1. Information on the study

Data point	CA 6.4.1
Report author	Shehata A.A. *et al.*
Report year	2014
Report title	Distribution of Glyphosate in Chicken Organs and its Reduction by Humic Acid Supplementation
Document No.	J. Poult. Sci., 2014, 51: 333-337
Guidelines followed in study	None stated
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:	Yes/ reliable with restrictions

2. Full summary of the study according to OECD format

Executive Summary

Glyphosate (N-(phosphonomethyl) glycine) is a most popular herb**icide in agricultural practices** throughout the world. It is possible that glyphosate spread in the ecosystems can reach plants, animals. The present work was directed to investigate the glyphosate residue in different organs of broiler chickens using ELISA and to study the possibility of its neutralisation using humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii*. Results showed that glyphosate residues could be detected in the animal feed and different organs as liver, spleen, lung, intestine, heart, muscles and kidney. Humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii* showed neutralization of the antimicrobial effect of glyphosate in vitro. Also, feed supplementation of commercial broiler with humic acid (0.2%) leads to a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, gastro-intestinal tract, heart, muscles and kidney, respectively. There were no significant effects of humic acid on the production parameters. This enlightenment will help to overcome the negative effect of glyphosate residues on gastrointestinal microbiota and protect consumers from glyphosate residues in chicken meat.

Materials and Methods

Distribution of Glyphosate in Feed and Tissues

A total of one hundred commercial broiler chickens collected from different farms were slaughtered at 30-day-old. Different organs as liver, spleen, lung, intestine, heart, muscles and kidney were collected and tested for presence of glyphosate using ELISA. Briefly, samples were collected from 10 chickens per farm at 39-day-old after slaughtering and cut to small pieces. In relation to its ability to retain water specimens were suspended in aqua distilled (Braun, Germany) at the rate of 1:1 (low water retention), 1:5 or 1:10 (high water retention). The specimens were heated at 100°C for 10 min, homogenized with ULTRA-TURRAX® (IKA, Wilmington, Germany) and frozen at minus 80°C for eight hours. Homogenized specimens were thawed at 40°C and centrifuged at 10000 x g for 10 min. The supernatant was filtered with an ultracentrifugal filter (3000 Da) to remove proteins and peptides. Filtrates were centrifuged again at 10000 x g for 10 min and the supernatant was tested for glyphosate concentration by ELISA using Glyphosate ELISA kits (Abraxis, Warminister, PA, USA) according to the manufacturer's protocol. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinische Labor (Bremen, Germany), the correlation coefficient between the two tests was 98%.

In vitro Neutralization of Glyphosate

The minimal inhibitory concentration (MIC) of glyphosate (Roundup UltraMax®, Monsanto, USA) on

E. faecalis, *Bacillus badius* (isolated from algae *Chlorella vulgaris*, Ökologische Produkte Altmark Co., Germany) and *Bifidobacterium adolescentis* (isolated from chickens), as indicators, was determined according to the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, the lowest concentration of glyphosate which shows bactericidal or bacteriostatic effects was determined in a 24-well micro-titre plate. Serial dilutions of glyphosate (5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 mg/ml) were made in reinforced clostridial medium (RCM, Sifin, Germany). Tested bacteria was added at a final concentration of 10^4 CFU/ml and the test plates containing diluted glyphosate and tested bacteria were incubated overnight at 37°C. The MIC value was evaluated by quantitative analysis of bacterial growth on Citrat-Azid-Tween-Carbonat Agar (CATC, Oxoid, Germany). The neutralizing effect of humic acid RB4, composed of different molecular weights molecules ranged from 1500 Da to 200000 Da, (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany), was tested. The MIC value of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid RB4 (1 mg/ml), *Chlorella vulgaris* extract (Ökologische Produkte Altmark Co., Germany) at a concentration of 1 mg/ml and *Saccharomyces boulardii* at a concentration of 10^9 CFU/ml (UCB Pharma GmbH, Monheim, Germany) determined.

In vivo Neutralization of Glyphosate Using Humic Acid

The experiment was performed in two chicken broiler barns, designated A and B, each barn accommodated for 22000 broiler chicks. Chickens kept in house A were fed the basic diet without supplementation of humic acid, while chickens kept in house B were fed the same diet with humic acid RB4 (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany) supplementation (0.2%) from the first day till slaughtering. The ration was formulated as follow: starter (21% corn, 40% wheat, 29% soya bean and 4.5% fat), grower (22% corn, 47% wheat, 19% soya bean and 5% fat), and finisher (17% corn, 48% wheat, 17% soya bean and 4.9% fat). Chickens were allowed to have free access to feed and water until the end of experiment. All