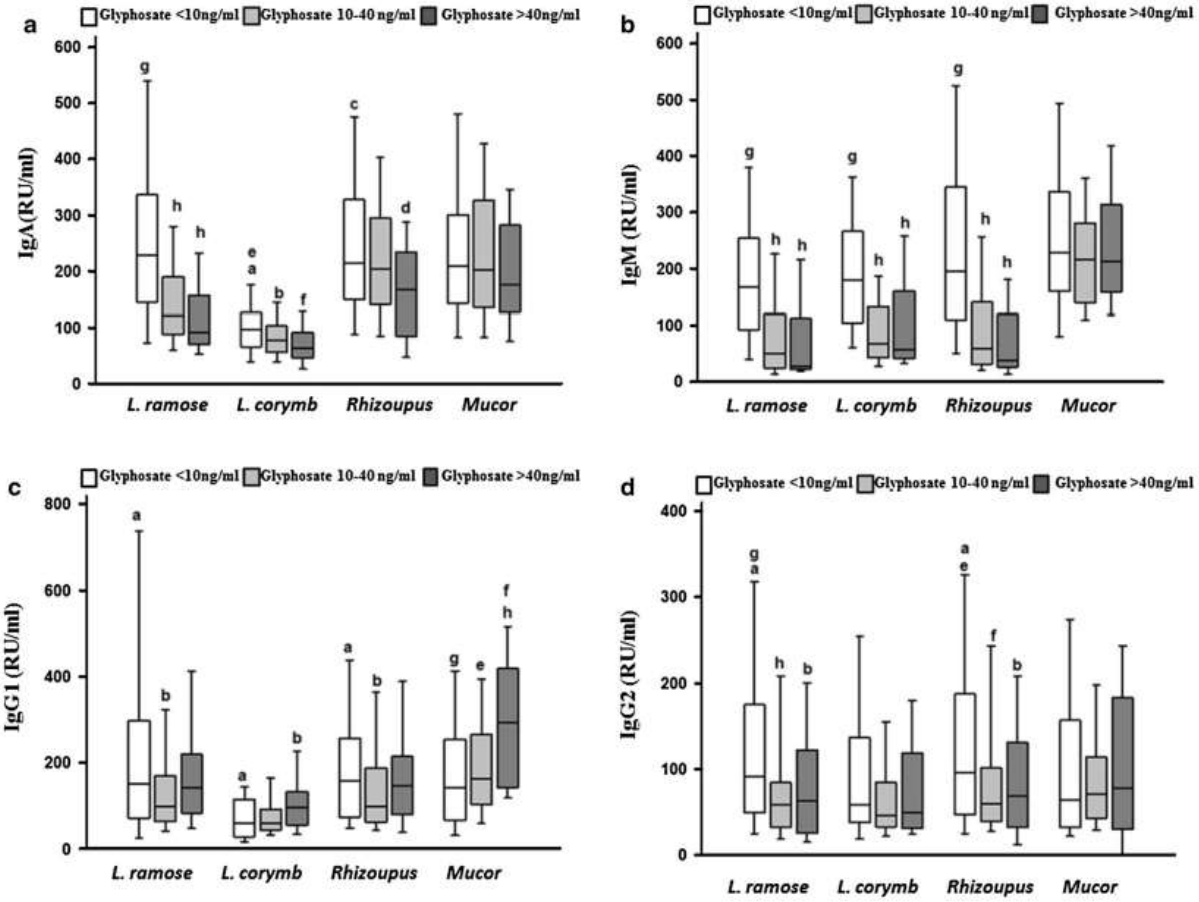
Detection of Antibodies Against Lichtheimia spp., Mucor *spp., and* Rhizopus *spp. in Blood Serum*

The IgA, IgM, IgG1, and IgG2 against *L. corymbifera*, *L. ramosa*, *Mucor* spp., and *Rhizopus* were measured in serum using ELISA (Fig. 2). IgA antibodies against L. corymbifera and L. ramosa decreased significantly in cows in the higher glyphosate groups; however, IgA antibodies against *Mucor* spp. and *Rhizopus* were not correlated with glyphosate concentrations in urine. On the other hand, except for *Mucor*, there was a significant decrease of IgM antibodies against *L. corymbifera*, *L. ramosa*, and *Rhizopus* in animals excreting higher glyphosate in their urine. Negative correlations between IgM-anti-fungi in blood and glyphosate concentrations in urine were observed, the correlation coefficients (Spearman) were -0.463, -0.496, and -0.513, for IgM against *L. corymbifera*, *L. ramosa*, and *Rhizopus*, respectively. On the other hand, IgG1 antibodies against *Mucor* spp. increased significantly in the highest glyphosate group. In contrast, IgG1 of *L. ramosa* significantly decreased in animals excreting 10–40 ng/mL glyphosate in their urine.

Acute Phase Protein LBP

LBP significantly decreased relative to increasing levels of glyphosate concentrations in urine. The mean values of LBP were 15.2, 11.1, and 9.3 ng/ml in animals excreting <10 ng ml-1 glyphosate, 10–40 ng/ml glyphosate, and >40 ng/ml glyphosate, respectively.

Figure 2. Antibody concentrations against members of the order *Mucorales* (*L. corymb.* = *L. corymbifera*, *L. ramosa*, *Rhizopus*, and *Mucor*) arranged in three different urinary glyphosate concentration groups of dairy cows (< 10, 10–40, and > 40 ng/mL). Different letters indicate significant differences as follows: a/b $r < 0.05$, c/d $p < 0.01$, e/f $p < 0.005$, and g/h $p < 0.001$.



Conclusion

The present study was initiated to investigate the mycological characteristics of dairy cows in relationship to glyphosate concentrations in urine. A total of 258 dairy cows on 14 dairy farms in Germany were examined. Different populations of *Lichtheimia corymbifera*, *Lichtheimia ramosa*, *Mucor*, and *Rhizopus* were detected. *L. corymbifera* and *L. ramosa* were significantly more abundant in animals containing high glyphosate (> 40 ng/mL) concentrations in urine. There were no significant changes in IgG1 and IgG2 antibodies toward isolated fungi that were related to glyphosate concentration in urine; however, IgA antibodies against *L. corymbifera* and *L. ramosa* were significantly lower in the higher glyphosate groups. Moreover, a negative correlation between IgM antibodies against *L. corymbifera*, *L. ramosa*, and *Rhizopus* relative to glyphosate concentration in urine was observed. LBP also was significantly decreased in animals with higher concentrations of glyphosate in their urine. In conclusion, glyphosate appears to modulate the fungal community. The reduction of IgM antibodies and LBP indicates an influence on the innate immune system of animals.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: Article investigate the correlation of glyphosate concentration in urine and fungal profile in rumen fluid of dairy cows. No administration of glyphosate.

The article investigates the changes in the intestinal mycobiota in relation to glyphosate concentrations in urine. It was found that glyphosate appears to modulate the fungal community. The reduction of IgM antibodies and LBP indicates an influence on the innate immune system of animals.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. Methodological shortcomings of the approaches used reduce the significance of the results: 1) rumen fungi are strictly anaerobic, but aerobic cultures

were used) 2) spot-urine concentrations are highly affected by the level of milk production 3) the ELISA is not validated and the LOD was not used. In addition no validation is described for other assays.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:
This article investigates the relation between glyphosate in urine and mycological characteristics of dairy cows. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. In addition, as described by the applicant, there are also some deficits regarding the study set up. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.7. Study 7

1. Information on the study

Data point:	CA 6.4
Report author	Shehata A. A. *et al.*
Report year	2014
Report title	Neutralization of the antimicrobial effect of glyphosate by humic acid in vitro.
Document No	Chemosphere (2014), Vol. 104, pp. 258
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

In the present study, the neutralization ability of the antimicrobial effect of glyphosate by different humic acids was investigated. The minimal inhibitory concentrations of glyphosate for different bacteria such as *Bacillus badius*, *Bifidobacterium adolescentis*, *Escherichia coli*, *E. coli* 1917 strain Nissle, *Enterococcus faecalis*, *Enterococcus faecium*, *Salmonella enteritidis* and *Salmonella typhimurium* were determined in the presence or absence of different concentrations of humic acid (0.25, 0.5 and 1.0 mg/mL). Our findings indicated that humic acids inhibited the antimicrobial effect of glyphosate on different bacteria. This information can help overcome the negative impact of glyphosate residues in feed and water.

Materials and methods

Indicator microorganisms

The following bacteria were used in the present study: *Bacillus badius*, *Bifidobacterium adolescentis*, *Escherichia coli*, *E. coli* 1917 strain Nissle, *Enterococcus faecalis*, *Enterococcus faecium*, *Salmonella enteritidis* and *Salmonella typhimurium*. Cultural conditions and the origin of these strains are shown in Table 1.

Table 1. Target strains used, their origin and medium used for glyphosate experiments.

Genus/species	Reference/source	Lab. No.	Culture media		Incubation conditions
			Agar	Broth	
Bacillus badius	Shehata et al. (2013a)	1/12	Blood agar[a]	RCM[b]	37 °C/24 h aerobic
Bifidobacter adolescentis	Shehata et al. (2013a)	4/12	MRS[c]	MRS	37 °C/48 h anaerobic
E. coli	Shehata et al. (2013a)	5/12	Blood agar	RCM	37 °C/24 h aerobic
E. coli 1917 strain Nissle	Ardeypharm GmbH, Herdecke, Germany	Nissle	Blood agar	RCM	37 °C/24 h aerobic
Enterococcus faecalis	Shehata et al. (2013a)	6/12	CATC[d]	RCM	37 °C/24 h aerobic
Enterococcus faecium	Shehata et al. (2013a)	7/12	CATC	RCM	37 °C/24 h aerobic
Salmonella enteritidis	Shehata et al. (2013a)	9/12	Caso agar[e]	RCM	37 °C/24 h aerobic
Salmonella typhimurium	Shehata et al. (2013a)	10/12	Caso agar	RCM	37 °C/24 h aerobic

[a] Blood agar (Fluka, Germany).
[b] RCM: reinforced clostridial medium (RCM, SIFIN, Germany).
[c] MRS: deMan, Rogosa and Sharpe *Lactobacillus* Agar (Oxoid, Germany).
[d] CATC: citric-acid-Tween-carbonate agar (Oxoid, Germany).
[e] Caso agar (3.5% casein–soya, 0.3% yeast extract, 0.1% glucose, 1.5% agar–agar).

Minimal inhibitory concentration

The minimal inhibitory concentrations (MIC) of glyphosate (Roundup UltraMax®, Monsanto, USA) for these pathogens were tested according to the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, the lowest concentration of glyphosate which showed bactericidal or bacteriostatic effects was determined in a 24-well micro-titre plate. Serial dilutions of glyphosate (10, 5, 2.5, 1.2, 0.6, 0.3, and 0.15, 0.075 mg/mL) were made in broth culture media. After the addition of bacteria (105 cfu/mL) to the plates, they were incubated at 37 °C. The MIC value was evaluated by quantitative analysis of bacterial growth on suitable agar media (Table 1).

Neutralization of antibacterial activity of glyphosate using humic acids

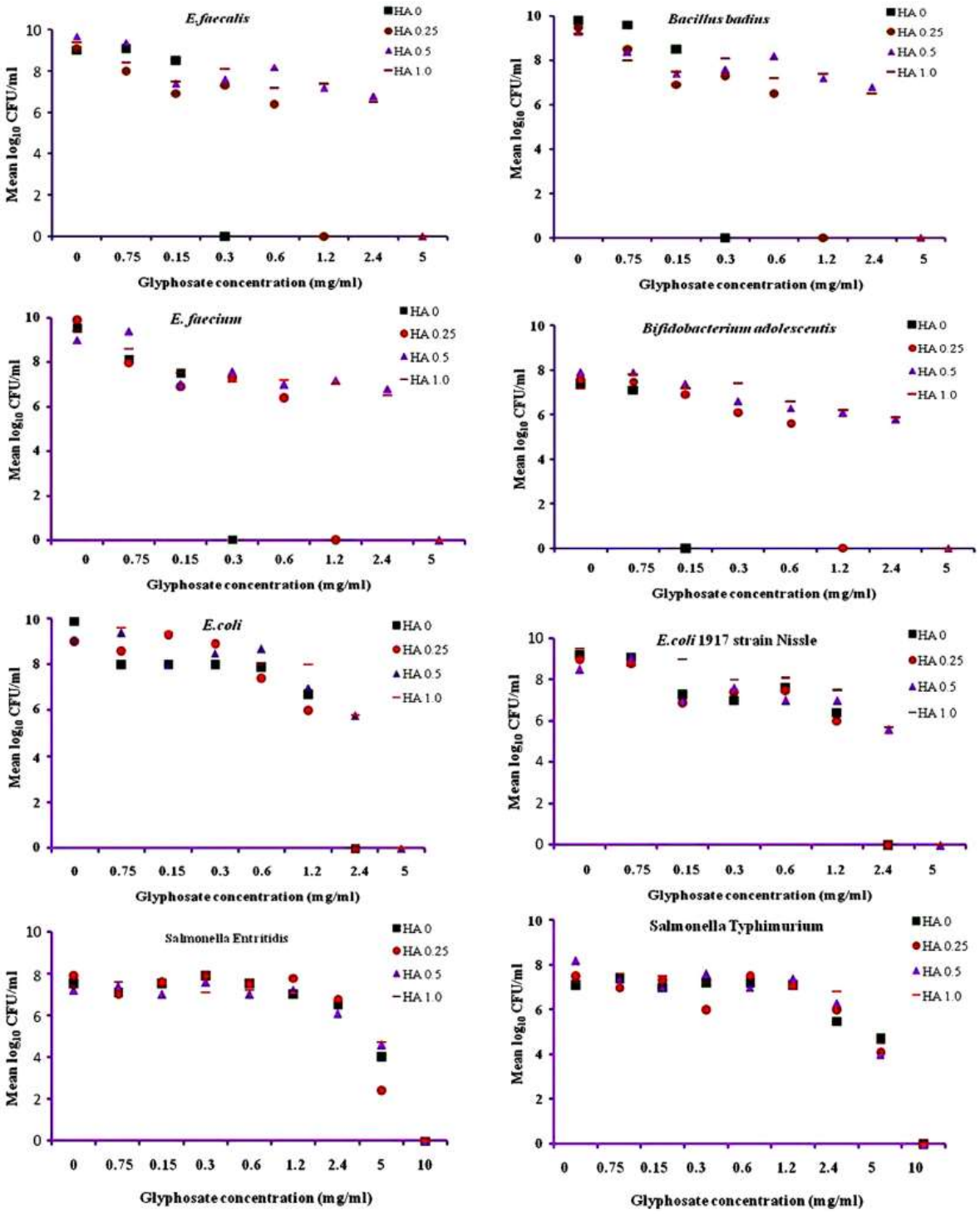
The MIC value of glyphosate (10, 5, 2.5, 1.2, 0.6, 0.3, 0.15, 0.075 mg/mL) on *E. faecalis*, *B. badius* and *B. adolescentis* in the presence of 1 mg mL^{-1} each of the following humic acids; WH67/ 1, WH67/2, WH67/3, WH67/4, WH67/5, WH67/6.1, WH67/6.2, WH67/7, WH67/8.1, WH67/8.2 and WH67/9 (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany) was evaluated. Different concentrations (1 mg, 0.5 and 0.25 mg/mL) of the most effective humic acid (WH67/4) were tested with *B. badius*, *B. adolescentis*, *E. coli*, *E. coli* 1917 strain Nissle, *E. faecalis*, *E. faecium*, *S. enteritidis* and *S. typhimurium* in the presence of different glyphosate concentrations (10, 5, 2.5, 1.2, 0.6, 0.3, 0.15, 0.075 mg/mL).

Results

The MIC value of glyphosate for *E. faecalis*, *B. badius* and *B. adolescentis* were 0.3, 0.3 and 0.15 mg/mL, respectively. Humic acids neutralized the antimicrobial effect of glyphosate in different patterns. The WH67/2, WH67/4/3, and WH67/4 humic acids at 1 mg/mL showed the highest neutralization of the antimicrobial effect of glyphosate. The MIC-value of glyphosate for *E. faecalis*, *B. badius* and *B. adolescentis* in the presence of WH67/2, WH67/3, and WH67/4 humic acids (1 mg/mL) were more than 2.4 mg/mL. The other humic acids had less neutralizing activity and MIC values ranged from 0.3–0.6 mg/mL. This indicates that glyphosate adsorption on humic substances varies considerably depending on their macromolecular structure. These data support the results of previous studies that indicated humic acids could adsorb glyphosate and lead to the formation of relatively stable complexes (Albers et al., 2009; Banta et al., 2009; Mazzei and Piccolo, 2012).

The WH67/4 humic acid neutralized the antimicrobial effect of glyphosate on *B. badius*, *B. adolescentis*, *E. coli*, *E. coli* 1917 strain Nissle, *E. faecalis*, *E. faecium* in a dose dependent manner (Figure. 1). Even at a low concentration of WH67/4 (0.25 mg/mL), glyphosate-sensitive *Enterococci*, *Bifidobacteria* and *B. badius* bacteria grow well in a culture medium containing 0.6 mg glyphosate (Figure. 1). In contrast, WH67/4 in a concentration of 1 mg/mL could not neutralize the antimicrobial effect on glyphosate-resistant *Salmonella* spp. The neutralization of high concentrations of glyphosate are of no value since they have no biological relevance and exceed by many fold the expected glyphosate residues in the feed and environment. It is worthy to mention that glyphosate also has an inhibitory effect on microbial growth and antibiotics effect at lower concentrations than those found in agriculture (Clair et al., 2012b). Glyphosate could disrupt the bacterial community due to differences in sensitivity between microorganisms (Clair et al., 2012; Shehata et al., 2013a; Krüger et al., 2013). Any modification of the environment which leads to a response by living organisms may be considered as a stress (Missous et al., 2007). The biotic stress observed in biology, is considered a global phenomenon, and can be extended to anthropogenic pressure such as genetic engineering or xenobiotic (including glyphosate) pollution (Thammavongs et al., 2008). Protection of *Enterococci* and *Bifidobacteria* from glyphosate residues by humic acids could help reduce the incidence of glyphosate- induced dysbiose and to reduce the risk of *C. botulinum* infection hence an antagonistic effect of *Enterococcus* spp. on *Clostridia* was proved (Shehata et al., 2013b). *Bifidobacteria* is sensitive to glyphosate and thought to create conditions unfavourable to the growth of pathogens such as *Salmonella* (Isolauri et al., 2001). The tested humic acids showed no direct significant effect on the growth of the tested bacteria (Figure. 1).

Figure 1. Neutralization of the antimicrobial effect of glyphosate to different bacteria with WH67/4 humic acid (HA). Bacterial counts were expressed as mean log10 CFU/mL.



Conclusion

In conclusion supplementation of humic acids in feed not only substantially reduces mycotoxicoses (Sabater-Vilar et al., 2007) and improves the performance, carcass, gastrointestinal tract and meat quality traits (Ozturk et al., 2011); but also neutralize the antimicrobial effect of glyphosate and reduces glyphosate accumulation in animal products. Moreover, the use of humic acids in environmental clean-up also serves to promote the microbial diversity in ecosystems.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: In the present study, the neutralization ability of the antimicrobial effect of glyphosate by different humic acids was investigated. The minimal inhibitory concentrations of glyphosate for different bacteria such as Bacillus badius, Bifidobacterium adolescentis, Escherichia coli, E. coli 1917 strain Nissle, Enterococcus faecalis, Enterococcus faecium, Salmonella enteritidis and Salmonella typhimurium were determined in the presence or absence of different concentrations of humic acid. The findings indicated that humic acids inhibited the antimicrobial effect of glyphosate on different bacteria.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. In the absence of a suitable dossier datapoint it was allocated to point KCA 6.4 as it concerns livestock. However, it is important to note that it is not a residue study and does not provide any data on the transfer of residues from feed to food of animal origin.

Assessment and conclusion by RMS:

In this public literature paper, the influence of humic acids on the antimicrobial effect of glyphosate has been investigated *in vitro*. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.8. Study 8

1. Information on the study

Data point	CA 6.4
Report author	Shehata A.A. *et al.*
Report year	2013
Report title	The Effect of Glyphosate on Potential Pathogens and Beneficial Members of Poultry Microbiota In Vitro
Document No.	Curr Microbiol (2013) Vol. 66, pp. 350-358
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

The use of glyphosate modifies the environment which stresses the living microorganisms. The aim of the present study was to determine the real impact of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. The presented results evidence that the highly pathogenic bacteria as *Salmonella* Entritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Clostridium perfringens* and *Clostridium botulinum* are highly resistant to glyphosate. However, most of beneficial bacteria as *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus badius*, *Bifidobacterium adolescentis* and *Lactobacillus* spp. were found to be moderate to highly susceptible. Also *Campylobacter* spp. were found to be susceptible to glyphosate. A reduction of beneficial bacteria in the gastrointestinal tract microbiota by ingestion of glyphosate could disturb the normal gut bacterial community. Also, the toxicity of glyphosate to the most prevalent *Enterococcus* spp. could be a significant predisposing factor that is associated with the increase in *C. botulinum*-mediated diseases by suppressing the antagonistic effect of these bacteria on clostridia.

Materials and Methods

Glyphosate

Roundup UltraMax® (Monsanto, USA) which contains 450 mg/mL of glyphosate was used in this study.

Strains and Cultural Conditions

Experiments were carried out with *Bacillus badius*, *Bacillus cereus*, *Bacteriodes vulgatus*, *Bifidobacterium adolescentis*, *Campylobacter coli*, *Campylobacter jejuni*, *C. perfringens*, *C. botulinum* type A, C. *botulinum* type B, *Escherichia coli*, *E. coli* 1917 strain Nissle, *E. faecalis*, *E. faecium*, *Lactobacillus buchneri*, *L. casei*, *L. harbinensis*, *Riemerella anatipestifer*, *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus lentus* were used. Cultural conditions and origin of these strains are described in Table 1.

Table 1. Target strains used, their origin and medium used for glyphosate experiments.

Genus/species	Origin	Reference no. and related collections	Lab. no.	Culture media		Incubation conditions
				Agar	broth	
Bacillus badius	*Green algae* (Altmark Co, Germany)	Isolated in this study	1/12	Blood agar[a]	RCM[b]	37 °C/24 h aerobic
Bacillus cereus	*Green algae* (Altmark Co, Germany)	Isolated in this study	2/12	Blood agar	RCM	37 °C/24 h aerobic
Bacteriodes vulgatus	Chickens	Isolated in this study	3/12	Blood agar	RCM	37 °C/48 h anaerobic
Bifidobacterium adolescentis	Chickens	Isolated in this study	4/12	MRS[c]	MRS	37 °C/48 h anaerobic
Campylobacter coli	Institute of Bacteriology, Leipzig University	11151/03	11151/03	Caso-modified agar	Caso-modified broth	37 °C/48 microaerophilic
Campylobacter jejuni	Institute of Bacteriology, Leipzig University	188	188	Caso-modified agar	Caso-modified broth	37 °C/48 microaerophilic
C. perfringens	RIPAC-LABOR GmbH, Germany	884/2	884/2	NeoP[d]	RCM	37 °C/24 h anaerobic
C. botulinum type A	National collection of type culture (NCTC)	7272	7272	Blood agar	RCM	37 °C/48 h anaerobic
C. botulinum type B	National collection of type culture (NCTC)	7273	7273	Blood agar	RCM	37 °C/48 h anaerobic
E.coli	Chickens	Isolated in this study	5/12	Blood agar	RCM	37 °C/24 h aerobic
E. coli 1917 strain Nissle	Ardeypharm GmbH, Herdecke, Germany	Nissle	Nissle	Blood agar	RCM	37 °C/24 h aerobic
Enterococcus faecalis	*Chlorella vulgaris*	Isolated in this study	6/12	CATC[e]	RCM	37 °C/24 h aerobic
Enterococcus faecium	Chickens	Iisolated in this study	7/12	CATC	RCM	37 °C/24 h aerobic
Lactobacillus buchneri	Effective microorganisms (Multikraft Co, Austria)	12/25	12/25	MRS	MRS	37 °C/48 h anaerobic
Lactobacillus casei	Effective microorganisms (Multikraft Co, Austria)	12/26	12/26	MRS	MRS	37 °C/48 h anaerobic
Lactobacillus harbinensis	Chickens	Isolated in this study	8/12	MRS	MRS	37 °C/48 h anaerobic
Riemerella anatipestifer	Institute of Poultry Diseases, Berlin University	12109	12109	Blood agar	RCM	37 °C/24 h aerobic
Salmonella Enteritidis	Chickens	Isolated in this study	9/12	Caso agar[f]	RCM	37 °C/24 h aerobic
Salmonella Gallinarum	Institute of Poultry Diseases, Berlin University	Z34/11	Z34/11	Caso agar	RCM	37 °C/24 h aerobic
Salmonella Typhimurium	Chickens	Isolated in this study	10/12	Caso agar	RCM	37 °C/24 h aerobic
Staphylococcus aureus	Chickens	Isolated in this study	11/12	Blood agar	RCM	37 °C/24 h aerobic
Staphylococcus haemolyticus	Chickens	Isolated in this study	12/12	Blood agar	RCM	37 °C/24 h aerobic
Staphylococcus lentus	Chickens	Iisolated in this study	13/12	Blood agar	RCM	37 °C/24 h aerobic

[a] Blood agar (Fluka, Germany)

[b] Reinforced clostridial medium (RCM, SIFIN, Germany)

[c] deMan, Rogosa and Sharpe Lactobacillus Agar (Oxoid, Germany)

[d] Neomycin–polymixin blood agar (neomycin (100 mg/l, Roth, Germany)/polymyxin B (50 mg/l Fluka, Germany)

[e] Citrat-Azid-Tween-Carbonat agar (Oxoid, Germany)

[f] Caso agar (3.5 % casein-soya, 0.3 % yeast extract, 0.1 % glucose, 1.5 % agar)

Bacterial Identification

Bacterial strains were tested using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF). In brief, about 10 mg of cell material of the cultured strains were suspended in 300 µl of sterile water. 900 µl of absolute ethanol was added, and the mixture was centrifuged at 10000 rpm for 2 min. The supernatant was discarded, and the

pellet was suspended in 50 µl formic acid (70% v/v). After adding 50 µl acetonitrile (AN), the mixture was centrifuged at 10000 rpm for 2 min. 1 µl of the clear supernatant was transferred to the MALDI target and allowed to dry. A saturated solution of α-cyano-hydroxy-cinnaminic acid (Bruker Daltonik GmbH) in a standard organic solvent mixture (2.5% trifluoroacetic acid to AN 50% in water was added to resuspended 1 µl of the dried material. All chemicals used were of the highest quality (Merck, designated to be especially suitable for HPLC or MALDI-based techniques). Before each MALDI run, *E. coli 1*917 strain Nissle was analysed to serve as the positive control and calibration standard. The MALDI–TOF MS analysis was performed using a Bruker microflex LT mass spectrometer (Bruker Daltonik Co), and the spectra were automatically identified using the Bruker BioTyper™ 1.1 software. *C. perfringens* was identified by MALDI-TOF and multiplex PCR, data are not shown.

Effect of Glyphosate on Different Bacteria

The minimal inhibitory concentration (MIC) of Roundup UltraMax® was determined in triplicate in a 24-well microtiter plate. 100 µl of tested bacteria (10^5 cfu/ml) was added to 900 µl broth media containing different concentrations of glyphosate (5.0, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml). Plates containing diluted glyphosate and bacteria were incubated at 37°C (Table 1). Bacterial growth was evaluated on suitable agar medium. Culture condition for each bacterium is shown in Table 1. The MIC value was evaluated by quantitative analysis of bacteria on agar plate. The morphology of bacteria was examined microscopically.

Effect of Glyphosate on C. botulinum Type A and B

Clostridium botulinum was cultured anaerobically in a cooked meat broth at 37°C for 5 days, followed by cultivation in reinforced clostridial medium (RCM, Sifin, Germany) anaerobically at 37°C for 3 days. *C. botulinum* types A and B were heated at 80°C for 10 min and left at room temperature under aerobic condition. Cultures were tested daily for sporulation using a Gram or Rakette stain. To study the effect of glyphosate on *C. botulinum* strains, heat treated spores or vegetative cells were added to RCM medium at a final concentration of 10^4 cfu/ml. The inhibitory effect of glyphosate was determined using the following concentrations: 5, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml. The mixture was incubated anaerobically at 37°C for 5 days. *C. botulinum* was quantified using the most probable number (MPN) estimation method. *C. botulinum* type A and B neurotoxins (BotNT) were analysed using ELISA. In brief, ELISA was performed in flat-bottomed ELISA plates (96 wells, high binding; Costar, Corning, New York, USA). All standard volumes were 100 µl/well and the standard incubation condition was 1 h at room temperature (1 h at RT) on a microtitre plate shaker (400 rpm). The coating buffer was 0.1 M $NaHCO_3$ and the wash solution (WS) was 0.9% NaCl with 0.1%. Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). After coating the wells with capture antibodies (3 µg ml, rabbit IgG against *C. botulinum* neurotoxin type A or B, Institute of Bacteriology and Mycology, University of Leipzig), the plates were incubated overnight at 4–6°C. ELISA plates were washed twice with WS and loaded with the diluted specimens. Supernatants of the cultures were diluted 1:10 or higher in assay buffer (assay buffer: 20 mM Tris, pH 8.0 [adjusted with 1 M HCl], 0.9% NaCl, 5 mM EDTA, 1.0% gelatine from cold water fish skin, 0.2% bovine serum albumin, 0.1 % rabbit IgG and 0.2 % Tween 20 (all from Sigma-Aldrich or Fluka, Taufkirchen, Germany). The plates were incubated for 1 h at RT, washed five times with WS and loaded with the detection antibodies conjugated with horseradish peroxidase, diluted in assay buffer. BotNT types A and B were detected with 2.5 µg/ml horse ($[Fab]_2$ from IgG) against *C. botulinum* types A and B, respectively (Novartis Vaccines and Diagnostics GmbH, Marburg, Germany). After 1 h incubation at RT, the plates were washed four times with WS. All washing steps were done by a Nunc-Immuno-Washer 12 (Nunc, Wiesbaden, Germany). The antibody bound marker enzyme, horseradish peroxidase, was detected by adding 3 mM H_2O_2 and 1 mM 3,3',5,5'-tetramethylbenzidine (TMB) in 0.2 M citrate-buffer to each well (pH 4.0). The substrate reaction was stopped with 1 M H_2SO_4 (50 µl/well). The optical density (OD) was measured using a microplate ELISA reader at 450 nm. The supernatant with known concentrations of the BotNT types A and B (Institute of Bacteriology and Mycology, University of Leipzig) were used as a standard.

Effect of Glyphosate on Campylobacter spp.

The effect of glyphosate on *C. coli* and *C. jejuni* was tested in a 10 ml tissue culture flask in Caso-modified broth media containing 3.5% caso broth 0.3% yeast extract, 0.3% casein hydrolysate, 0.03% $FeSO_4$ and 0.03% natrium pyruvate. Glyphosate was added with different concentrations (5.0, 2.40, 1.20, 0.60, 0.30 and 0.15 mg/ml). Tested bacteria was added at a final concentration of 10^4 cfu/ml under microaerophilic conditions at 37°C for 48 h. Bacterial growth in the presence of different concentrations of glyphosate was tested on Caso-modified agar (3.5% caso agar, 0.3% yeast extract, 0.3% casein hydrolysate, 0.03% $FeSO_4$ and 0.0.3% natrium pyruvate, 0.4% active charcoal and 1% agar).

Effect of Glyphosate on Sporozoites

Sporulated *Eimeria tenella* oocysts (isolate LE-01 Eten-05/1) were kindly supplied by Institute of Parasitology, Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany. Sporozoites of *E. tenella* were excysted as described. In brief, after surface sterilization with bleach, the oocyst walls were broken using 0.5-mm glass beads

(Biospec product, Bartleville, OK, USA). Thereafter, the sporozoites were recovered from sporocysts by enzymatic excystation using 0.025 trypsin (w/v) (Carl Roth, Karlsruhe, Germany), 10 mM $MgCl_2$ (w/v) and 1% sodium taurocholic acid (w/v) (Sigma, Taufkirchen, Germany) at 41°C for 60–90 min. The excysted sporozoites were purified by anion exchange method. The pellet sporozoites were collected carefully from the bottom of the micro-tubes and washed three times with phosphate-buffered saline (PBS). To study the effect of glyphosate on *E. tenella*, sporozoites were incubated at 37°C overnight with 1.20, 0.60, 0.30, 0.15 and 0.075 mg glyphosate in 1 ml Dulbecco's modified Eagle's medium (DMEM) containing 5% newborn calve serum. The viability of sporozoites was determined by the trypan blue staining method. In short, sporozoites suspensions were exposed to the same volume of 0.5% trypan blue in 0.1 M phosphate buffer, pH 7.0. The non-stained parasites were regarded as viable and those stained as dead. Examination of morphological alterations and/or viability was done and images were captured using an inverted microscope (Leica DM IRB, Bensheim, Germany).

Results

Effect of Glyphosate on Potential Pathogens and Beneficial Bacteria

Most of tested pathogenic bacteria were highly resistant to glyphosate; however, most of tested beneficial bacteria were found to be moderate to highly susceptible (Table 2). The herbicide formulated glyphosate at a concentration of 1.2 mg/ml inhibited the growth and BotNT expression (Tables 2, 3). Supplementation of the medium with 1.2 mg/ml glyphosate reduced the cell numbers of *C. botulinum* type A and B after 5 days of cultivation. *C. perfringens*, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Salmonella* Entritidis and *E. coli* showed to be highly resistance to glyphosate (MIC value 5 mg/ml) (Table 2). *L. casei*, *L. buchneri*, *L. harbinensis*, *Staphylococcus aureus* and *Staphylococcus lentus* were moderately resistant to glyphosate (MIC value 0.60 and 0.30 mg/ml, respectively). On the other hand, with the exception of *Lactobacillus* spp., all tested beneficial bacteria including *E. faecalis*, *E. faecium* and *B. badius*, *B. cereus* and *B. adolescentis* were highly sensitive to glyphosate with MIC value of 0.15, 0.15, 0.30 and 0.075 µg/ml, respectively. Colonies morphology showed no significant differences between control and glyphosate-treated bacteria with glyphosate. Microscopically, *Lactobacillus* spp. *B. vulgatus* and *E. tenella* showed swelling and corrugated cell wall.

Table 2. Inhibitory effect of glyphosate on different bacteria.

Genus/species	MIC value of glyphosate (mg/ml)[a]	Bacterial count[b]	
		Treated at MIC value (mean ± SD, $n = 3$)	Untreated (mean ± SD, $n = 3$)[c]
Bacillus badius	0.150	2.24 ± 0.49	8.90 ± 0.44
Bacillus cereus	0.300	2.75 ± 0.68	8.08 ± 0.12
Bacteriodes vulgatus	0.600	3.54 ± 0.31	7.37 ± 0.10
Bifidobacterium adolescentis	0.075	3.87 ± 0.50	8.67 ± 0.48
Campylobacter coli	0.150	3.07 ± 0.50	9.00 ± 0.70
Campylobacter jejuni	0.150	3.90 ± 0.50	9.54 ± 0.97
C. perfringens	5.000	3.37 ± 0.89	8.30 ± 0.28
C. botulinum type A	1.200	4.00 ± 0.50	8.16 ± 0.32
C. botulinum type B	1.200	3.56 ± 0.45	7.60 ± 0.57
E. coli	1.200	3.15 ± 0.24	8.00 ± 0.34
E. coli 1917 strain Nissle	1.200	2.35 ± 0.24	7.26 ± 0.21
Enterococcus faecalis	0.150	2.00 ± 0.45	8.49 ± 0.58
Enterococcus faecium	0.150	2.01 ± 0.34	7.06 ± 0.95
Lactobacillus buchneri	0.600	4.00 ± 0.88	8.00 ± 0.22
Lactobacillus casei	0.600	4.74 ± 0.56	8.28 ± 0.35
Lactobacillus harbinensis	0.600	5.30 ± 0.44	8.40 ± 0.32
Riemerella anatipestifer	0.150	4.00 ± 0.50	7.88 ± 0.50
Salmonella Enteritidis	5.000	2.35 ± 0.26	8.28 ± 0.16
Salmonella Gallinarum	5.000	2.15 ± 0.33	8.68 ± 0.20
Salmonella Typhimurium	5.000	2.75 ± 0.68	8.03 ± 0.16
Staphylococcus aureus	0.300	5.74 ± 0.58	9.00 ± 0.10
Staphylococcus haemolyticus	0.300	5.74 ± 0.32	8.08 ± 0.16
Staphylococcus lentus	0.300	3.90 ± 0.44	8.08 ± 0.14

[a] Minimal inhibitory concentration (MIC) of Glyphosate

[b] Mean of quantitative bacterial counts expressed as reciprocal $\log_{10}$

[c] Bacterial counts without glyphosate treatment (control)

Effect of Glyphosate on E. tenella Sporozoites

In comparison to untreated control, glyphosate at concentrations of 0.6 mg/ml clearly affected the sporozoites morphology after exposure to glyphosate for 24 h. *E. tenella* sporozoites showed morphological changes including swelling and corrugated cell wall. At concentration of 0.3 mg/ml, few sporozoites were affected. However, at 0.15 mg/ml, there was no visible effect on sporozoites morphology. At 1.20 mg and 2.4 mg/ml glyphosate, all sporozoites were destroyed.

Table 3. Effect of different glyphosate concentrations on C. botulinum types A and B growth and BotNT expression.

Glyphosate concentration (mg/ml)	*C. botulinum* type A[a]		*C. botulinum* type B	
	CFU[b] (mean ± SD, $n = 3$)	BotNT type A[c] (mean ± SD, $n = 3$)	CFU (mean ± SD, $n = 3$)	BotNT type B (mean ± SD, $n = 3$)
0	8.16 ± 0.32	2,400 ± 378	7.60 ± 0.57	1,350 ± 270
0.6	6.12 ± 0.67	2,250 ± 126	5.50 ± 0.18	470 ± 60
1.2	4.00 ± 0.50	130 ± 89	3.56 ± 0.45	70 ± 19
2.4	$<10^3$	0.0	$<10^3$	0.0
5.0	$<10^3$	0.0	$<10^3$	0.0

[a] *Clostridium botulinum* spores (10^5 cfu/ml) in reinforced clostridial medium (RCM) broth incubated with different glyphosate concentrations anaerobically for 5 days at 37 °C

[b] *Clostridium botulinum* quantified using the most probable number (MPN) estimation method and expressed as reciprocal $\log_{10}$

[c] Botulinum neurotoxines measured by ELISA [30]

Conclusion

The aim of the present study was to determine the real impact of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. The presented results evidence that the highly pathogenic bacteria as *Salmonella* Entritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Clostridium perfringens* and *Clostridium botulinum* are highly resistant to glyphosate. However, most of beneficial bacteria as *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus badius*, *Bifidobacterium adolescentis* and *Lactobacillus* spp. were found to be moderate to highly susceptible. Also *Campylobacter* spp. were found to be susceptible to glyphosate. A reduction of beneficial bacteria in the gastrointestinal tract microbiota by ingestion of glyphosate could disturb the normal gut bacterial community. Also, the toxicity of glyphosate to the most prevalent *Enterococcus* spp. could be a significant predisposing factor that is associated with the increase in *C. botulinum*-mediated diseases by suppressing the antagonistic effect of these bacteria on clostridia.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The study investigate the influence of glyphosate (Roundup Ultramax) on potential pathogens and beneficial members of poultry microbiota *in vitro*. Glyphosate showed differences in sensitivity between potential pathogens and beneficial microbiota in chicken. The toxicity of glyphosate to *Enterococcus spp.* and *B. badius* could be a significant predisposing factor that is associated with the increase in *C. botulinum* mediated diseases by suppressing the antagonistic effect of these bacteria on *C. botulinum*.

The study represents pure *in vitro* experiments and no monitoring data. The formulation tested is not the EU representative formulation. Moreover, the study has as systemic error, given that gut bacteria of poultry are not exposed to formulated product, except it would be administered to poultry. Hence, gut bacteria of poultry would be exposed to residues of glyphosate and AMPA at a worst-case feed burden below 0.08 mg/kg DM. In conclusion, all trials are overapplied, the effect of other formulation components is not excluded, and conclusion were transferred from artificial single cultures to a complex microbiome.

Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. The publication does not provide new information (potential effects on microorganims with sensitive EPSPS are well known) and real world conditions of the gut are not replicated (study conducted on minimal media; microorganisms exposed to extremely high doses of glyphosate (1000x); aged cultures inducing additional stress).

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:

The present paper investigates the impact of glyphosate on both pathogenic as well as beneficial poultry microbiota *in vitro*. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. In addition, as described by the applicant, there are also some deficits regarding the study set up. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.9. Study 9

1. Information on the study

Data point:	CA 6.4
Report author	Vicini J. L. *et al.*
Report year	2019
Report title	Glyphosate in livestock: feed residues and animal health
Document No	Journal of animal science (2019), Vol. 97, No. 11, pp. 4509
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

Glyphosate is a nonselective systemic herbicide used in agriculture since 1974. It inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway present in cells of plants and some microorganisms but not human or other animal cells. Glyphosate-tolerant crops have been commercialized for more than 20 yr using a transgene from a resistant bacterial EPSP synthase that renders the crops insensitive to glyphosate. Much of the forage or grain from these crops are consumed by farm animals. Glyphosate protects crop yields, lowers the cost of feed production, and reduces CO_2 emissions attributable to agriculture by reducing tillage and fuel usage. Despite these benefits and even though global regulatory agencies continue to reaffirm its safety, the public hears conflicting information about glyphosate's safety. The U.S. Environmental Protection Agency determines for every agricultural chemical a maximum daily allowable human exposure (called the reference dose, RfD). The RfD is based on amounts that are 1/100th (for sensitive populations) to 1/1,000th (for children) the no observed adverse effects level (NOAEL) identified through a comprehensive battery of animal toxicology studies. Recent surveys for residues have indicated that amounts of glyphosate in food/feed are at or below established tolerances and actual intakes for humans or livestock are much lower than these conservative exposure limits. While the EPSP synthase of some bacteria is sensitive to glyphosate, in vivo or in vitro dynamic culture systems with mixed bacteria and media that resembles rumen digesta have not demonstrated an impact on microbial function from adding glyphosate. Moreover, one chemical characteristic of glyphosate cited as a reason for concern is that it is a tridentate chelating ligand for divalent and trivalent metals; however, other more potent chelators are ubiquitous in livestock diets, such as certain amino acids. Regulatory testing identifies potential hazards, but risks of these hazards need to be evaluated in the context of realistic exposures and conditions. Conclusions about safety should be based on empirical results within the limitations of model systems or experimental design. This review summarizes how pesticide residues, particularly glyphosate, in food and feed are quantified, and how their safety is determined by regulatory agencies to establish safe use levels.

Materials and methods

This paper reviews studies related to the effect of glyphosate on animal health and, more specifically, on gut microbes by inhibition of EPSP synthase. Emphasis will be given to ruminants, due to their reliance on rumen microbes for the efficient digestion of fibrous feedstuffs and their conversion of fibrous feedstuffs to nutritious meat or milk.

Results

Mechanism of Action and Development of Glyphosate Tolerant Crops

Glyphosate is a broad-spectrum herbicide because it inhibits plant EPSP synthase, an enzyme in the shikimate pathway responsible for the de novo synthesis of aromatic amino acids (Phe, Trp, and Tyr). This pathway is critical in plants, not only for the amino acids required for protein synthesis, but also for the synthesis of other abundant plant compounds such as lignin (Tzin and Galili, 2010). Because human and other animal cells do not have this pathway, these amino acids must be obtained from the diet and this enzyme is not a target for these species (Giesy et al., 2000).

EPSP synthase catalyzes the conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. Glyphosate blocks this step by competing with PEP for binding to the enzyme's active site. Crop scientists screened for an EPSP synthase that was resistant to glyphosate, i.e. Class II enzymes from microbial species resistant to glyphosate, to enable the use of a nonselective herbicide with crops that would be

resistant to the herbicide. A suitable candidate was discovered from the CP4 strain of *Agrobacterium tumefaciens* that was isolated from wastewater at a glyphosate manufacturing facility (Barry et al., 1997).

The major soil degradation pathway for glyphosate results in the formation of aminomethylphosphonic acid (AMPA) and CO_2 (Rueppel et al., 1977). Von Soosten (2016) detected AMPA in feed but glyphosate is not the only source of AMPA (Nowack, 2003). AMPA does not compete with PEP for enzyme binding (Reddy et al., 2004; Duke et al., 2012).

Residues, Exposures, and Risk Assessment

The EPA relies on well-defined process to determine safe exposure levels and establish allowable residues in food and feed. A series of chronic toxicological tests is used to establish a no observed adverse effect level (NOAEL). The NOAEL is the highest dose in collective toxicological studies that does not produce any adverse effect in the most sensitive species of test animals. A reference dose (RfD), expressed as daily pesticide exposure per body weight (BW) (mg/ kg/ d), is the maximum allowable exposure intended to provide a "reasonable certainty of no harm" to humans. EPA derives the RfD by dividing the NOAEL by 100 or 1000, to account for animal to human extrapolation (factor of 10), sensitive human populations (factor of 10) and effects specific to children (factor of 10, not always used). The sum of the most conservative or greatest possible exposures resulting from all uses cannot exceed the RfD.

Based on empirical data, tolerances (in some countries referred to as maximum residue limits), are then determined for each crop or animal products. These data are the result of multiple field trials in which the maximum rate given on the product label and the residues are measured. Tolerances by definition are not safety standards. Instead, they are the highest level of residues allowed for legal use of a pesticide regardless of whether even greater residue amounts might be acceptable from a safety perspective (Winter and Jara, 2015).

Recently, The European Food Safety Agency (EFSA) and the U.S. Food and Drug Administration (FDA) published results of surveys of foods and feeds in which glyphosate residues were measured using highly sensitive methods (EFSA, 2018b; FDA, 2018). They detected glyphosate residues in 3.6% of food samples. Of these foods, glyphosate was detected in commodities that are commonly used in feeds such as soybean (16%), barley (19%), and wheat (13%) and none of these amounts exceeded established tolerances for the European Union. Likewise, FDA-tested animal feeds and nonviolative residues of glyphosate were found in 63% of corn samples and 67% of soybean samples.

It is important to understand the analytical methods available to test for glyphosate in foods/feed. The most sensitive and selective method that has been validated for multiple feeds and other matrices is liquid chromatography tandem mass spectrometry (LC-MS/MS) (Jensen et al., 2016). A cheaper **antibody-based** ELISA method was developed to test glyphosate in water (a simple matrix), but this method has generated some questionable results when applied to complex matrices. An example is a report of glyphosate detected in human milk that was posted on a website (summarized by Bus (2015)). This study presented no validation information for the milk matrix and was in contrast to other studies that used validated LC-MS/MS methods with selectivity for glyphosate and did not detect glyphosate in milk from humans and cows (NZ Ministry for Primary Industiries, 2012; Ehling and Reddy, 2015; McGuire et al., 2016; Steinborn et al., 2016; von Soosten et al., 2016; EFSA, 2018b; FDA, 2018; Zoller et al., 2018).

Another questionable result from use of the ELISA was the alleged detection of glyphosate in deformed piglets (Krüger et al., 2014). Validation for this matrix was not reported in detail and there was no control group. In contrast, metabolism studies with high doses of glyphosate in feed detected no glyphosate in meat (muscle). These data combined with other physical-chemical properties of glyphosate suggest that glyphosate should not accumulate in the body (Bus, 2015) or be detectable in meat, milk or eggs (Van Eenennaam and Young, 2017). FDA recently monitored food for residues of glyphosate and glyphosate was not detectable in milk nor was it detected in eggs (FDA, 2018). Zoller et al. (2018) also found no glyphosate on most samples of meat, fish and samples that appear to be only meat or fish. Three samples had glyphosate residues that were slightly above the LOQ (limit of quantification), but these 3 were sausages or meat loaf could have nonmeat ingredients derived from grain.

The theoretical maximum exposure is based on a person consuming all possible food items containing the highest level of permissible pesticide residue and having the maximum possible contact through home uses or water. The theoretical maximum exposure must be less than or equal to the RfD.

EFSA conducted a risk assessment for glyphosate residues in animal feed and calculated that the maximum dietary burdens for cattle and swine are 13.2 and 2.85 mg/kg BW/d, respectively (EFSA, 2018a). Even at the maximum dietary burden based on tolerances, cattle or swine could consume 4 or 18 times as much glyphosate before their intakes would be at the level of the NOAEL. As stated previously, the issue of exposure is further complicated in that tolerances of individual feed ingredients are all based on the application regimens that result in the greatest amounts of herbicide residue. This exposure value will therefore over predict the average or typical herbicide residue for the feedstuff.

Understanding the possible residues in a crop is of practical significance when designing studies and selecting appropriate doses. An alternative would be to calculate the maximum reasonably balanced diet (OECD, 2009). And still another approach is to use empirical data, such as urine values, for a specific population of animals. Validated

assay data on urine values for livestock are not as extensively available as values for human exposure (Niemann et al., 2015). Examples of these measurements of toxicity and resulting calculated glyphosate exposures are listed in Table 1. When calculating the exposure of ingested glyphosate residues on the ruminant's gut microbes, the intake, metabolism and absorption, and volume/turnover of the rumen or other compartments of the digestive tract all must be taken into consideration.

Table 1. *Measurements of toxicity and range of glyphosate exposures that can be estimated for dairy cows.*

Measurements of toxicity or exposure	Value	
Acute toxicity (LD 50)	>5,000 mg/kg BW	
Chronic EPA NOAEL (EPA, 2019)	100 mg/kg BW	
Chronic EFSA NOAEL (EFSA, 2018a)	50 mg/kg BW	
Calculated exposures for dairy cow:	**Value**	**Assumptions**
Most conservative estimate (100% grass hay with highest tolerance)	20 mg/kg	
Maximum Reasonably Balanced Diet	11 mg/kg	1) 24 kg DMI and 600 kg cow; 2) diet with highest tolerances of a roughage source, a carbohydrate grain and a protein concentrate.
Based on urine data (von Soosten et al., 2016)	0.007 mg/kg	1) 600 kg cows; 2) all AMPA from glyphosate; and 3) uses highest urinary values reported.

Glyphosate and Gut Microbiota

Glyphosate tolerant crops have been commercialized since 1996 and are widely adopted in the United States with no apparent effects on animal productivity (Van Eenennaam and Young, 2014). Moreover, experimentally determined NOAELs reflect the lack of adverse finding, including within the full length of the gastrointestinal tract. Notwithstanding these conclusions, some have speculated whether gut microbes could be affected by the inhibition of microbial Class I EPSP synthase from glyphosate residues in the digesta. Microbial fermentation and digestive physiology are complex processes and are intricately interwoven. Therefore, conclusions about animal health, or even gut health, are not just a matter of the presence of Class 1 or Class 2 EPSP synthase. For instance, some or many strains of bacteria may not need the shikimate pathway to synthesize amino acids de novo when amino acids are present in their environment. The impact of glyphosate on microorganisms would be dependent on several factors, such as 1) the concentration of glyphosate within the gastrointestinal tract to allow for competitive inhibition with PEP; 2) the need for, or flux through, the shikimate pathway; and 3) the availability of Trp (usually the least abundant of the aromatic amino acids).

Studies show that there are multiple means to amino acid incorporation/synthesis and the importance of the interaction (cross-feeding) among mixed populations of intestinal microorganisms. So, rather than base possible glyphosate toxicity to bacteria on a single mechanism, it is critical that in vivo studies or model systems accurately replicate the conditions of particular sections of the gastrointestinal tract.

Batch culture studies

The simplest study design for testing effects of glyphosate or other compounds on gut microbes is to test individual strains for growth on media using batch culture systems. The single strain exposed to a compound inside a glass tube is cultured in conditions that are quite unlike in vivo conditions. Inside the rumen, metabolism is carried out by a complex interaction of thousands of types of microbes. Yet, these single-strain systems have been used to test the effects of adding glyphosate to media.

Due to the conditions of some studies (Krüger et al., 2013; Shehata et al., 2013) they are difficult to extrapolate to actual conditions in the gastrointestinal tract. One study, for example, was performed in aerobical conditions, although many intestinal bacteria only grow anaerobically. Furthermore, these studies used formulated glyphosate that contained surfactant, which are known for their bactericidal properties when applied in large amounts to unprotected cells. Therefore, these in vitro, batch culture studies do little to demonstrate that gut microbes are affected by glyphosate via EPSP synthase. The batch culture studies cited above used incubation times of 24 to 48 h. During this time, microorganisms typically go through a lag phase and an exponential growth phase, but growth curves were not presented to indicate the growth phase of these cultures. Culture conditions are critical as diet changes are known to result in adaptation of not only species of bacteria, but also in adaptive changes within a bacterial species (Saluzzi et al., 2001)

In vivo studies

Studies have been conducted to examine the feeding of glyphosate-tolerant GE crops with various farm animals. These studies involved dairy cows (Grant et al., 2003; Ipharraguerre et al., 2003; Castillo et al., 2004; Combs and Hartnell, 2008), beef cattle (Erickson et al., 2003), sheep (Hartnell et al., 2005), and broilers (Taylor et al., 2003; Kan and

Hartnell, 2004; Taylor et al., 2005; Taylor et al., 2007a, 2007b; McNaughton et al., 2011). None of these studies found that feeding crops sprayed during cultivation with glyphosate had an impact on animal productivity.
In an in vitro setting, glyphosate can affect EPSP synthase of some bacteria, and thus has the potential to impact gut microbes, but the critical question is whether the normal use of glyphosate in vivo results in changes in digestive function, altered performance or impaired animal health. The rumen ecosystem is complex and highly adaptable (McSweeney and Mackie, 2012); therefore, other studies were conducted to more specifically examine microbial populations using either a more dynamic in vitro system or animal models. These systems allow for longer incubation times and use of mixed populations of rumen microbes. In addition to livestock studies, some rodent models have also been used to study impact of glyphosate on gut microbes. A study performed by Nielsen et al. (2018) where, the mice received 5 and 50 times the RfD showed no deleterious effects on the gut microbes.

Glyphosate in the rumen
Theoretical concentrations of glyphosate in the rumens of cattle can be calculated to put the experimental conditions of the above cited studies into context. Von Soosten (2016) measured glyphosate intake from the feed for dairy cattle and found a range of glyphosate intakes from 0.08 to 6.67 mg/d. Assuming an average BW of 680 kg, intakes would have ranged from 0.0001 to 0.01 mg/kg and the no effect level from EFSA is 50 mg/kg (EFSA, 2018a). Estimates for rumen volume and turnover (Stokes et al., 1985) can be used to determine daily liquid flow through the rumen. Based on the amount of glyphosate found in the dairy cow feed above, the concentrations of glyphosate ranged from 0.0000004 to 0.00004 mg/mL. In the risk assessment done by EFSA (2018a), the calculated maximum dietary burden for glyphosate consumed by dairy cattle using the most conservative assumptions of the highest possible intakes for legal application of glyphosate (predominantly based on glyphosate applied to grass) is 13.17 mg/kg. Using the same assumptions for ruminal kinetics, calculations result in a glyphosate concentration of 0.05 mg/mL. These data suggest that in vitro studies that use glyphosate at concentrations greater than 0.05 µg/mL use concentrations greater than ruminal bacteria would be exposed.

Chelating Properties of Glyphosate
Glyphosate is a zwitterion with 3 acidic protons that make it a tridentate chelating agent of divalent and trivalent metals, forming either 1:1 or 1:2 complexes. Many publications suggest that glyphosate was patented originally as a chelator in a patent issued to the Stauffer Chemical Company in 1964 and critics cite knowledge of this characteristic as an example of corporate malfeasance. However, glyphosate was discovered and patented as a herbicide in 1969 and was never even part of a claim in the Stauffer patent (Swarthout et al., 2018). This chemical property is often overstated as a mechanism whereby glyphosate application to plants limits mineral availability either by limiting uptake from the soil or limiting mineral transport in the phloem. This claim is not corroborated by the commercial viability of herbicide tolerant crops, since the EPSP synthase transgene does not provide any protection from chelation of minerals and yet these transgenic varieties have not shown yield losses when sprayed with glyphosate (Duke et al., 2012). Likewise, some have claimed that absorption of minerals in the digestive tract is perturbed by chelation from ingested residues of glyphosate. Most dietary minerals are fed at levels that greatly exceed the amounts of glyphosate residue that would be consumed. For ruminants, cobalt is required for microbial synthesis of vitamin B12 and the recommended daily amount in the diet is the least of all the minerals, thus Co would be at the lowest concentrations of the minerals in the rumen. Other ubiquitous anionic chemicals, such as amino acids and phytic acid can form complexes with cationic minerals in the rumen (Durand and Kawashima, 1980) and some amino acids are more prevalent and more potent chelators than glyphosate (Harris et al., 2012). Formation constants measure the strength of complexes between ions and ligands. By comparing these values for complexes of Co^{2+} with glyphosate (Motekaitis and Martell, 1985) and Co^{2+} with amino acids it is apparent that Co^{2+} is more likely to be bound to amino acids than glyphosate. Furthermore, glyphosate is more likely to bind Fe than Co^{2+} because the formation constant for glyphosate and Fe^{2+} is similar to that of Co^{2+}, and that with Fe^{3+} is signifcantly higher.

Conclusion
The mode of action for the herbicidal effect of glyphosate is through EPSP synthase. This enzyme does not exist in the cells of humans and other mammals, which is why aromatic amino acids are considered essential nutrients that must be supplied in the diet. Although some microbes have an EPSP synthase that is susceptible to glyphosate, it does not mean that glyphosate alters their ability to compete or function in the gut. Important factors when designing or interpreting model systems of gut microbes are to consider the impacts of single vs. multiple strains, batch vs. semicontinuous or continuous systems, turnover rates (i.e., growth rate), the concentration of glyphosate in the digesta, aerobic vs. anaerobic, the duration of culture and the relevance of endpoints to function. As with any model system, conclusions should be based on empirical results within the limitations of the model system. Likewise, the ability to form complexes with certain metal ions is a property of glyphosate, but it has not been found to impact animal nutrition due to the concentrations and interplay among competing ligands and ions, and the relative stabilities of alternative chelators to form complexes. The weight of the evidence suggests that glyphosate use in crops fed to poultry and

livestock has not affected animal health, rumen/gut microbes or production without affecting the safety of consuming meat, milk, and eggs.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: This paper reviews studies related to the occurrence and effects of glyphosate. It states the review of EFSA and FDA published results of surveys of foods and feeds, states the importance of validated and selective analytical methods used to determine glyphosate in food of plant and animal origin, discusses consumer risk assessment and approaches for calculation of animal exposure. The influence of glyphosate on gut microbes by inhibition of EPSP synthase is reviewed. The authors state, it is critical that in vivo studies or model systems which are used to investigate this issues accurately replicate the conditions of particular sections of the gastrointestinal tract. Batch culture studies are stated as difficult to extrapolate to actual conditions in the gastrointestinal tract.

In addition to livestock studies, some rodent models are mentioned which studied the impact of glyphosate on gut microbes and showed no deleterious effects on the gut microbes. It is stated that in vitro studies that use glyphosate at concentrations greater than 0.05 µg/mL use concentrations greater than ruminal bacteria would be exposed. Chelating properties of glyphosate are discussed in context of other ubiquitous anionic chemicals, such as amino acids and phytic acid, in context of formation constants and the high level of dietary minerals are fed.

Based on these points the paper concluded that the weight of the evidence suggests that glyphosate use in crops fed to poultry and livestock has not affected animal health, rumen/gut microbes or production without affecting the safety of consuming meat, milk, and eggs.

The publication represents a review and discussion of publically available literature focusing on the effects of glyphosate to gut microbes which is not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear.

Assessment and conclusion by RMS:

This paper concerns a review article, in which, among other things, the effect of glyphosate on the animal gut microbiota is reviewed. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.4.10. Study 10

1. Information on the study

Data point	CA 6.5
Report author	Clair E. *et al.*
Report year	2012
Report title	Effects of Roundup® and Glyphosate on Three Food Microorganisms: *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*
Document No.	Curr. Microbiol. (2012) Vol. 64, pp. 486-491
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

2. Full summary of the study according to OECD format

Use of many pesticide products poses the problem of their effects on environment and health. Amongst them, the effects of glyphosate with its adjuvants and its by-products are regularly discussed. The aim of the present study was to shed light on the real impact on biodiversity and ecosystems of Roundup®, a major herbicide used worldwide, and

the glyphosate it contains, by the study of their effects on growth and viability of microbial models, namely, on three food microorganisms (*Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) widely used as starters in traditional and industrial dairy technologies. The presented results evidence that Roundup® has an inhibitory effect on microbial growth and a microbicide effect at lower concentrations than those recommended in agriculture. Interestingly, glyphosate at these levels has no significant effect on the three studied microorganisms. Our work is consistent with previous studies which demonstrated that the toxic effect of glyphosate was amplified by its formulation adjuvants on different human cells and other eukaryotic models. Moreover, these results should be considered in the understanding of the loss of microbiodiversity and microbial concentration observed in raw milk for many years.

Materials and Methods

Strains and Culture Conditions

Experiments were performed with *G. candidum* ATCC 204307, *Lactobacillus delbrueckii* subsp. bulgaricus CFL1 and *Lactococcus lactis* subsp. *cremoris* ATCC 19257. *G. candidum* was cultured in MSF, pH 5.6; *L. lactis* in M17 containing lactose, pH 7.1 (AES Laboratoire, Combourg, France); and *L. bulgaricus* in MRS, pH 6.4 (AES Laboratoire). Cultures recovered in stationary phase of growth were used to inoculate new media at 2% (volume of liquid culture/volume of culture broth). The initial cell concentrations before treatments are $1.95 \times 10^4 \pm 0.36$, $6.31 \times 10^5 \pm 2.03$ and $6.70 \times 10^8 \pm 2.52$ UFC/mL for *G. candidum*, *L. bulgaricus* and *L. cremoris*, respectively.

Roundup and Glyphosate Treatments

Roundup R400 (400 g/Lof glyphosate) and R450 (450 g/L of glyphosate) (Monsanto, Anvers, Belgium) were diluted in autoclaved culture media, pH adjusted to each medium and 0.2 µm filtered. A solution of glyphosate (Sigma-Aldrich, Saint-Quentin Fallavier, France), equivalent in glyphosate concentration and pH to R450, was diluted in different media.

Turbidimetry Measurement and Colony Counting

Experiments were performed with 96-well plates. The minimal inhibitory concentration (MIC) was evaluated, after treatment, by turbidimetry measurement at 600 nm using a microplate reader (Metertech R960, Taipei, Taiwan). The minimal microbicide concentration (MMC) which corresponds to the minimal treatment leading to 99.99% of lethality was evaluated by colony counting, after plating the previously treated micro-organisms. Concentrations between the MIC value and the MMC value correspond to cells that do not growth but are not dead. For these experiments, it is not necessary to wash the cells before plating. The final dilution is so important that the herbicide residues are then negligible.

Colonies Observations

Each microorganism was plated on Petri dishes containing agar and Roundup, and then incubated during 48 h. Colonies were macroscopically observed using a Canon EOS 350D camera and microscopically using a Leica DMLB microscope (magnification 500x for *G. candidum* and 1000x for *L. bulgaricus* and *L. cremoris*) after coloration with cotton blue for *G. candidum* and methylene blue for the two bacteria.

Statistical Analysis

Data were expressed as mean ± SEM for three independent determinations (n = 9). Significant differences were determined by Student t test with $P < 0.05$*, $P < 0.01$** and $P < 0.001$***.

Results

Our first observation is the relatively comparable toxicity profiles on three essential food microorganisms, in a short 24-h period (Fig. 1a–c). Roundup is always more potent than glyphosate, and in all cases, toxic from levels 10–100 times below the lowest agricultural uses (10’000 ppm). *G. candidum* and *L. cremoris* are more Roundup sensitive than *L. bulgaricus*. Similar impacts have not been observed for glyphosate alone (except for *G. candidum* at 10,000 ppm). Roundup effect was not proportional to glyphosate concentration in the Roundup formulation, since R400 is almost 10-fold more inhibitory than R450 (Fig. 1a). A specific biphasic inhibitory effect was transiently observed at low doses (around 100 ppm) for *L. cremoris*, and possibly for *G. candidum*. Microbicide effect of Roundup was obtained at concentrations 1.2, 1.6 and 2 times higher than the measured MICs for *L. bulgaricus*, *G. candidum* and *L. cremoris*, respectively (Table 1).

Figure 1. Growth inhibition of the three microorganisms (**a** *Geotrichum candidum*, **b** *Lactococcus lactis* subsp. *cremoris* and **c** *Lactobacillus delbrueckii* subsp. *bulgaricus*) after 24 h of incubation in growth media supplemented with Roundup (grey circles 400 and black circles 450) or equivalent amount of glyphosate (triangles) evaluated by turbidimetry (600 nm). The herbicides' concentrations are up to the lowest agricultural uses (10'000 ppm). SEMs are shown in all instances (n = 3); Student test (P < 0.05*; P < 0.01** and P < 0.001***).

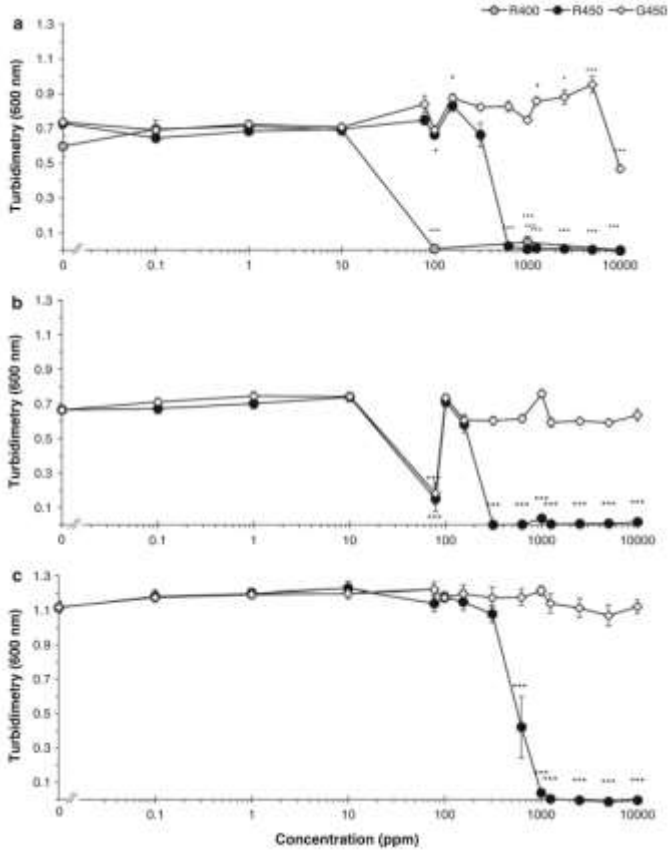


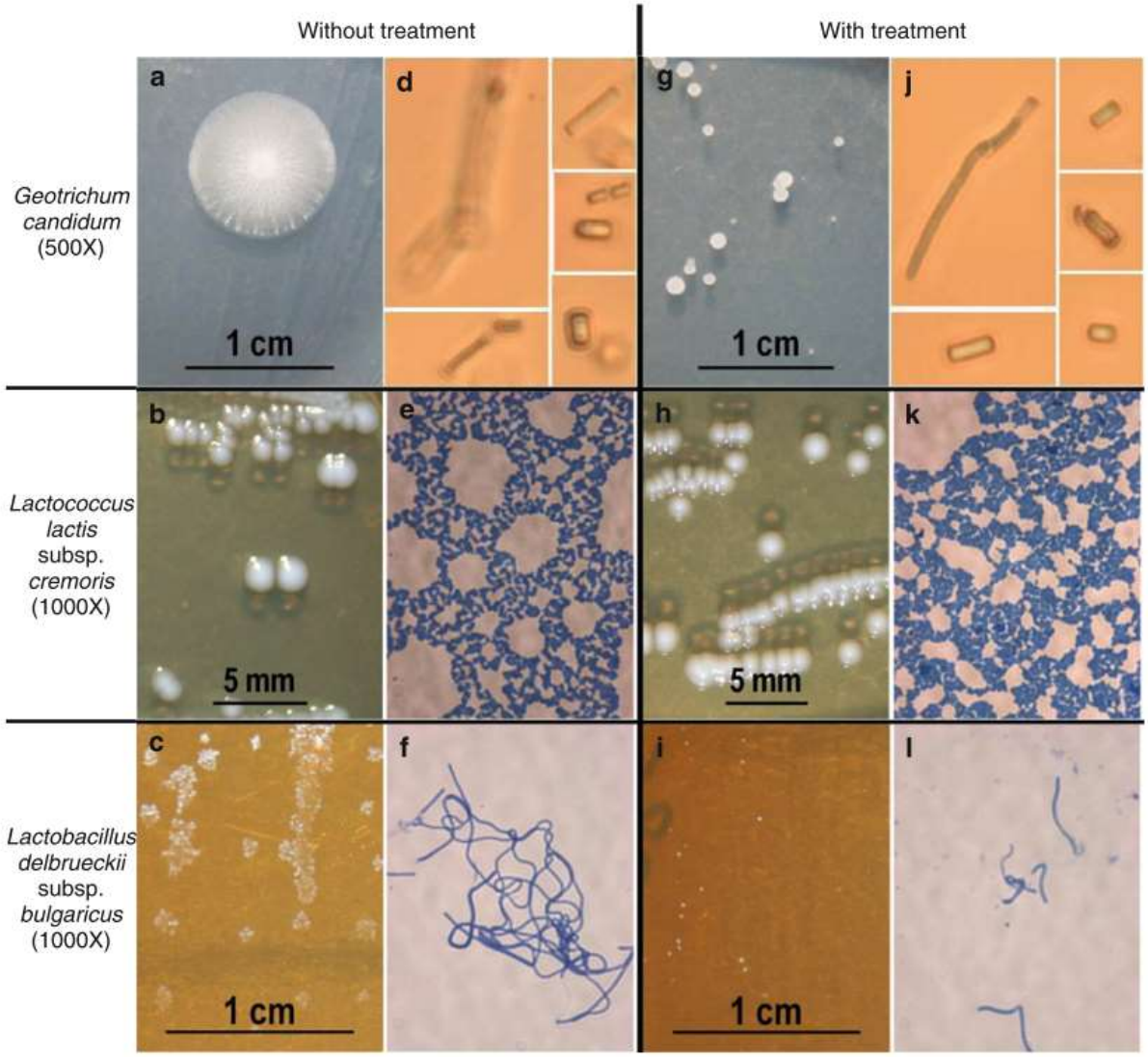
Table 1. MIC and MMC for three microorganisms (*Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) after 24 h of incubation in growth media supplemented with Roundup or equivalent amount of glyphosate.

Strain	Glyphosate in Roundup® (g/l)	MIC (ppm)	MMC (ppm)
G. candidum ATCC204307	400	100	1,000
	450	625	1,000
L. lactis subsp. *cremoris* ATCC19257	450	312	625
L. delbrueckii subsp. *bulgaricus* CFL1	450	1,000	1,250

The minimal agricultural use of the herbicide is 10,000 ppm

The macroscopic observations (Fig. 2) showed differences in sensitivity between the three micro-organisms: *L. cremoris, G. candidum* and *L. bulgaricus*, and no colonies were observed at 200, 1,000 and 2'000 ppm of Roundup, respectively. Considering the size, colonies of *G. candidum* and *L. bulgaricus* decrease depending on the concentration of Roundup (Fig. 2g, i) present. Microscopically, no significant differences were observed between controls and treated colonies. However, it seems that cell contents of *L. bulgaricus* leaks out (Roundup 1'000 ppm; Fig. 2l).

Figure 2. Macroscopic (**a**–**c**, and **g**–**i**) and microscopic (**d**–**f** and **j**–**l**) observations of *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, after 48 h of incubation on solid growth media supplemented with Roundup (500 ppm for **g** and **j**, 100 ppm for **h** and **k**, 1'000 ppm for **i** and **l**) or without (for a, d, b, e, c and f). The herbicide concentrations are up to the lowest agricultural uses (10'000 ppm).



Conclusion

The relatively comparable toxicity profiles on these three essential food microorganisms is surprising since the fungus is phylogenetically far from the other two. It has previously been shown that glyphosate and Roundup are toxic and can reduce fungal growth at concentrations above 10 ppm for mycorrhizal fungi (*Hebeloma crustuliniforme*, *Laccaria laccata*, *Thelephora americana*, *T. terrestris* and *Suillus tomentosus*). Similarly, some adverse effects were observed at concentrations below those found in the soil after typical applications. Moreover, differences in the sensitivities of the different species have also been observed towards the herbicide. The inhibitory and microbicide effects observed at agricultural sub-doses in this work are consistent with those previously observed in fungi. Glyphosate is believed to be the major active principle in the herbicide through its inhibition of EPSPS. Glyphosate metabolism varies by organism. Glyphosate blocks EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), a key enzyme involved in the biosynthesis of aromatic amino acids, naturally present in plants, fungi and some bacteria. EPSPS, which exists in two classes-glyphosate-sensitive and glyphosate-tolerant is essential in plants for protein production via the shikimic acid pathway. There could be an enzyme highly tolerant to glyphosate, which would have some genetical homologies with the first and/or the second classes of this enzyme. This enzyme is absent in mammals. However, other inhibition pathways are involved, like Cyp450 aromatase inhibition, since glyphosate is weakly responsible of the cytotoxicity on eukaryotic cells (human placental, embryonic or umbilical cord cells) and cellular endocrine disruption. This is in agreement with the lethality observed on the three food microorganisms studies in the present work. Moreover, like on eukaryotic cells, impact of glyphosate is not proportional to its concentration in Roundup formulations, confirming

adjuvants are not inert—an observation that supports the findings of previous studies. Similar effects on microorganisms have been reported previously in the literature; in fact, it appeared that *Ichthyophthirius multifiliis* and *T. thermophila* tolerate glyphosate but not Roundup. The commercial formulation was then 100 times more toxic than the active ingredient (G). Amongst these adjuvants, POEA which promotes a xenobiotic penetration into cells, is more toxic than glyphosate. Microorganisms studied here are 10 times less sensitive than human embryonic, placental and hepatic cells. At non-toxic concentrations, some endocrine biphasic disrupting effects have already been observed in human cells with Roundup. This could be the case of a receptor-mediated phenomenon with a stimulation followed by internalization and desensitization. Thus, if there is a stress reaction widely distributed in evolution, we could suggest that biphasic inhibitory effects at non-toxic doses of Roundup and glyphosate on *L. cremoris* and probably *G. candidum* could be receptor-mediated.
In conclusion, the pesticide Roundup sprayed on Roundup tolerant GMOs and on non-agricultural soils could thus impact on specific microbiodiversity including food interest microorganisms. This is illustrated by the fact that actual food processing requires industrial food starters for milk fermentation. Furthermore, unpredictable consequences of Roundup on soil microorganisms have to be considered.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: The article investigate the influence of glyphosate and Roundup on three food microorganisms (Geotrichum candidum, Lactococcus lactis subsp. cremoris and Lactobacillus delbrueckii subsp. bulgaricus) widely used as starters in traditional and industrial dairy technologies (milk processing). It was found evidence that Roundup® has an inhibitory effect on microbial growth and a microbicide effect at lower concentrations than those recommended in agriculture. Interestingly, glyphosate at these levels had no significant effect on the three studied microorganisms. Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. However, based on the results presented, it is not possible to reach a scientifically sound conclusion that the ability to make cheese using these organisms has been compromised by Roundup formulations. Application of dilutions (1%) of glyphosate were shown to inhibit a yeast-like organism, which is unsurprising. Surfactant solutions are routinely used to sanitize food processing equipment at concentrations at or above those tested by Clair et al. These concentrations are vastly higher than the concentrations of glyphosate or possible surfactant present (if any) in incoming milk.

This article does not provide any regulatory endpoints for metabolism and/or residues.

Assessment and conclusion by RMS:

This publicly available article investigated the effect of glyphosate on three food microorganisms, used in dairy industry. Since investigation of the gut microbiota is currently not part of the European assessment framework for pesticides, it is hard to assess the relevance of the findings. Until there are data requirements and accompanying guidelines to study pesticide effects on microbiota, and while there currently appear to be no adverse effects regarding glyphosate and its possible influence on the microbiota up to the calculated maximum animal exposure within the EU (EFSA Journal 2018;16(5):5283), the article is considered as having no further impact on the existing risk assessment parameters.

B.7.8.5. Studies excluded after detailed assessment, but included on request of the RMS

B.7.8.5.1. Study 1

Data point	CA 9
Author	Bandana B. *et al.*
Year	2015
Title	Dissipation kinetics of glyphosate in tea and tea-field under northwestern mid-hill conditions of India
Document source	Journal of Pesticide Science (2015), Vol. 40, No. 3, pp. 82-86
Short description of literature article	A study was conducted to determine the dissipation of glyphosate applied at three dose levels in tea crop in mid hill conditions of northwest Himalaya, India in two consecutive seasons. More than 65% of the initial residues in the soil were found to have dissipated within 30 days following application to the tea

	irrespective of the dose. Glyphosate persisted in the soil for up to 30, 45 and 60 days at application doses of 0.5, 1.0 and 2.0 kg/ha, respectively. Glyphosate residues in the tea leaves were detected for up to 15 days with all three treatments. Half lives of glyphosate ranged from 5.80 to 19.10 days in the soil of the tea fields and 5.82 to 7.91 days in the tea leaves at the three doses. Glyphosate concentrations in the tea leaves were found to be below the maximum residue limit (1 mg/kg).
Short description of findings	Glyphosate persisted in the soil for 30 to 60 days, following applications of 0.5 to 2.0 kg/ha in tea crops. Glyphosate residues in the tea leaves were detected up to 15 days at all three treatment doses. The above findings indicate that glyphosate dissipated in tea plants within 15 days of application and that it is safe to use glyphosate in tea plantations at a recommended dose of 1.0 kg/ ka in tea gardens. However, on the basis of the present study the mechanism of the degradation behavior of glyphosate could not be confirmed and needs further investigation.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The article concerns a crop that is not a representative crop for the glyphosate EU renewal.
Assessment and conclusion by RMS	The article investigates the glyphosate decline in soil and tea leaves after foliar application to the crop in Asia. Since tea is not among the crops of the defended use for the current renewal of glyphosate, and also foliar applications are not within the requested use, and it concerns a field investigation outside the EU, there is no further impact expected on the existing risk assessment parameters.

B.7.8.5.2. Study 2

Data point	CA 9
Author	Barker A. L. *et al.*
Year	2019
Title	Fate of Glyphosate during Production and Processing of Glyphosate-Resistant Sugar Beet (*Beta vulgaris*).
Document source	Journal of agricultural and food chemistry (2019), Vol. 67, No. 7, pp. 2061-2065
Short description of literature article	Glyphosate is a widely used herbicide in commercial crop production for both conventional and herbicide-resistant crops. Herbicide-resistant crops, like glyphosate-resistant sugar beet, are often exposed to multiple applications of glyphosate during the growing season. The fate of this herbicide in resistant crops has not been publicly documented. The researchers investigated the fate of glyphosate and main metabolite aminomethylphosphonic acid in glyphosate-resistant sugar beet grown in northern Colorado. Glyphosate residues were measured via directed ultra-high-performance liquid chromatography tandem mass spectrometry analysis of sugar beet shoots and roots throughout the growing season, from samples collected at various steps during sugar beet processing, and from flow-through samples of greenhouse-grown beets. Sugar beet rapidly absorbed glyphosate after foliar application, and subsequently translocated the herbicide to its roots, with between 2 and 3 μg/g fresh weight measured in both tissue types within 1 week of application. However, only trace amounts of glyphosate remained in either the shoots or the roots 2 weeks after application. Analysis of irrigation flow-through in pot assays confirmed that the herbicide readily exuded out of the roots. Processing of the beets removed glyphosate and herbicide levels were below the limit of detection in the crystalline sugar final product.
Short description of findings	The article investigates the fate of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in glyphosate-resistant sugar beet. The results show that sugar beet rapidly absorbed glyphosate after foliar application, and subsequently translocated the herbicide to its roots, with

	between 2 and 3 mg/kg fresh weight measured in both tissue types within 1 week of application. Only trace amounts of glyphosate remained in either the shoots or the roots 2 weeks after application. Analysis of irrigation flow-through in pot assays confirmed that the herbicide readily exuded out of the roots. In conclusion, glyphosate is an outstanding tool to manage weeds in sugar beet production. Its ability to translocate and exude from the roots ensures that beets have very low levels of the herbicide by harvest time. Furthermore, processing of the juice into crystalline sugar removes any trace of this herbicide from the final product.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The article provides information on processing of glyphosate in glyphosate tolerant sugar beet. This is not a relevant use for the glyphosate EU renewal.
Assessment and conclusion by RMS	This public article investigated the residue levels of glyphosate and AMPA in glyphosate-resistant sugar beets, also after processing. Although, indeed glyphosate-resistant sugar beets are not among the crops of the defended use for the current renewal of glyphosate; and also foliar applications are not within the requested use; and the full reliability of the results is to be assessed; and studies investigating the magnitude of the residue after processing are not required for sugar beets; it is, however, of interest to note that no glyphosate ended up in the final processed product sugar. There is no further impact expected on the existing risk assessment parameters.

B.7.8.5.3. Study 3

Data point	CA 9
Author	Gélinas P. *et al.*
Year	2018
Title	Wheat preharvest herbicide application, whole-grain flour properties, yeast activity and the degradation of glyphosate in bread
Document source	International journal of food science & technology (2018), Vol. 53, No. 7, pp. 1597-1602
Short description of literature article	The aim of this study was to determine the effects of wheat preharvest application of a glyphosate-based herbicide (Roundup WeatherMax® with Transorb® 2 Technology) on whole-grain flour composition and properties, including yeast activity. The effect of dough fermentation on the degradation of herbicide residues was also estimated. Grain samples from two hard red spring wheat varieties exceeded the maximum residue limits (5 mg/kg) in Canada. Glyphosate had minor effects on wheat kernels composition and properties, including fructans content and yeast gassing power. No degradation of Roundup® or pure glyphosate was seen after dough fermentation for up to 4 h and baking. These results call for more scientific studies on glyphosate residues in wheat.
Short description of findings	The article investigates the effect of pre-harvest applications of glyphosate on wheat whole-grain composition and dough properties. Only minor effects of glyphosate were observed. Yeast gassing power was not inhibited in dough spiked with glyphosate, and no degradation of the herbicide was seen during dough fermentation.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The artile provides information on effects of glyphosate on flour properties in baking following pre-harvest application in wheat. This is not a relevant use for the glyphosate EU renewal. In addition, the formulation tested is not the representative formulation for the glyphosate EU renewal.
Assessment and conclusion by RMS	The study investigated the effect of glyphosate on whole-grain composition after application on wheat, and the subsequent dough properties. Although, indeed cereals are not among the crops of the defended use for the current

	renewal of glyphosate; and also pre-harvest applications are not within the requested use; and the full reliability of the results is to be assessed; and studies investigating the magnitude of the residue after processing are not required for cereals; it is, however, of interest to note the levels of spiked glyphosate in dough during baking. There is no further impact expected on the existing risk assessment parameters.

B.7.8.5.4. Study 4

Data point	CA 9
Author	Perboni L. T. *et al.*
Year	2018
Title	Yield, germination and herbicide residue in seeds of preharvest desiccated wheat
Document source	Journal of Seed Science (2018), Vol. 40, No. 3, pp. 304-312
Short description of literature article	The goals of this study were to evaluate herbicide application rates at different timings for preharvest desiccation of wheat (Trial 1), as well as to evaluate the effect of the timing of herbicide desiccation at preharvest and harvest timing (Trial 2) on yield, germination, and herbicide residue in wheat seed. In Trial 1, treatments consisted of two application rates of glufosinate, glyphosate, paraquat, or paraquat + diuron and a control without application; application time periods were in the milk grain to early dough stage, soft dough to hard dough stage, and hard dough stage. In Trial 2, treatments consisted of different application time periods (milk grain to early dough stage, and soft dough to hard dough stage), different herbicides (glufosinate, 2,4-D + glyphosate, and untreated control), and different harvest times (5, 10 and 15 days after herbicide application). One thousand seeds weight, yield, first and final germination count, and herbicide residue on seeds were evaluated. Preharvest desiccation with paraquat, glufosinate, and 2,4-D + glyphosate at the milk grain to early dough stage reduces wheat yield. Regardless of the herbicide and application rate, application in the milk grain to early dough stage and soft dough to hard dough stage provides greater germination of wheat seeds, except at the lower dose of paraquat. Systemic herbicides accumulate more in wheat seeds.
Short description of findings	The article investigates the effect of pre-harvest herbicide applications (glufosinate, glyphosate (± 2,4-D), paraquat (± diuron)) on desiccation of wheat and on yield, germination, and herbicide residue in wheat seed. Pre-harvest desiccation with paraquat, glufosinate, and glyphosate + 2,4-D at the milk grain to early dough stage reduced wheat yield. Regardless of the herbicide and application rate, application in the milk grain to early dough stage and soft dough to hard dough stage provided greater germination of wheat seeds, except at the lower dose of paraquat. Preharvest desiccation with systemic herbicides leads to greater accumulation of residues in wheat seeds.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The article provides information on glyphosate pre-harvest use in wheat. This is not a relevant use for the glyphosate EU renewal.
Assessment and conclusion by RMS	This article studies the effect of application rates and timing of application of a glyphosate pre-harvest use on wheat, including its residue levels in grain. Since cereals are not among the crops of the defended use for the current renewal of glyphosate, and also pre-harvest applications are not within the requested use, there is no further impact expected on the existing risk assessment parameters.

B.7.8.5.5. Study 5

Data point	CA 9

Author	Zhao J. *et al.*
Year	2018
Title	Detection of glyphosate residues in companion animal feeds
Document source	Environmental pollution (2018), Vol. 243, No. Pt B, pp. 1113-1118
Short description of literature article	The widespread adoption of genetically modified, glyphosate-tolerant corn and soybean varieties in US crop production has led to a dramatic increase in glyphosate usage. Though present at or below regulatory limits currently set for human foodstuffs, the concentration of glyphosate in companion animal feed is currently unknown. In the present study, 18 commercial companion animal feeds from eight manufacturers were analyzed for glyphosate residues using ELISA. Every product contained detectable glyphosate residues in the range of 7.83×10^1 - 2.14×10^3 µg/kg dry weight, with the average and medians being 3.57×10^2 and 1.98×10^2 mg/kg respectively. Three products were tested for within-bag variation and six were tested for lot to lot variation. Little within-bag variation was found, but the concentration of glyphosate varied by lot in half of the products tested. Glyphosate concentration was significantly correlated with crude fiber content, but not crude fat or crude protein. Average daily intakes by animals consuming feeds containing the median glyphosate concentration are estimated to result in exposures that are 0.68-2.5% of the Allowable Daily Intake (ADI) for humans in the US and EU, which are 1750 and 500 mg kg^{-1} respectively. Consumption of the most contaminated feed, however, would result in exposure to 7.3% and 25% of the above ADIs, though the relevance of such an exposure to companion animals is currently unknown. Companion animal feeds contained 7.83×10^1 - 2.14×10^3 µg/kg glyphosate which is likely to result in pet exposure that is 4-12 times higher than that of humans on a per Kg basis.
Short description of findings	Glyphosate was found in all 18 companion animal feeds examined. The concentrations ranged from 7.83×10^1 - 2.14×10^3 µg/kg. The mean and median concentrations were 3.57×10^2 and 1.98×10^2 mg/kg respectively. Glyphosate concentration correlated with the fiber content of the feeds, but not with either fat or protein, suggesting a plant origin. The glyphosate concentrations in the companion animal foods the researchers tested were higher than those reported for average human diets, but within the maximum residue limits currently set for human foodstuffs (European Food Safety Authority, 2015; Office of the Federal Registry, 2017). The tendency of companion animals to eat more as a percent of their body weight than humans do, combined with higher glyphosate levels in their food suggests that their exposure to glyphosate may be 2.7-28 times higher on a body weight basis depending on the feed. The impact of such exposures on companion animals is currently unknown. Future studies should focus on understanding the long-term impacts of these low-dose exposures on companion animals.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Analysis of glyphosate residues in companion animal feed. Transgenic crop is not relevant for the glyphosate EU renewal.
Assessment and conclusion by RMS	This study investigated the presence of glyphosate in companion animal feed. The impact of such glyphosate exposure on pets is currently unknown, and not within the scope of the current renewal framework of glyphosate. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6. Studies considered as non-relevant after a rapid assessment, but included on request of the RMS

B.7.8.6.1. Study 1

Data point	CA 9
Author	Anonymous

Year	2015
Title	On the trail of "uninvited guests"
Document source	Brot + Backwaren (2015), pp. 72-73, No. 4
Short description of literature article	During the European Cereal Monitoring for the recently completed 2014/2015 grain production year, 98 mills as well as six large agricultural trading groups have provided samples. The uniform standard programme provides for investigations of hundreds pesticides, mycotoxins such as aflatoxins, ochratoxin A etc., heavy metals such as lead and cadmium, as well as investigations of the microbiological status of the relevant batch. The varying investigations address topics specified annually by a European Cereal Monitoring Task Force. This year the spotlight is on ergot alkaloids and glyphosate. In addition, checks were made for stalk reducing substances, other plant protection products, mycotoxins, for which there are so far no maximum values, GMO components, accidentally present allergens such as soya, lupins or mustard, as well as for the possible presence of antibiotics, which may, for example, enter into or onto the cereal via sludge or livestock manure inputs.
Short description of findings	The investigations conducted by Biotask clearly demonstrate that, since then, no glyphosate was detected in almost 90% of the investigated samples and that consequently cereals and milled products are not significant sources of entry of this herbicide into human food.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of cereals is not a representative use for renewal.
Assessment and conclusion by RMS	The article concerns monitoring data of, among others, glyphosate in wheat and wheat flour. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6.2. Study 2

Data point	CA 9
Author	Belter A.
Year	2016
Title	Long-term monitoring of field trial sites with genetically modified oilseed rape (*Brassica napus* l.) in Saxony-Anhalt, Germany. fifteen years persistence to date but no spatial dispersion
Document source	Genes, (2016) Vol. 7, No. 3 doi:10.3390/genes7010003
Short description of literature article	Oilseed rape is known to persist in arable fields because of its ability to develop secondary seed dormancy in certain agronomic and environmental conditions. If conditions change, rapeseeds are able to germinate up to 10 years later to build volunteers in ensuing crops. Extrapolations of experimental data acted on the assumption of persistence periods for more than 20 years after last harvest of rapeseed. Genetically-modified oilseed rape-cultivated widely in Northern America since 1996-is assumed not to differ from its conventional form in this property. Here, experimental data are reported from official monitoring activities that verify these assumptions. At two former field trial sites in Saxony-Anhalt genetically-modified herbicide-resistant oilseed rape volunteers are found up to fifteen years after harvest. Nevertheless, spatial dispersion or establishment of GM plants outside of the field sites was not observed within this period.
Short description of findings	Long-term persistence of viable rape seeds at agronomic sites is exceedingly promoted by high quality soil conditions. It is very difficult to remove rape seeds that once reached the soil seed bank and hinder them to germinate years later. The observations showed no spatial dispersion effects of genetically-modified herbicide resistant OSR in the environment of the former release sites over many

	years despite of the persistence. All these findings are able to prove the substantial equivalence of conventional and GM OSR, except the trait.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Transgenic crop is not relevant for the glyphosate EU renewal.
Assessment and conclusion by RMS	In this publication, persistence of genetically modified oilseed rape in agronomic sites is being investigated. Since glyphosate-resistant oilseed rape is not among the crops of the defended use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.3. Study 3

Data point	CA 9
Author	Bernal J. *et al.*
Year	2012
Title	Development and application of a liquid chromatography-mass spectrometry method to evaluate the glyphosate and aminomethylphosphonic acid dissipation in maize plants after foliar treatment
Document source	Journal of Agricultural and Food Chemistry, (2012) Vol. 60, No. 16, pp. 4017-25.
Short description of literature article	A simple and fast method has been developed and validated to measure glyphosate (GLYP) and aminomethylphosphonic acid (AMPA), which were previously derivatized with 9-fluorenylmethylchloroformate (FMOC-Cl), in maize plants using liquid chromatography (LC) coupled to fluorescence (FLD) and electrospray ionization mass spectrometry (ESI-MS) detection. The method has shown to be consistent, reliable, precise, and efficient. Moreover, the limits of detection (LOD) and quantification (LOQ) reached with the proposed method for GLYP and AMPA are lower than the established maximum residue levels (MRLs). The validated method was applied to quantify GLYP and AMPA in genetically modified (GM) maize foliar treated with the herbicide. It has been found that the GLYP dissipation was mainly due to the progressive dilution effect after herbicide treatment. Finally, it was also observed that the GLYP residue dissipation trend in maize shoot (leaves and stem) tissue determined by LC-ESI-MS matched that determined by liquid scintillation
Short description of findings	In this work, a fast and simple LC-FLD-ESI-MS method has been developed to determine GLYP and AMPA, which were online derivatized with FMOC-Cl before passing through the column, in maize plants. Finally, it could be postulated that the GLYP residue dissipation trend in maize shoot obtained using ESI-MS was comparable with those determined by LSS. However, it must be mentioned that the proposed LC-ESI-MS method permitted one to distinguish between GLYP and AMPA and the use of radiolabelled GLYP was not required; meanwhile, the LSS had the advantage of being more economic than the LC-ESI-MS system, and at the same time the consumption of reagents and solvents was lower.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Analytical method development or validation without information useful for the risk assessment (determination of spiked samples etc.)
Assessment and conclusion by RMS	The article describes primarily the development of an analytical method for the determination of glyphosate and AMPA in maize plants. In addition, the developed method has been applied to genetically modified maize plants, which were treated with glyphosate. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6.4. Study 4

Data point	CA 9

Author	Bhattacherjee A. K. *et al*.
Year	2016
Title	High performance liquid chromatographic determination of chlorpyriphos and glyphosate residues in mango orchard soil
Document source	Natural Resource Management: Ecological Perspectives, Volume 1. Proceedings of the Indian Ecological Society International Conference, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, India, 18-20 February (2016), 839 p
Short description of literature article	Chlorpyriphos, an organophosphate insecticide with contact mode of action, is used widely in mango ecosystem for the management of various insect pests and glyphosate, a broad spectrum systemic herbicide, is widely used to control various weeds. Chlorpyriphos (2.5 and 5.0 mL/L of water) and glyphosate (8.0 and 16.0 mL/L of water) were applied to mango (*Mangifera indica* L.) (cv. Dashehari) orchard soil to study their dissipation pattern in soil and subsequent uptake in fruit. Soil samples were collected periodically at 10 days interval up to 50 days and fruit samples were collected at 15 days interval from second week of May to third week of June, 2013 (up to harvest) for both the pesticides. Chlorpyriphos and glyphosate were extracted from soil samples with hexane: acetone (1:1, v/v) and water: dichloromethane (4:1, v/v) mixtures, respectively, by mechanical agitation with a shaker. Both the pesticides were analyzed by HPLC coupled with a photodiode array detector and rheodyne injector.
Short description of findings	Chlorpyriphos persisted in mango orchard soil up to 50 days and dissipated from 5.05 and 8.30 mg/kg after 1h of application to 0.75 and 1.07 mg/kg after 50 days of application at single and double doses, respectively. Glyphosate dissipated in mango orchard soil from 3.37 and 6.12 mg/kg after 1h of treatment to 0.61 and 1.04 mg/kg after 30 days of treatment at single and double doses, respectively. No residue was detected in soil beyond 30 days after its application. The residual half-lives in mango orchard soil were calculated as 18 and 17 days for chlorpyriphos, and 13 and 12 days for glyphosate from normal and higher doses, respectively. The rate of their dissipation followed first-order kinetics in soil at both the concentrations. No residues of either chlorpyriphos or glyphosate were detected in unripe as well as ripe mango fruits. Hence, both the pesticides can be considered safe for their respective use.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: This is a conference abstract only. Analytical method development or validation without information useful for the risk assessment (determination of spiked samples etc.)
Assessment and conclusion by RMS	This publication is a conference contribution. Therefore, no original data and only an undetailed summary is available. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6.5. **Study 5**

Data point	CA 9
Author	Bøhn T. *et al*.
Year	2014
Title	Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans
Document source	Food Chemistry (2014), Vol. 153, pp. 207-215
Short description of literature article	This article describes the nutrient and elemental composition, including residues of herbicides and pesticides, of 31 soybean batches from Iowa, USA. The soy samples were grouped into three different categories: (i) genetically modified, glyphosate-tolerant soy (GM-soy); (ii) unmodified soy cultivated using a conventional ‘‘chemical’’ cultivation regime; and (iii) unmodified soy cultivated

	using an organic cultivation regime. Organic soybeans showed the healthiest nutritional profile with more sugars, such as glucose, fructose, sucrose and maltose, significantly more total protein, zinc and less fibre than both conventional and GM-soy. Organic soybeans also contained less total saturated fat and total omega-6 fatty acids than both conventional and GM-soy. GM-soy contained high residues of glyphosate and AMPA (mean 3.3 and 5.7 mg/kg, respectively). Conventional and organic soybean batches contained none of these agrochemicals. Using 35 different nutritional and elemental variables to characterise each soy sample, we were able to discriminate GM, conventional and organic soybeans without exception, demonstrating ''substantial non-equivalence'' in compositional characteristics for 'ready-to-market' soybeans.
Short description of findings	The study demonstrated that Roundup Ready GM-soy may have high residue levels of glyphosate and AMPA, and also that different agricultural practices may result in a markedly different nutritional composition of soybeans. In the present study organic soybean samples had a more profitable nutritional profile than industrial conventional and GM soybeans. We argue that pesticide residues should have been a part of the compositional analyses of herbicide tolerant GM plants from the beginning. Lack of data on pesticide residues in major crop plants is a serious gap of knowledge with potential consequences for human and animal health. The authors therefore recommend (i) increased effort on sampling and testing crop material from the market; (ii) testing for possible dose–response effects of chemical residues in long-term feeding studies; (iii) inclusion of pesticide residue measurements and safety testing in the regulatory system for risk-assessment and (iv) further research on the indirect ecological effects of herbicides and pesticides, i.e., on ecological interactions in the soil community with possible effects on nutrient uptake and plant composition.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Transgenic crop is not relevant for the glyphosate EU renewal.
Assessment and conclusion by RMS	This paper investigated the nutrient and elemental composition, including glyphosate and AMPA levels, of different categories of soybeans (genetically modified, conventional and organic). Since genetically modified crops are not among the crops of the defended use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.6. Study 6

Data point	CA 9
Author	Flachowsky G.
Year	2013
Title	Feeding studies with first-generation GM plants (input traits) with food-producing animals
Document source	Flachowsky, G. (2013) pp. 72-93. Animal Nutrition with Transgenic Plants. Publisher: CABI PUBLISHING, CABI Biotechnology Series. ISBN-13: 978 1 78064 176 8
Short description of literature article	Nutritionists distinguish genetically modified (GM) plants mostly into first-generation plants and second-generation plants. This designation is purely pragmatic or historical; it does not reflect any particular scientific principle or technological development. The first generation of GM plants is generally considered to be crops carrying simple input traits such as increased resistance to pests or tolerance against herbicides. Other inputs, such as more efficient use of water and/or nutrients or an increased resistance against heat and drought are not expected to cause any substantial change in composition and nutritive value. Such plants could also be considered from the nutritional point of view as plants of the first generation. The newly expressed proteins that confer these effects occur in modified plants at very low concentrations and do not change their composition

	or feeding value significantly when compared with isogenic lines. The paradigm of the so-called 'substantial equivalence' (OECD, 1993) is the frame of the assessment and the first step of the assessment based on composition, but the substantial equivalence is not relevant for biotech crops of the next generation (crops with output traits; Llorente *et al*., 2011). Some authors consider this traditional assessment (comparison with 'known' traditional/historical counterparts) as not really science based and propose a registration and assessment of newly expressed criteria. In the case of plants with input traits, the gene products are functional proteins that affect a plant pest adversely or confer herbicide tolerance. These genes are not normally expected to affect biochemical pathways or cascades (Herman *et al*., 2009). Therefore, some authors (e.g. Matten *et al*., 2008; Herman *et al*., 2009; Giddings *et al*., 2012) criticize the present regulations and suggest that compositional assessment and feeding studies with feed from first-generation GM plants are no more necessary for evaluating the safety of transgenic crops than they are for plants bred traditionally.
Short description of findings	Since 1996 (Hammond *et al*., 1996), about 150 feeding studies with feeds from first generation GM plants (GM plants with input traits) in food-producing animals have been reported in the scientific literature (see FASS, 2013). Such plants did not show biologically relevant effects on the composition of the feed. Therefore, no biologically relevant effects on animal health and welfare, animal yields and the quality of products of animal origin are expected. In summary, feeding studies with feeds from first-generation GM plants in food-producing animals do not add substantial knowledge to feed science and animal nutrition because of the substantial equivalence of such plants/feeds to their isogenic counterparts. From the present perspective, there is no reason to use other feed value tables for such feeds in animal feeding. Feeds from first-generation GM plants can be used as traditional feeds under consideration of their composition to meet the energy and nutrient requirements (NRC, 1994, 1998, 2001; GfE, 1999, 2001, 2008) of food-producing animals.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Transgenic crop is not relevant for the glyphosate EU renewal.
Assessment and conclusion by RMS	This publication concerns a book chapter, and as such it is more considered as a review of studies, related to animal feed based on genetically modified crops. Since genetically modified crops are not among the crops of the defended use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.7. **Study 7**

Data point	CA 9
Author	Juraske R. *et al*.
Year	2011
Title	Pesticide uptake in potatoes: model and field experiments
Document source	Environmental Science & Technology (2011), Vol. 45, No. 2, pp. 651-657
Short description of literature article	A dynamic model for uptake of pesticides in potatoes is presented and evaluated with measurements performed within a field trial in the region of Boyaca´, Colombia. The model takes into account the time between pesticide applications and harvest, the time between harvest and consumption, the amount of spray deposition on soil surface, mobility and degradation of pesticide in soil, diffusive uptake and persistence due to crop growth and metabolism in plant material, and loss due to food processing. Food processing steps included were cleaning, washing, storing, and cooking. Pesticide concentrations were measured periodically in soil and potato samples from the beginning of tuber formation until harvest. The model was able to predict the magnitude and temporal profile

	of the experimentally derived pesticide concentrations well, with all measurements falling within the 90% confidence interval. The fraction of chlorpyrifos applied on the field during plant cultivation that eventually is ingested by the consumer is on average10^{-4}-10^{-7},depending on the time between pesticide application and ingestion and the processing step considered.
Short description of findings	As for future application, the model developed could be combined with application data of local farmers in order to identify risks and, if necessary, measures to mitigate human exposure to pesticides. The model could furthermore serve as a pest management tool in advising farmers in regard to their pesticide application schemes and in the calculation of sufficient waiting times before harvest.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Residue trial conducted outside the EU, climatic conditions not comparable. Exposure assessment based on non-EU consumption data.
Assessment and conclusion by RMS	This paper investigated a model on pesticide uptake in potatoes in Colombia. The study didn't show any results on glyphosate. In addition, it concerns a field investigation outside the EU. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6.8. Study 8

Data point	CA 9
Author	Kniel B. *et al.*
Year	2015
Title	Studies examining glyphosate's introduction pathways into cereals and cereal-based milled grain products and baked goods
Document source	Cereal Technology (2015), No. 1, pp. 21-27
Short description of literature article	Glyphosate is the most popular herbicide used worldwide. Within the past 10 years, the number and range of applications have clearly increased in Germany. Annual domestic sales are currently between 5,000 and 6,000 t. According to calculations, 39% of arable land is treated with glyphosate, particularly land used for the cultivation of winter oilseed rape, winter barley and grain legumes. Some of the areas where wheat and rye are cultivated are also treated. For these crops, the focus is placed on treatment at the time of sowing, while pre-harvest treatment (crop desiccation) is conducted with a clearly reduced scope. The use of this cross-spectrum herbicide for grain crops has increasingly been discussed over the last few years due to the wealth of critical publications in print media and television reports on the use of glyphosate in grain cultivation and on residues in cereals and the food made from such raw materials. Currently, there are no published findings available that explain how glyphosate migrates into grain and cereal food. The introduction pathways of this herbicide and its major metabolite AMPA into bread cereal and its role in the production of milled grain products and baked goods have been investigated in the context of this study. The results of the study suggest that glyphosate is introduced into the grain mainly via pre-harvest treatments and not via the treatment applied prior to sowing. Shorter waiting periods prior to harvest tend to result in lower concentrations than a waiting time of seven days or more (as is common practice). As expected, during the milling process glyphosate accumulates in the bran fraction, while in the refined flour it is considerably depleted. Glyphosate in grain and milled cereal products will not be degraded during storage or in the production process of baked goods. The herbicide tolerates the usual dough production methods for wheat and rye bread and is heat-stable in the baking process.
Short description of findings	The results of the glyphosate application trials suggest that this herbicide is primarily introduced into cereals by means of pre-harvest treatment rather than via pre-sowing treatments. Shorter waiting periods tended to result in lower levels

	compared to the usual waiting period of about seven days or even longer waiting periods. Obviously, the cereal crop can absorb more glyphosate in active metabolic phases than in the phase just before harvesting, when metabolic activity is already restricted. As expected, during milling glyphosate is enriched in the bran fraction, which is rich in hulls, while in contrast it is significantly depleted in superfine flour. Glyphosate in cereals and milled grain products does not degrade during storage. This does not happen either during the production of baked goods. The herbicide withstands the usual dough leavening processes for wheat and rye breads and is heat-stable during the baking process.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of cereals is not a representative use for renewal.
Assessment and conclusion by RMS	This study investigated how/when glyphosate is taken up in cereal grains, and how glyphosate and AMPA are being processed in grain products. Although, cereals are not among the crops of the defended use for the current renewal of glyphosate; and also pre-harvest applications are not within the requested use; and the full reliability of the results is to be assessed; and studies investigating the magnitude of the residue after processing are not required for cereals; it is, however, of interest to read on the findings that glyphosate would not degrade in processed grain products during storage or processing. There is no further impact expected on the existing risk assessment parameters.

B.7.8.6.9. Study 9

Data point	CA 9
Author	Lindemann da Silva I. *et al.*
Year	2017
Title	Foliar desiccators glyphosate, carfentrazone, and paraquat affect the technological and chemical properties of cowpea grains
Document source	Journal of Agricultural and Food Chemistry, (2017) Vol. 65, No. 32, pp. 6771-6778.
Short description of literature article	The effects of the use of glyphosate (GLY), glyphosate plus carfentrazone (GLY/CAR), and paraquat (PAR) as plant desiccators on the technological and chemical properties of cowpea grains were investigated. All studied desiccants provided lower cooking time to freshly harvested cowpea. However, the coat color of PAR- and GLY/CAR-treated cowpea was reddish in comparison to the control treatment. Principal component analysis (PCA) from liquid chromatography-mass spectrometry (LC-MS) data sets showed a clear distinction among cowpea from the different treatments. Catechin-3-glucoside and epicatechin significantly contributed for discriminating GLY-treated cowpea, while citric acid was responsible for discriminating GLY/CAR-treated cowpea. Quercetin derivative and gluconic acid were responsible for discriminating control treatment. Residual glyphosate and paraquat content was higher than the maximum limits allowed by Codex Alimentarius and the European Union Commission. Improvements in the technological and chemical properties of cowpea may not be overlapped by the risks that those desiccants exhibit when exceeding the maximum limits of tolerance in food.
Short description of findings	Transcriptome, proteome, and metabolome studies may help to deeply understand the metabolic responses of cowpea to the different commercial desiccants. Moreover, studies dealing with other types of herbicides, desiccant doses, period of desiccant application, and conditions will help farmers and bean industries to find alternatives for a safe cowpea harvesting in large areas.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of pulses is not a representative use for renewal.

Assessment and conclusion by RMS	The article investigated the effect of glyphosate as plant desiccator on cowpea grains. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.10. Study 10

Data point	CA 9
Author	Marcinkowska K.
Year	2017
Title	The residues of glyphosate in grain and straw of spring wheat and germination of grain after pre-harvest application of herbicide
Document source	Progress in Plant Protection (2017), Vol. 57, No. 1, pp. 95-100
Short description of literature article	The influence of six herbicidal ionic liquids include glyphosate [*N*-(phosphonomethyl)glycine] using in spring wheat as the preharvest application (BBCH 87) was studied in 2013–2014. Tested compounds had not negative impact on seed germinative energy and germination capacity. There were not residues of glyphosate and its main metabolite AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propionic acid] in grains of spring wheat. Residues of these compounds were below the limit of quantification (< 0.01 mg/kg). The presence of AMPA in the straw was not detected, while the residues of glyphosate were negligible (0.01-0.02 mg/kg).
Short description of findings	The application of herbicidal ionic liquids containing glyphosate as well as commercial forms of this herbicide during the hard dough stage (BBCH 87) of spring wheat development showed no negative impact on the germination energy or the germination capacity of grain. After the preharvest application of glyphosate in the form of ionic liquids in spring wheat, no contamination of the grain with this compound and its main metabolite AMPA was found. The presence of AMPA in the straw was not detected, while the residues of glyphosate were negligible (0.01-0.02 ppm).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of cereals is not a representative use for renewal.
Assessment and conclusion by RMS	The paper investigated the effect of pre-harvest use of glyphosate on wheat. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.11. Study 11

Data point	CA 9
Author	McNaughton K. E. *et al*.
Year	2015
Title	Effect of application timing of glyphosate and saflufenacil as desiccants in dry edible bean (*Phaseolus vulgaris* L.).
Document source	Canadian Journal of Plant Science, (2015) Vol. 95, No. 2, pp. 369-375.
Short description of literature article	Early application of desiccants in dry edible bean may cause yield reductions and unacceptable herbicide residue levels, resulting in rejection of exported shipments. The effect of application timing of two registered desiccants, glyphosate and saflufenacil, was examined in 12 field trials conducted over a 4-yr period (2009-2012) at Exeter, Ontario, Carman, Manitoba, and Lethbridge, Alberta. Desiccants were applied alone and in combination at five crop maturation stages. When glyphosate or saflufenacil alone, or in combination, was applied at 100% crop maturity, herbicide residue levels were acceptable (less than 2.0 and 0.01 ppm for glyphosate and saflufenacil, respectively) and there was no

	reduction in yield or hundred seed weight. Glyphosate residues remained below 2.0 ppm when the desiccant was applied alone or with saflufenacil at 75% crop maturity, but crop yield decreased by 16% compared with the untreated control when glyphosate and saflufenacil were combined. Residue levels were unacceptable when glyphosate was applied at 0, 25, and 50% maturity; generally the earlier glyphosate was applied, the greater the residue concentration in the seeds at harvest. Although no application timing resulted in saflufenacil residues above 0.01 ppm, crop yield was reduced when the desiccant was applied at 0, 25, 50, and 75% crop maturity. This information will provide dry bean processors with the necessary information to design guidelines concerning the application timing of glyphosate and saflufenacil so that bean yield and quality remain unaffected and seed residues remain below accepted levels.
Short description of findings	The maturity at which glyphosate is applied as a desiccant to dry bean does affect seed residue levels for that herbicide. If glyphosate is applied prior to beans reaching 75% maturity then glyphosate residues may exceed acceptable limits of 2.0 ppm. Application timing of saflufenacil also affects saflufenacil seed residues, although none of the application timing treatments had residues above 10 ppb. Similar to glyphosate residue results, earlier applications of saflufenacil resulted in the highest saflufenacil seed residues. Although dry bean seed residue values were acceptable for all application timings with saflufenacil, crop yield and quality were negatively impacted by saflufenacil applications at 75% crop maturity or earlier, making early applications of the desiccant unattractive to producers. Therefore, to ensure that foreign markets continue to accept Canadian dry bean, and crop yields remain unaffected, processors and producers must ensure that saflufenacil and glyphosate desiccant use adheres to label recommendations.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of pulses is not a representative use for renewal.
Assessment and conclusion by RMS	The study investigated the effect of the timing of a pre-harvest glyphosate application on dry edible beans. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.12. **Study 12**

Data point	CA 9
Author	McNaughton K. E. *et al*.
Year	2015
Title	Effect of five desiccants applied alone and in combination with glyphosate in dry edible bean (*Phaseolus vulgaris* L.).
Document source	Canadian Journal of Plant Science, (2015) Vol. 95, No. 6, pp. 1235-1242.
Short description of literature article	Application of dry bean desiccants just prior to crop maturity is common practice by Canadian producers. As dry beans are grown for human consumption it is critical that producers pick desiccants that do not affect crop yield, seed quality, or result in desiccant seed residue levels above accepted levels. In this study the efficacy of glyphosate, diquat, glufosinate, carfentrazone, flumioxazin, and saflufenacil as desiccants was examined for navy, cranberry, pinto, and great northern dry bean. Seed herbicide residues were also tested for each of the dry bean classes tested. Navy, cranberry, pinto, and great northern dry bean yields were not impacted by use of the desiccants diquat, carfentrazone, flumioxazin, or saflufenacil when applied at labelled rates and application timings. Additionally, herbicide residues in seed following application remained lower than maximum residue limits (MRL) established by primary Canadian dry bean export partners. Generally, dry bean colour, irrespective of class, was not altered by desiccant use; diquat and flumioxazin caused minor increases in the degree of red and yellow seed pigmentation for cranberry bean only. Although colour differences were

	noted using a Chroma meter the differences were slight and would not likely be of economic importance. Application of glyphosate did not affect crop yield, and seed residue levels were below MRLs for navy, pinto, and great northern bean. However, seed glyphosate residue levels were above the MRL for cranberry bean when glyphosate was applied alone or tankmixed with carfentrazone, flumioxazin, or saflufenacil. Seed residue levels were also above listed MRLs for some export countries when glufosinate was applied to navy, cranberry, and pinto bean, although crop yield and seed quality remained unaffected. These findings suggest that growers and contractors should avoid using glufosinate as a dry bean desiccant at least for some markets and that care should be taken when selecting glyphosate as a desiccant, especially for cranberry bean. Across all market classes desiccation progress of bean leaf, stem, and pod tissue was slowest when glyphosate and carfentrazone were used.
Short description of findings	Generally, the use of glyphosate as a desiccant did not appear to affect yield or the bean seed quality parameters examined in this study for navy, pinto, and great northern bean market classes. However, glyphosate seed residues were above 2.0 ppm in cranberry bean for several examined treatments, suggesting producers need to exercise caution when using glyphosate as a desiccant. The elevated cranberry residue levels identified in this trial may have resulted from the slightly early application of glyphosate to the crop (90% pod colour turn instead of the labelled 100% pod turn). McNaughton *et al*. (2015) previously found that early application of glyphosate to dry edible bean increased seed glyphosate residues across the market classes examined. Other studies have documented differences in dry bean market class responses to applied herbicides (Wilson and Miller 1991; Urwin *et al*. 1996), which may explain why only the cranberry market class displayed elevated glyphosate levels. Apparent variation between seed residue values for bean treated with the same desiccant, across the four market classes may also have been caused by differing environmental conditions at each location at the time of application. Absorption and translocation of foliar herbicides are known to be affected by light, temperature, and relative humidity (Wanamarta and Penner 1989).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of pulses is not a representative use for renewal.
Assessment and conclusion by RMS	The study investigated the effect of (among other active substances) a pre-harvest glyphosate application on dry beans. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.13. Study 13

Data point	CA 9
Author	Nordmeyer H. *et al*.
Year	2017
Title	Using glyphosate with a different application technique - late application in winter wheat Original Title: Glyphosatanwendung mit unterschiedlicher Applikationstechnik - Spaetanwendung im Winterweizen
Document source	Journal fuer Kulturpflanzen (2017), Volume 69, Number 8, pp. 264-270
Short description of literature article	Glyphosate was used in a field trial in winter wheat shortly before harvesting for weed control and desiccation purposes. It was applied with the conventional application technique and the dropleg application technique at the winter wheat growth stage BBCH 89. The analyses covered glyphosate and AMPA residues at harvesting, seven and 16 days after application on the outside of the wheat grain, chaff and straw. The results demonstrate varying residues depending on the application technique. The highest residues with up to 6.4 mg/kg were found on the wheat grain following the conventional application technique. Significantly

	lower residues were found with the dropleg application technique. In straw, glyphosate residues were higher for the dropleg application technique (at 280 mg/kg) when compared to the conventional application technique (up to 160 mg/kg). The level of residues on grain and straw decreased as the waiting period increased. The results obtained suggest that using the dropleg application technique may reduce residue amounts on grain. However, the dropleg application technique can only be used for upright cereals.
Short description of findings	The results show glyphosate residues on wheat grain, chaff and straw following application of glyphosate for the purpose of winter wheat desiccation. The residue levels depend on both the application technique and the waiting period. The conventional application technique leads to higher residues, as the wheat ear is sprayed with more liquid during application than with the dropleg application technique, since less than half of the crop's ear is treated. This can be seen very clearly in the straw residues during both mechanical and manual harvesting. Straw's residue values are higher compared to the conventional application technique. When glyphosate is applied during the crops' growth stage BBCH 89 to full maturity, no or only very small amounts of the active substance are absorbed by the crops. The active substance essentially remains on the crops' surfaces. There is no absorption and displacement in the crop. On the other hand, the possibility of negative effects cannot be ruled out when the grain is treated at lactic ripeness. As more time passes from the application date, residues are likely to decrease. Significant differences were found between the two sampling dates. The residues are generally lower 16 days post-application than seven days post-application. The waiting period is therefore highly significant for the residue levels. It can also be assumed that, following application, the weather conditions play a crucial role in determining the residue level. Precipitation increases active substance wash-off and reduces the surface residue level of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) on grain, chaff and straw.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of cereals is not a representative use for renewal.
Assessment and conclusion by RMS	The present study investigated different pre-harvest applications techniques of glyphosate on wheat. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.14. **Study 14**

Data point	CA 9
Author	Rodrigues N. R. *et al.*
Year	2018
Title	Occurrence of glyphosate and AMPA residues in soy-based infant formula sold in Brazil
Document source	Food Additives & Contaminants (2018), Vol. 35, No. 4, pp. 724-731
Short description of literature article	The presence of glyphosate and AMPA residues in soy-based infant formulas was evaluated during the years 2012–2017, totalising 105 analyses carried out on 10 commercial brands from different batches. Glyphosate and AMPA were determined by liquid chromatography with fluorescence detection after derivatization reaction. The method was validated and showed accuracy and precision with a limit of quantification (LOQ) of 0.02 mg/kg. Among those samples that contained levels above the LOQ, the variation of glyphosate residues was from 0.03 mg/kg to 1.08 mg/kg and for AMPA residues was from 0.02 mg/kg to 0.17 mg/kg.
Short description of findings	The proposed method for analysing residues of glyphosate and AMPA in soy-based infant formula was validated and considered sensitive and reliable and

	its application is relevant for samples analyses. The method allowed the quantification of glyphosate and AMPA. GLY was present in higher concentrations in samples based on soybean extract with 1.08 mg/kg, while in soybean protein samples the highest GLY result was 0.11 mg/kg.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: The article does not concern a representative use for the renewal of EU approval.
Assessment and conclusion by RMS	This paper studied the presence of glyphosate and AMPA in soy-based infant formula. Soybean is not among the crops of the defended use for the current renewal of glyphosate. No further impact expected on the existing risk assessment parameters.

B.7.8.6.15. **Study 15**

Data point	CA 9
Author	Stark P. B. *et al.*
Year	2019
Title	Open-source food: Nutrition, toxicology, and availability of wild edible greens in the East Bay
Document source	PloS one, (2019) Vol. 14, No. 1, e-0202450
Short description of literature article	Field observations, soil tests, and nutritional and toxicology tests on plant tissue were conducted for three sites, each roughly 9 square blocks, in disadvantaged neighbourhoods in the East San Francisco Bay Area in 2014-2015. The sites included mixed-use areas and areas with high vehicle traffic.
Short description of findings	Wild edible greens harvested in industrial, mixed-use, and high-traffic urban areas in the San Francisco East Bay area are abundant and highly nutritious. Even grown in soils with elevated levels of heavy metals, tested species were safe to eat after rinsing in tap water. Pesticides, glyphosate, and PCBs were below detection limits.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Residues of glyphosate in foraged greens in urban environment in the US. Not relevant to EU risk assessment.
Assessment and conclusion by RMS	In this publication, edible wild greens were investigated on their nutritional and toxicological characteristics, including monitoring of glyphosate. Since this article concerns wild greens in the US, and regarding glyphosate the article is more a monitoring exercise, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.16. **Study 16**

Data point	CA 9
Author	Wang Y. *et al.*
Year	2010
Title	Residue and field decline study of glyphosate-ammonium in ramie field
Document source	Nongyaoxue Xuebao, (2010) Vol. 12, No. 2, pp. 201-206
Short description of literature article	A simple, sensitive and selective method using gas chromatography equipped with flame photometric detector (GC-FPD) was developed to determine residues of glyphosate ammonium in soil and ramie root.
Short description of findings	Soil samples were extracted with 0.01 mol/L sodium hydroxide and other samples were extracted with water and acetone. Glyphosate was previously derived with trimethylorthoacetate (TMOA) in the presence of acetic acid. Combination of AGl-X8 anion exchange chromatography with Florisil cartridge clean-up process was favourable for the GC-PFPD analysis. The recovery ranged from 73.6% to 102.6% and 85.9% to 105.1% with the relative standard deviations of 2.3% to 8.1% and 5.4% to 13.0%, respectively. The limit of detection (LOD) of the

	method was $0.5x10^{-10}$g. The limit of quantification (LOQ) was 0.05 mg/kg. The half-life of glyphosate-ammonium was 1.6-2.6, 1.0 – 1.8 d and 1.1-1,5 d in soil of Hunan, Guangxi and Fujian Province at two years, respectively. No glyphosate-ammonium residue were detected in ramie and soil samples at treatments of 2250–3375 g (a.i.)/ha at harvest season (60 days after the treatment).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Non-EU study conducted in China in Chinese soil and on ramie root.
Assessment and conclusion by RMS	The article investigated the effect of glyphosate on ramie and Chinese soil. Since ramie is s not among the crops of the defended use for the current renewal of glyphosate, and it concerns a field investigation outside the EU, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.17. **Study 17**

Data point	CA 9
Author	Zhang T. *et al*.
Year	2015
Title	Evaluation of harvest-aid herbicides as desiccants in lentil production
Document source	Weed Technology (2016), Vol. 30, No. 3, pp. 629-638
Short description of literature article	Desiccants are currently used to improve lentil dry-down prior to harvest. Applying desiccants at growth stages prior to maturity may result in reduced crop yield and quality, and leave unacceptable herbicide residues in seeds. There is little information on whether various herbicides applied alone or as a tank-mix with glyphosate have an effect on glyphosate residues in harvested seed. Field trials were conducted at Saskatoon and Scott, Saskatchewan, Canada, from 2012 to 2014 to determine whether additional desiccants applied alone or tank mixed with glyphosate improve crop desiccation and reduce the potential for unacceptable glyphosate residue in seed. Glufosinate and diquat tank mixed with glyphosate were the most consistent desiccants, providing optimal crop dry-down and a general reduction in glyphosate seed residues without adverse effects on seed yield and weight.
Short description of findings	In summary, tank mixes of glufosinate + glyphosate or diquat + glyphosate applied at 30% to 40% seed moisture content provided excellent crop desiccation while reducing glyphosate seed residues, with no effect on yield or seed weight. Therefore, these tank mixes would be good options for growers seeking to manage weeds at harvest and where the application of glyphosate alone will not provide rapid crop desiccation. Saflufenacil provided good crop desiccation without yield losses, but failed to reduce glyphosate seed residues consistently when compared to glyphosate applied alone. Other contact herbicides, such as flumioxazin and pyraflufen-ethyl, did not provide efficacious crop desiccation, nor did they consistently decrease glyphosate residues. Moreover, some of the glyphosate residues observed for these treatments exceeded the MRL of Japan, an importer of Canadian lentils and thus, these herbicides should not be considered for lentil desiccation.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of pulses is not a representative use for renewal.
Assessment and conclusion by RMS	The publication studied the effect of glyphosate, among other active substances, as pre-harvest application to lentils. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.6.18. **Study 18**

Data point	CA 9

Author	Zhang T. *et al*.
Year	2017
Title	Early application of harvest aid herbicides adversely impacts lentil
Document source	Agronomy Journal, (2017) Vol. 109, No. 1, pp. 239-248
Short description of literature article	The objective of this study was to determine the response of lentil to various application timings of glyphosate, saflufenacil, and the combination of these two herbicides. A field experiment consisting of a randomized complete block design was run at Saskatoon and Scott, SK, Canada in 2012, 2013, and 2014 to address the objective. Application of harvest aid herbicides before 30% seed moisture content reduced seed yield and thousand seed weight up to 25 and 8%, respectively. Moreover, application timings before 30% seed moisture resulted in lentil seed samples exceeding residue levels of 2.0 and 0.03 mg/kg for glyphosate and saflufenacil, respectively.
Short description of findings	Application of desiccants below 30% seed moisture content, when lentil was close to physiological maturity, did not impact seed yield or thousand seed weight (TSW), and did not result in lentil seed samples that exceeded residue levels of 2 and 0.03 mg/kg for glyphosate (sampled 7 DAA) and saflufenacil (sampled 21 DAA), respectively. Although glyphosate residue levels were substantially lower in the tank mixture, adding saflufenacil to glyphosate did not reduce glyphosate residue in lentil seeds compared to glyphosate applied alone. It did, however, improve crop desiccation and reduce seed residues of saflufenacil compared with either glyphosate or saflufenacil applied alone. This tank mixture should also improve weed control over using either herbicide alone and offers two distinct modes of action, which is important to delay the evolution of herbicide resistance.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Desiccation of pulses is not a representative use for renewal.
Assessment and conclusion by RMS	This article investigated the effect of different pre-harvest application timings of glyphosate on lentils. Since pre-harvest applications are not within the requested use for the current renewal of glyphosate, there is no further impact expected on the existing risk assessment parameters.

B.7.8.7. Public literature from previous RAR (2015) for completeness

Public literature considered for the residues evaluation during the previous renewal procedure for glyphosate (RAR B.7 by Germany of 2015) has been copied into the current RAR for completeness. This information is an exact copy, and as such numbering of tables has not been changed; and highlighting has not been adjusted.

Assessment and conclusion by RMS:
In the M-doc from the applicant, the following was stated:
During the AIR 2 evaluation process of glyphosate, in the Renewal Assessment Report 2015 version, the RMS Germany included public literature articles as part of the B.7 section. All articles included in that RAR Vol 3 2015 version, have been included in this annex for sake of completeness, with the aim to present to the EU authorities during the AIR 5 EU process, all information available for glyphosate from previous EU evaluations.
The RMS has also copied this information into the current RAR, although it is considered not necessary. None of the articles, retrieved in the RAR from 2015, are considered to have any impact on the existing risk assessment parameters. In addition, most articles were more than 10 years old, and are as such not required to be included. The literature from the 2015 RAR, which was published within the last 10 years, can be retrieved in the literature search, conducted for the current renewal of glyphosate. There is one exception: Krüger *et al.* (2014). Therefore, there is a data requirement for the applicant to provide a summary of this article, and include their assessment. It is acknowledged that the applicant does refer to the respective article of Krüger *et al* in the white paper they provided (CA 6.10.1/002), however, the article should also be handled in the current RAR as a separate article.

Renewal Assessment Report, Revised 29 January 2015 Vol 3, Annex B.7

B.7.1.1.1 Fruit crops

Reference:	OECD KIIA 6.2.1
Report:	Hasegawa, L. S.; Kumamoto, J.; Jordan, L. S. Degradation of Glyphosate in avocado fruit 10.04.1995, L365, ASB2011-13642
Guidelines:	no
Deviations:	not applicable
GLP:	no
Acceptability:	The study is considered to be additional information.

Material and Methods:
In this study conducted in a research project supported by the California Avocado Advisory Board the bahaviour of ^{14}C-radiolabelled glyphosate was investigated in avocado.
The study contained two parts. First a selected leaf was painted with ^{14}C-glyphosate. After up to 10 days samples of leaves near the treated leaf were analysed for radioactive residues.
In the second part of the experiment a mature avocado fruit was picked and a cavity was drilled into the end of the peduncle. The cavity was filled with an aqueous solution containing ^{14}C-glyphosate (453000 cpm) and then kept filled with destilled water for the remainder of 10 days. The fruit was placed in a respiration chamber, monitoring the formation of $^{14}CO_2$.
Analysis of the radioactivity was conducted by LSC and ion exchange HPLC with radiodetection agains glyphosate and AMPA as reference substances.

Findings:
In the first part of the experiment no translocation of radioactivity from the treated leaf into other parts of the plant was observed.
The treated avocado fruit was separated into its different segments and analysed for radioactivity. Results are presented in the following table:

Table B.7.1-16: Recovery of radiolabel from ^{14}C-glyphosate treated avocado fruit

Matrix (weight)	Glyphosate	AMPA	Unasssigned
	cpm (%)	cpm (%)	cpm (%)
Avocado, mesocarp (190.4 g)	290378 (64.1)	1179 (0.26)	4330 (0.9)
Avocado, exocarp (24.9 g)	31029 (6.8)	0 (0)	363 (<0.1)
Avocado, seed (53.9 g)	202 (<0.1)	0 (0)	2107 (0.47)

Avocado, peduncle (2.6 g)	69057 (15.2)	340 (<0.1)	2162 (0.48)
Filter paper	4225 (0.9)	0 (0)	98 (<0.1)
Total recovered	394891 (87.1)	1519 (0.34)	9060 (2)
CO_2	-	-	16239 (3.6)

Conclusions:
The information provided by this study are limited. No translocation of radioactivity from one treated leaf into other parts of the branch were observed. In one avocado fruit directly treated with glyphosate via a drilled cavity in the peduncle, most of radioactivity was present in the mesocarp and peduncle. AMPA was present in very low amounts <1 % of the TRR.

B.7.1.4 Public literature

In several studies the effect of glyphosate on the concentration of secondary plant metabolites was investigated. Bresnahn et al (2003, ASB2012-12365) investigated the levels of shikimic acid, which is involved in the main mode of action for glyphosate in plants, in wheat. It was observed that following glyphosate application an approximately 2-fold increase in the shikimic acid concentration in the grain was observed compared to control samples or wheat treated with other herbicides.
In studies published by Bohm et al (2008, ASB2012-12366) and Duke et al (2003, ASB2012-12400) the effect of glyphosate to isoflavones in soya beans was investigated. No significant influence was identified.
The activity of gluthathione-S-transferase in maize was investigated by Cataneo et al (2003, ASB2012-12384). The results indicate an increase in the activity of the molecule, probably due to its role in the plant metabolism of the active substance.

In a literature review Duke (2011, ASB2012-12401) comapred the mode of glyphosate resistance in weed, showing that only a minor share of the weeds express a GOX gene. Most of the weeds contained a unchanged glyphosate, suggesting a modification of the EPSPSenzyme. However, the level of resistance in weeds was found to be lower than in genetically modified crops.
Further investigations on secondary plant metabolites in organic, conventional and GM-soya beans and the residues of glyphosate and AMPA were published by Bøhn *et al.* (2014, ASB2014-6353). Organic soybeans showed a nutritional profile with more sugars, such as glucose, fructose, sucrose and maltose, significantly more total protein, zinc and less fibre than both conventional and GM-soya. Organic soybeans also contained less total saturated fat and total omega-6 fatty acids than both conventional and GM-soya. The ration of residues of glyphosate and AMPA (mean 3.3 and 5.7 mg/kg, respectively) confirmed the findings from ^{14}C-radiolabelled plant metabolism studies provided by the applicant.

The metabolism of glyphosate in *Mucuna pruriens var. utilis* (velvet beans) was investigated by Rojano-Delgado et al (2012, ASB2012-12462). The velvet beans are a plant species exhibiting an innate, very high resistance to glyphosate. Using ^{14}C-radiolabelled glyphosate, the uptake and metabolism in velvet beans compared to *Amaranthus retroflexus* was observed. It was shown that velvet beans had a much lower uptake of the radioactivity via the leaves. While amaranthus accumulated up to 94 % of the applied dose within 24h, an uptake of less than 40 % was observed for the velvet beans. After 72 hours 52 % of the applied dose were taken up.
Besides the investigation of the uptake, degradation products found in the velvet bean leaves were analysed to identify the mode of tolerance for this species. In treated plants glyphosate, AMPA, sarcosine and formaldehyde were identified. In comparision to non-tolerant amaranthus, where mainly glyphosate and traces of AMPA were found, a secondary metabolic pathway via sarcosine, which is normally found in bacteria only, was postulated by the author:

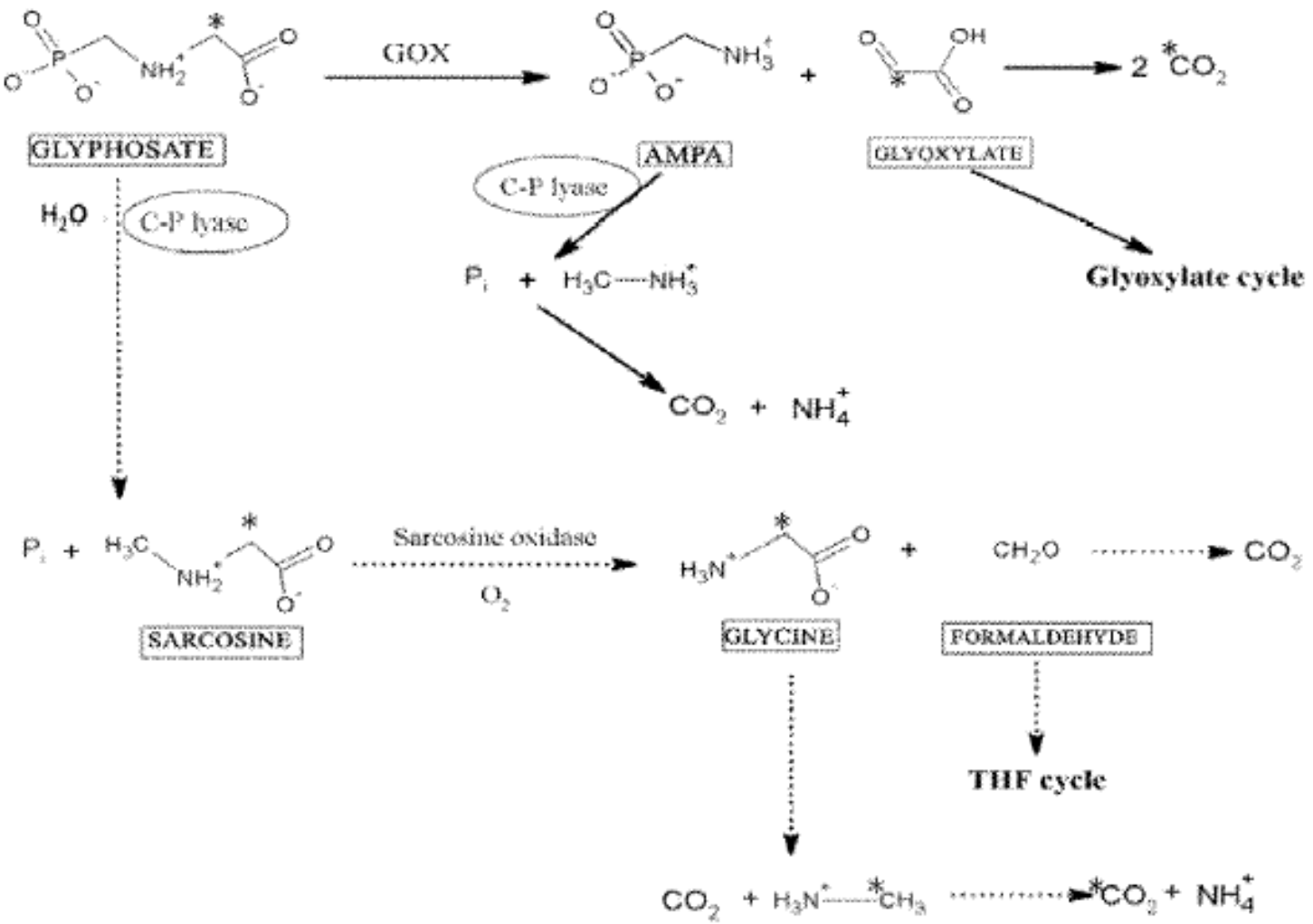


Fig. 6. Potential degradation pathway for glyphosate (⟶ primary pathway in plants and soils, ⋯⋯▸ primary pathway in bacteria).

In summary it was concluded, that the natural tolerance of velvet beans is based on three modes of action: a natural GOX modification resulting in AMPA, a high-tolerance EPSPS enzyme and the additional degradation into sarcosine, finally resulting in glycine, formaldehyde and their natural products.
In view of the residue situation the observation of an additional metabolic pathway is relevant. However, all degradation products observed in this pathway are identical to already known metabolites (AMPA) or represent natural products commonly available in bichemical cycles (sarcosine, glycine, formaldehyde, glyoxylate). Further investigation in addition to the information already available are not necessary to describe the metabolism of glyphosate in terms of residues.

The sensitivity of different plant species to glyphosate was investigated by Reddy et al (2008, ASB2012-12463). Besides these phytotoxic effects it was investigated, if the metabolism of glyphosate into AMPA is a common factor in the natural resistance of plants against glyphosate. Although non-tolerant crops (especially soya beans) showed increased concentrations of shikimic acid, no correlation of glyphosate or AMPA concentration to the tolerance were found.

The uptake of glyphosate into maize seedlings was investigated by Wagner *et al.* (2003, ASB2012-12484) using ^{14}C-radiolablled active substance. The seedlings were grown in ^{14}Cglyphosate solutions with concentrations of 0-30 mg as/kg). After 26h of exposure the plants were transferred into fresh nutrient solution and grown for 5 additional days. The glyphosate uptake was observed to be 11 % of the theoretical mass flow. If more than 0.6µg/g glyphosate were observed, a decrease in the growth was observed. It could be shown, that radioactivity taken up by the plants was mainly located in the new leaves, suggesting symplastic distribution in the plants.

B.7.6.6.6 Public literature

A study published by Ando C. et al (2003, ASB2012-12350) investigated the residue situation on typical herbs collected by native Americans in the California National Forest. The results are not related to the representative uses and the situation in the EU.

Arregui et al (2004, ASB2012-12351) reported monitoring results of glyphosate residues in transgenic soya beans from Argentina from 1997-1999. An additional study was provided by Lorenzatti et al (2004, ASB2012-12448). The representative uses evaluated within this document do not involve transgenic plants or import tolerances. The respective study was not taken into account.

In Denmark, the residue levels of glyphosate in cereal grain were monitored 1998 and 1999 by Granby et al (2001, ASB2012-12423). In 1998the average concentration was 0.08 mg/kg (n=49) and increased to 0.11 mg/kg in 1999 (n=46). No MRL violations were identified.

B.7.7.3 Open literature

The influence of glyphosate residues during malting after desiccation of barley was investigated by Caierao et al (2007, ASB2012-12382). The residue concentration of glyphosate showed no effect to the malting of barley.

Low et al (2005, ASB2012-12449) investigated the inpact of Saccharomyces cerevisiae on the stability of glyphosate during bread leavening. It was shown that the approximately 20 % of the initial glyphosate concentration was degraded within 1 hour. However, no analysis on the metabolites formed was conducted.

B.7.8.4 Public literature

In paper primarily dealing with determinations of glyphosate in the urine of humans and cattle, Krüger *et al.* (2014, ASB2014-5024) reported data from Danmark also including findings in various tissues. The samples obtained from cows on “conventional husbandry” (compared to “organic husbandry”) were analysed by means of a not further specified ELISA (Abraxis, USA) but an LOD or LOQ were not mentioned. No numeric values such as mean and standard deviation are given in this very brief paper but only figures. Based on these figures, maximum glyphosate concentrations of up to 0.06 mg/kg in kidney (n = 26), 0.04 mg/kg in liver (n = 41) and 0.02 mg/kg in muscle (n = 6) can be estimated.
The study confirms the findings from livestock animal feeding studies that liver and kidney are the target tissues for glyphosate residues. However, since no linkage to a potentical dietary burden can be made, the study is of limited value for the estimation of maximum residue levels in animal commodities or the dietary intake assessment.

B.7.15.3 Public literature

The chronic dietary intake of numerous pesticides was investigated by Nougadère, A. et al (2011, ASB2012-11982) to introduce a ranking and scoring method for the active substances. The exposure of the Cammeroonian against glyphosate was investigated in a total diet study by Gimou et al (2008, ASB2012-12422). Harris C. et al published a case study to predict the chronic dietary intake of glyphosate based on several intake models in 2004 (2004, ASB2012-12428).
In view of the representative use based evaluation of glyphosate within this document, the provided literature was not applicable to support the dietary intake assessment for glyphosate.

B.7.8.8. References relied on, reference list

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.1-001	[redacted]	2020	ILV of method ME-2220-01 and short term storage stability of glyphosate and its metabolite AMPA in honey Report No.: S19-04663 Document No.: M-681330-01-1 Eurofins Agroscience Services EcoChem GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.1-002	[redacted]	2012	Storage stability of residues of Glyphosate and AMPA in citrus fruit Report No.: REG-09-234 Document No.: MSL0023608 Monsanto Company GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-003	[redacted]	2010	Storage stability of residues of Glyphosat and AMPA in various plant materials Report No.: FCS-0707 Document No.: ASB2012-12488 Eurofins Dr. Specht GLP GmbH GLP/GEP: Y Published: N	N	N	--	ADM	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-004	[redacted]	2009	Stability of Glyphosate and Metabolites in corn green plant, forage, grain, and stover containing the GAT and ZM-HRA genes during frozen storage - Final Report Report No.: 60874 Document No.: ASB2008-2656 ABC Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-005	[redacted]	2009	Stability of Glyphosate, N-Acetylglyphosate, Aminomethyl phosphonic acid and N-Acetyl AMPA in GAT soybean forage, seed, and hay stored frozen Report No.: 49990 Document No.: ASB2008-2654 ABC Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-006	[redacted]	2007	Stability of Glyphosate, N-Acetylglyphosate and Aminomethyl phosphonic acid in GAT Corn Forage, Grain, and Stover, Stored Frozen Report No.: 49991 Document No.: ASB2008-2655 ABC Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.1-007	[redacted]	1997	Determination of the storage stability of Glyphosate in beans, oilseed rape and linseed Report No.: IF-94/13882-00 Document No.: 394 GLY Institut Fresenius, Chemische und Biologische Laboratorien GmbH GLP/GEP: Y Published: N	N	N	--	FMC	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-008	[redacted]	1996	Determination of Glyphosate in soybean raw agricultural commodities (RAC) - Stability report Report No.: 91210 Document No.: 455 GLY Landis International, Inc ; and Huntingdon Life Sciences (Analytical Facility) GLP/GEP: Y Published: N	N	N	--	FMC	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-009	[redacted]	1996	Determination of Glyphosate in pasture grasses - Stability report Report No.: 91212 Document No.: 456 GLY Landis International, Inc ; and Huntingdon Life Sciences (Analytical Facility) GLP/GEP: Y Published: N	N	N	--	FMC	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-010	[redacted]	1996	Storage Stability of Residues of N-(phosphonomethyl)glycine and Trimethylsulphonium Cation in Banana Report No.: RJ2161B Document No.: 33010290 (94JH232) Zeneca Agrochemicals GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: Not accepted in RAR (2015) Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-011	[redacted]	1995	Storage stability of Glyphosate and AMPA in wheat grain and straw and in rye grain and straw Report No.: 303614 Document No.: 325 GLY RCC Umweltchemie AG GLP/GEP: Y Published: N	N	N	--	FMC	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.1-012	[redacted]	1991	Storage stability of Glyphosate residues in crop commodities Report No.: MSL-10843 Document No.: M-644183-01-1 Monsanto Agricultural Company GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: EG:AIIA-6.3 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.1-013	[redacted]	1989	Storage stability validation for ICIA 0224 in raw agricultural commodities Report No.: WRC 89-22 Document No.: VV-320945 ICI Americas Inc. GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: -- Monograph Trimesium: AIIIA-8.2
KCA 6.1-014	[redacted]	1988	Storage stability of Glyphosate and AMPA in swine tissues, dairy cow tissues and milk, laying hen tissues and eggs Report No.: MSL-7515 Document No.: M-645906-01-1 Monsanto Agricultural Company GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.1.1 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.1-015	[redacted]	1987	Magnitude of SC-0224 residues in eggs and poultry Report No.: [redacted] 87-43 Document No.: - [redacted] GLP/GEP: N Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.4.2 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.1-016	[redacted]	1987	Magnitude of SC-0224 Residues in Meat and Milk Report No.: [redacted] 87-44 Document No.: - [redacted] GLP/GEP: N Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.4.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.2.1-001	[redacted]	1975	The metabolism of CP 67573 by citrus - February 1973 - October 1974 Report No.: 328 Document No.: - Monsanto Agricultural Products Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.1-002	[redacted]	1987	The nature of the residue of SC-0224 in citrus Report No.: PMS-158R Document No.: VV-497772 Stauffer Chemical Company GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: -- Monograph 1998: -- Monograph Trimesium: AIIA-6.1
KCA 6.2.1-003	[redacted]	1976	Absorption, translocation and metabolism of Roundup herbicide in walnut, almond and pecan trees Report No.: 403 Document No.: - Monsanto Agricultural Research Dept. GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-004	[redacted]	1974	CP 67573 Residue and metabolism Part 23: The metabolism of CP 67573 in apple trees Report No.: 342 Document No.: M-649026-01-1 Monsanto Commercial Products Co. GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-005	[redacted]	1991	Glyphosate-trimesium: Uptake and metabolism in USA grape vines (Volume I and II) Report No.: RJ1002B Document No.: VV-323412 ICI Agrochemicals GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.1
KCA 6.2.1-006	[redacted]	1990	ICIA0224: Uptake and Metabolism in Grape-Vines Report No.: RJ0815B Document No.: - ICI Agrochemicals GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.1
KCA 6.2.1-007	[redacted]	1974	CP 67573 Residue and metabolism Part 20: The metabolism of CP 67573 in grape plants Report No.: 335 Document No.: M-649025-01-1 Monsanto Commercial Products Co. GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.1-009	[redacted]	1976	CP 67573 Residue and metabolism Part 29: The metabolism of CP 67573 in sugar beets Report No.: 394 Document No.: M-649164-01-1 Monsanto Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-010	[redacted]	1989	ICIA0224: Metabolism on wheat following a preharvest foliar spray Report No.: RJ0778B Document No.: - ICI Agrochemicals GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.1
KCA 6.2.1-011	[redacted]	1974	CP 67573 Residue and metabolism Part 22: The metabolism of N-phosphonomethylglycine in barley, oats, rice and sorghum Report No.: 341 Document No.: M-649027-01-1 Monsanto Agricultural Products Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-012	[redacted]	1973	CP 67573 Residue and Metabolism Part 10: The Metabolism of CP 67573 in soybeans, cotton, wheat, and corn Report No.: 304 Document No.: M-648850-02-1 Monsanto Commercial Products Co. GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-013	[redacted]	1976	The metabolism of glyphosate in pasture crops Report No.: 404 Document No.: - Monsanto Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-014	[redacted]	1992	[14C-Anion] ICIA0224: Nature of the residue: Soybeans (WRC-91-189) Report No.: RR 91-092B Document No.: - ICI Americas Inc. GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.1

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.1-015	[redacted]	1973	CP 67573 Residue and Metabolism Part 10: The Metabolism of CP 67573 in soybeans, cotton, wheat, and corn Report No.: 304 Document No.: M-648850-02-1 Monsanto Commercial Products Co. GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-016	[redacted]	1975	CP 67573 Residue and metabolism Part 24: The metabolism of CP 67573 in coffee plants Report No.: 344 Document No.: M-649024-01-1 Monsanto Agricultural Products Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-017	Anonymous	1976	Glyphosate residue and metabolism studies in sugarcane and soils Report No.: RD93 Document No.: M-651454-02-1 Monsanto Agricultural Products Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.3 Monograph Trimesium: --
KCA 6.2.1-018	[redacted]	2000	Metabolism of Glyphosate in Roundup Ready Sugarbeet Report No.: 861W Document No.: MSL-16247 PTRL West, Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: -- Monograph Trimesium: --
KCA 6.2.1-019	[redacted]	2000	Metabolism of Glyphosate in Roundup Ready Wheat Report No.: 811W Document No.: MSL-16028 PTRL West, Inc. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.2.1-020	[redacted]	1995	Nature of glyphosate residues in corn plants which are tolerant to Roundup® herbicide Report No.: MSL-14018 Document No.: M-650178-01-1 Monsanto Company; ABC Laboratories, Inc.; XenoBiotic Laboratories Inc.; Agvise Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: B.6.1.7.1 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.1-021	[redacted]	1994	Nature of Glyphosate Residues in Roundup® Herbicide Tolerant Canola Report No.: MSL-13318 Document No.: - The Agricultural Group of Monsanto Company; XenoBiotic Laboratories Inc ; Agvise Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-022	[redacted]	1994	Nature of Glyphosate Residues in Soybeans Tolerant to Roundup® Herbicide. Report No.: MSL-13520 Document No.: M-650176-01-1 The Agricultural Group of Monsanto Company; XenoBiotic Laboratories Inc ; Agvise Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: EG:AIIA-6.1 Monograph Trimesium: --
KCA 6.2.1-023	[redacted]	1997	Nature of Glyphosate Residues in Cotton Plants (Genotype Line #1445) Tolerant to Roundup® Herbicide Report No.: MSL-14113 Document No.: - Monsanto Company; ABC Laboratories, Inc.; Agvise Laboratories, Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: -- Monograph Trimesium: --
KCA 6.2.1-024	[redacted]	2007	The metabolism of [14C]Glyphosate in Optimum™ GAT™ (Event DP-Ø9814Ø-6) field corn Report No.: 807194 Document No.: DuPont-19529 Charles River Laboratories GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: -- Monograph Trimesium: --
KCA 6.2.1-025	[redacted]	2010	The Metabolism of [14C]Glyphosate in 0827 Canola Report No.: 808685 Document No.: DuPont-26109 Charles River Laboratories GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.2.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.2.1-026	[redacted]	2007	The Metabolism of [14C]Glyphosate in gat/gm-hra (DP-356Ø43-5, PHP20163a) Soybeans Report No.: 806960 Document No.: DuPont-19530 Charles River Laboratories GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.2.1; KIIA 4.3 (OECD) Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.2-001	[redacted]	1994	(14C-Glyphosate): Absorption, distribution, metabolism and excretion following repeated oral administration to the laying hen Report No.: 676/8-1011 Document No.: 276 GLY [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.2 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.2-002	[redacted]	1988	Metabolism study of synthetic 13C/14C-labeled Glyphosate and Aminomethylphosphonic acid in laying hens. Part I Report No.: [redacted] 6103-112 Document No.: [redacted]-7591 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.2 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.2-003	[redacted]	1988	Metabolism study of synthetic 13C/14C-labeled Glyphosate and Aminomethylphosphonic acid in laying hens. Part II Report No.: [redacted]-7420 Document No.: - [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.2 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.2-004	[redacted]	1994	[14C-PMG] Glyphosate-trimesium: Nature of the residue in tissues and eggs of laying hens Report No.: RR93-064B Document No.: - [redacted] GLP/GEP: Y Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.2 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.2.2-005	[redacted]	2007	The metabolism of [14C]-N-acetylglyphosate (IN-MCX20) in laying hens Report No.: 210573 Document No.: [redacted]-19795 [redacted] GLP/GEP: Y Published: N	Y	N	--	COR	Y RAR 2017: OECD: KIIA 6.2.2 Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.2.3-001	[redacted]	1994	(14C-Glyphosate): Absorption, distribution, metabolism and excretion following repeated oral administration to the dairy goat Report No.: 676/9-1011 Document No.: 279 GLY [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.3 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.3-002	[redacted]	1994	The nature of residues of orally administered [Phosphonomethylene-14C] glyphosate-trimesium in goat tissues and milk Report No.: RR 93-062B Document No.: WIN 8325, WRC-93-088, [redacted] 378 [redacted] GLP/GEP: Y Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.2.3 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.2.3-003	[redacted]	1988	Metabolism study of synthetic 13C/14C-labeled Glyphosate and Aminomethylphosphonic acid in lactating goats. Part I Report No.: [redacted] 6103-113 Document No.: [redacted]-7586 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.3 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.3-004	[redacted]	1988	Metabolism study of synthetic 13C/14C-labeled Glyphosate and Aminomethylphosphonic acid in lactating goats. Part II Report No.: [redacted]-7458 Document No.: [redacted]-7458 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.2.3 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.2.3-005	[redacted]	2007	Metabolism of [14C]-N-Acetylglyphosate (IN- MCX20) in the lactating goat Report No.: 210583 Document No.: [redacted]-19796 [redacted] GLP/GEP: Y Published: N	Y	N	--	COR	Y RAR 2017: KIIA 4.3 (OECD) Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.1-001	[redacted]	2014	Glyphosate-Residue Study on Mandarin Oranges in Spain in 2013 Report No.: S13-02531 Document No.: A12798QA_10348 Eurofins Agroscience Services Ltd. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-002	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in tree nuts (outdoor) at 2 sites in Southern Europe 2015 Report No.: S15-00018 Document No.: A12798QA_10340 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-003	[redacted]	2014	Glyphosate -Residue Study on Apple in the United Kingdom and Germany in 2013 Report No.: S13-03425 Document No.: A12798QA_10343 Eurofins Agroscience Services Ltd. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-004	[redacted]	2014	Glyphosate - Residue Study on Apple in Spain and Italy in 2013 Report No.: S13-03426 Document No.: - Eurofins Agroscience Services Ltd. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-006	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in apricots (outdoor) at 4 sites in Southern Europe 2015 Report No.: S15-00019 Document No.: MSL0027488 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-007	[redacted]	2014	Glyphosate - Residue Study on Cherry in Spain and Italy in 2013 Report No.: S13-03427 Document No.: A12798QA_10349 Eurofins Agroscience Services Ltd. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-008	[redacted]	2014	Glyphosate - Residue study on plum in Italy in 2013 Report No.: S13-03233 Document No.: A12798QA_10347 Eurofins Agroscience Services Ltd. GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.1-009	[redacted]	2013	Determination of Residue of Glyphosate in Stone Fruits Following One Application of Glyphosate SL 360g/L (CA2705) in Northern and Southern France, in 2012 Report No.: S12-03071 Document No.: NUA-1201 Eurofins Agroscience Services Chem GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-010	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in vine grapes (outdoor) at 2 sites in Germany 2015 Report No.: S15-00491 Document No.: MSL0027503 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-011	[redacted]	2015	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in vine grapes (outdoor) at 4 sites in Northern France Report No.: S14-04157 Document No.: MSL0027071 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-012	[redacted]	2015	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in vine grapes (outdoor) at 3 sites in Germany and 2 sites in Spain 2014 Report No.: S14-04158 Document No.: MSL0027070 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-013	[redacted]	2015	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in vine grapes (outdoor) at 4 sites in Southern Europe 2014 Report No.: S14-04226 Document No.: MSL0027069 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-015	[redacted]	1996	Residues of glyphosate and AMPA in olives and olive oil, following a soil treatment with Roundup® herbicide. Spanish field trials, 1995 Report No.: MLL-30469 Document No.: 95-GLY-20SP Monsanto Europe S.A., the Agricultural Group; Agrisearch UK GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: EG:AIIA-6.3; EG:AIIA-6.5 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.1-019	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in kiwi fruit (outdoor) at 2 sites in Southern Europe 2015 Report No.: S15-00469 Document No.: MSL0027501 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-020	[redacted]	2015	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in bananas (outdoor) at 4 sites in Spain (Canary Islands) 2014 Report No.: S14-04159 Document No.: MSL0027222 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-021	[redacted]	2018	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in citrus plants (outdoor) at 2 sites in Southern Europe 2017 Report No.: S17-02881 Document No.: MSL0029656 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.1-022	[redacted]	2018	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in plums (outdoor) at 4 sites in Southern Europe 2017 Report No.: S17-02878 Document No.: MSL0029654 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.2-001	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in potatoes (outdoor) at 4 sites in France, Germany and Italy 2011 Report No.: S11-00258 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-002	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in carrots (outdoor) at 4 sites in France, Spain and Poland 2011 Report No.: S11-00259 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.2-003	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in bulb onions (outdoor) at 4 sites in France, Spain and Bulgaria 2011 Report No.: S11-00260 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-004	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in tomato (outdoor) at 2 sites in Hungary and Germany 2011 Report No.: S11-00267 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-005	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in cucumber and zucchini (outdoor) at 3 sites in Italy, France and Germany 2011 Report No.: S11-00261 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-006	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in cauliflower (outdoor) at 4 sites in France, Hungary, Bulgaria and Italy 2011 Report No.: S11-00263 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-007	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in head cabbage (outdoor) at 4 sites in Hungary, France (North), Spain and Bulgaria 2011 Report No.: S11-00262 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-008	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in leaf and head lettuce (outdoor) at 4 sites in France, Spain, UK and Germany 2011 Report No.: S11-00264 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.2-009	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in leek (outdoor) at 4 sites in France, United Kingdom, Bulgaria and Italy 2011 Report No.: S11-00265 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.2-010	[redacted]	2012	Determination of residues of Glyphosate and AMPA after one application of MON 52276 in sugar beet (outdoor) at 2 sites in Spain and Italy 2011 Report No.: S11-00266 Document No.: - Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: -- Monograph Trimesium: --
KCA 6.3.3-001	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in carrots (outdoor) at 4 sites in Southern Europe 2015 Report No.: S15-00482 Document No.: MSL0027502 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-002	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in radish (outdoor) at 2 sites in Southern Europe 2015 Report No.: S15-00467 Document No.: MSL0027500 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-003	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in bulb onions (outdoor) at 4 sites in Southern and 2 sites in Northern Europe 2015 Report No.: S15-00466 Document No.: MSL0027499 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-004	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in tomato (outdoor) at 4 sites in Southern Europe 2015 Report No.: S15-00465 Document No.: MSL0027498 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.3.3-005	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in cucumber (outdoor) at 2 sites in Southern and 2 sites in Northern Europe 2015 Report No.: S15-00464 Document No.: MSL0027497 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-006	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in courgette (outdoor) at 2 sites in Southern and 2 sites in Northern Europe 2015 Report No.: S15-00463 Document No.: MSL0027496 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-007	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in head lettuce (outdoor) at 4 sites in Southern and 2 sites in Northern Europe 2015 Report No.: S15-00460 Document No.: MSL0027493 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-008	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in parsley (outdoor) at 2 sites in Southern and 2 sites in Northern Europe 2015 Report No.: S15-00459 Document No.: MSL0027492 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.3.3-009	[redacted]	2016	Determination of residues of glyphosate and its metabolite AMPA after one application of MON 79351 in green beans (outdoor) at 4 sites in Southern and 4 sites in Northern Europe 2015 Report No.: S15-00461 Document No.: MSL0027494 Eurofins Agroscience Services GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.4.1-001	[redacted]	2007	Magnitude of Residues of N-Acetylglyphosate and Degradates in Laying Hen Tissues and Eggs Report No.: 28212 Document No.: [redacted]-20088 [redacted] GLP/GEP: Y Published: N	Y	N	--	COR	Y RAR 2017: OECD: KIIA 6.4.1 Monograph 1998: -- Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.4.1-002	[redacted]	1987	Magnitude of SC-0224 residues in eggs and poultry Report No.: [redacted] 87-43 Document No.: - [redacted] GLP/GEP: N Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.4.2 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.4.1-003	[redacted]	1987	Residue determination of Glyphosate and AMPA in laying hen tissues and eggs following a 28 day feeding study Report No.: [redacted]-6676 Document No.: M-651048-01-1 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.4.1 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --
KCA 6.4.1-004	Shehata, A.A. *et al.*	2014	Distribution of Glyphosate in Chicken Organs and its Reduction by Humic Acid Supplementation Report No.: DOI 10.2141/ jpsa.0130169; ISSN 1346-7395 Document No.: - Journal of Poultry Sciences (2014) 51:333-337 GLP/GEP: N Published: Y	Y	N	--	LIT	N
KCA 6.4.2-001	[redacted]	2007	Magnitude of residues of N-Acetylglyphosate and degradates in dairy cow tissues and milk Report No.: 28210 Document No.: [redacted]-20087 [redacted] GLP/GEP: Y Published: N	Y	N	--	COR	Y RAR 2017: OECD: KIIA 6.4.2 Monograph 1998: -- Monograph Trimesium: --
KCA 6.4.2-002	[redacted]	1987	Residue determination of Glyphosate and AMPA in dairy cow tissues and milk following a 28 day feeding study Report No.: [redacted]-6729 Document No.: M-650790-02-1 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.4.2 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.4.2-003	[redacted]	1987	Magnitude of SC-0224 Residues in Meat and Milk Report No.: [redacted] 87-44 Document No.: - [redacted] GLP/GEP: N Published: N	Y	N	--	SYN	Y RAR 2017: OECD: KIIA 6.4.1 Monograph 1998: -- Monograph Trimesium: AIIA-6.2; AIIA-6.4
KCA 6.4.2-004	Shelver, W.L. *et al.*	2018	Distribution of chemical residues among fat, skim, curd, whey, and protein fractions in fortified, pasteurized milk Report No.: DOI 10.1021/acsomega.8b00762; ISSN 2470-1343 Document No.: - ACS Omega (2018) 3:8697-8708 GLP/GEP: N Published: Y	Y	N	--	LIT	N
KCA 6.4.2-005	Schnabel, K. *et al.*	2017	Effects of glyphosate residues and different concentrate feed proportions on performance, energy metabolism and health characteristics in lactating dairy cows. Report No.: DOI 10.1080/1745039X.2017.1391487; E-ISSN 1477-2817 Document No.: - Archives of Animal Nutrition (2017) 71:413-427 GLP/GEP: N Published: Y	Y	N	--	LIT	N
KCA 6.4.2-006	Von Soosten, D. *et al.*	2016	Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows. Report No.: DOI 10.3168/jds.2015-10585; E-ISSN 1525-3198 Document No.: - Journal of Dairy Science (2016) 99:5318-5324 GLP/GEP: N Published: Y	Y	N	--	LIT	N
KCA 6.4.3-001	[redacted]	1987	Residue determination of Glyphosate and AMPA in swine tissues following a 28-day feeding study Report No.: [redacted]-6627 Document No.: M-651049-01-1 [redacted] GLP/GEP: Y Published: N	Y	N	--	BCS	Y RAR 2017: OECD: KIIA 6.4.3 Monograph 1998: EG:AIIA-6.2; EG:AIIA-6.4 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.5.1-001	[redacted]	2020	AMPA and N-Acetyl AMPA Hydrolysis under typical conditions (pH, temperature and time) of processing Report No.: S19-22457 Document No.: M-680101-01-1 Eurofins Agroscience Services EcoChem GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.5.1-002	[redacted]	2010	Nature of [14C]Glyphosate residues in processed commodities - High temperature hydrolysis Report No.: 1925W-001 Document No.: MSL0023072 PTRL West, Inc. GLP/GEP: Y Published: N	N	N	--	GTF	Y RAR 2017: OECD: KIIA 6.5.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.5.1-003	[redacted]	2006	High temperature hydrolysis of [14C]IN-MCX20 in buffered aqueous solution at pH 4, 5, and 6 Report No.: DuPont-19797 Document No.: - E.I. du Pont de Nemours and Company GLP/GEP: Y Published: N	N	N	--	COR	Y RAR 2017: OECD: KIIA 6.5.1 Monograph 1998: -- Monograph Trimesium: --
KCA 6.5.3-004	[redacted]	1996	Residues of glyphosate and AMPA in olives and olive oil, following a soil treatment with Roundup® herbicide. Spanish field trials, 1995 Report No.: MLL-30469 Document No.: 95-GLY-20SP Monsanto Europe S.A., the Agricultural Group; Agrisearch UK GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: EG:AIIA-6.3; EG:AIIA-6.5 Monograph Trimesium: --
KCA 6.5.3-005	[redacted]	1993	Residues of Glyphosate/AMPA in olives and olive oil following a soil treatment with MON 65040 herbicide. Italian field trials, 1993 Report No.: MLL-30319 Document No.: 93GLY-01 Monsanto GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: EG:AIIA-6.3; EG:AIIA-6.5 Monograph Trimesium: --

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.5.3-006	[redacted]	1992	Residues of Glyphosate/AMPA in olives and olive oil following use of Sting SE - Spanish field trials 1990 / 1992 Report No.: MLL-30297 Document No.: 90-GLY-02 / 92-GLY-01SP Monsanto GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.3 Monograph 1998: EG:AIIA-6.3; EG:AIIA-6.5 Monograph Trimesium: --
KCA 6.6.1-001	[redacted]	1998	LX1146-02 (Glyphosate technical) Confined Rotational Crop Study on lettuce, radish, and wheat in California Report No.: 1651-91-146-01-09B-17 Document No.: 459-GLY Pharmaco LSR International Inc. GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.6.2 Monograph 1998: -- Monograph Trimesium: --
KCA 6.6.1-002	[redacted]	1993	[14C-Anion] Glyphosate-Trimesium: Confined Accumulation Studies on Rotational Crops Report No.: RR92-096B Document No.: - Zeneca Inc. GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: OECD: KIIA 6.6.2 Monograph 1998: -- Monograph Trimesium: AIIA-6.1
KCA 6.6.1-003	[redacted] (part I); [redacted] (part II)	1990	Confined rotational crop study of Glyphosate. Part I: In-field portion Confined rotational crops study of Glyphosate - Part II: Quantitation, characterization and identification of Glyphosate and its metabolites in rotational crops Report No.: MSL-9810 (part I); MSL-9811 (part II) Document No.: - Pan-Agricultural Laboratories, Inc. (part I); Monsanto Agricultural Company (part II) GLP/GEP: Y Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.6.2 Monograph 1998: EG:AIIA-6.6; EG:AIIIA-8.5 Monograph Trimesium: --
KCA 6.6.1-004	[redacted]	1989	[14C-Anion]ICIA0224 Confined Accumulation Studies on Rotational Crops Report No.: WRC 89-25 Document No.: VV-320956 ICI Americas Inc. GLP/GEP: Y Published: N	N	N	--	SYN	Y RAR 2017: -- Monograph 1998: -- Monograph Trimesium: AIIA-6.6; AIIIA-8. 5

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.6.1-006	[redacted]	1976	Uptake and Metabolism of CP 67573 in representative vegetables and rotation crops Report No.: 406 Document No.: - Monsanto Company GLP/GEP: N (Pre GLP) Published: N	N	N	--	BCS	Y RAR 2017: OECD: KIIA 6.6.2 Monograph 1998: EG:AIIA-6.6; EG:AIIIA-8.5 Monograph Trimesium: --
KCA 6.9-001	Zoller, O. *et al.*	2018	Glyphosate residues in Swiss market foods: monitoring and risk evaluation Report No.: DOI 10.1080/19393210.2017.1419509; E-ISSN 1939-3229 Document No.: - Food Additives and Contaminants: Part B (2018) 11:83-91 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.9-002	Panseri, S. *et al.*	2020	Occurrence of perchlorate, chlorate and polar herbicides in different baby food commodities Report No.: 'DOI 10.1016/j.foodchem.2020.127205 Document No.:- Food chemistry, (2020) Vol. 330, Art. No. 127205 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.10.1-001	[redacted]	2020	Determination of Residues of Glyphosate in Honey after one Application in *Phacelia tanacetifolia* at 4 Sites in Germany 2019 Report No.: S19-04329 Document No.: - Eurofins Agroscience Services Ecotox GmbH GLP/GEP: Y Published: N	N	Y	First submission in EU	GRG	N
KCA 6.10.1-002	[redacted]	2020	Residues of Glyphosate in Food, Feed and Urine Report No.: TRR0000298 Document No.: - Bayer Crop Science GLP/GEP: N Published: N	N	Y	First submission in EU	GRG	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.10.1-003	El Agrebi, N. *et al.*	2019	Honeybee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey Report No.: DOI 10.1016/j.scitotenv.2019.135312; E-ISSN 1879-1026 Document No.: - The Science of the total Environment (2020) 704:135312 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.10.1-004	Thompson, T.S. *et al.*	2019	Determination of glyphosate, AMPA, and glufosinate in honey by online solid-phase extraction-liquid chromatography-tandem mass spectrometry. Report No.: DOI 10.1080/19440049.2019.1577993; E-ISSN 1944-0057 Document No.: - Food Additives and Contaminants: Part A (2019) 36:434-446 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.10.1-005	Chiesa, L.M. *et al.*	2019	Detection of glyphosate and its metabolites in food of animal origin based on ion-chromatography-high resolution mass spectrometry (IC-HRMS) Report No.: DOI 10.1080/19440049.2019.1583380; E-ISSN 1944-0057 Document No.: - Food Additives and Contaminants: Part A (2019) 36:592-600 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.10.1-006	Berg, C.J. *et al.*	2018	Glyphosate residue concentrations in honey attributed through geospatial analysis to proximity of large-scale agriculture and transfer off-site by bees. Report No.: DOI 10.1371/journal.pone.0198876; E-ISSN 1932-6203 Document No.: - PloS One (2018) 13:e0198876 GLP/GEP: N Published: Y	N	N	--	LIT	N
KCA 6.10.1-007	Karise, R. *et al.*	2017	Are pesticide residues in honey related to oilseed rape treatments? Report No.: DOI 10.1016/j.chemosphere.2017.09.013; E-ISSN 1879-1298 Document No.: - Chemosphere (2017) 188:389-396 GLP/GEP: N Published: Y	N	N	--	LIT	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If Yes, for which data point?
KCA 6.10.1-008	Rubio, F.M. *et al.*	2014	Survey of glyphosate residues in honey, corn, and soy products Report No.: DOI 10.4172/2161-0525.1000249 Document No.: - Journal of Environmental and Analytical Toxicology (2014) 5:1000249 GLP/GEP: N Published: Y	N	N	--	LIT	N

[1] In order to facilitate the compilation of the final list of the tests and studies relied upon and the corresponding data protection, indicate whether the study was used in the previous DAR/RAR or, when the information is available, whether the study was already submitted in the framework of national authorisations.

[2] See Art.3 of Annex of Regulation No 283/2013 and 284/2013

[3] The RMS shall check that the GLP statement has been properly signed in the study report, that the study results are properly reported in accordance with GLP standards and following the relevant guidance by OECD on the review of the GLP status of non-clinical safety data (currently under development).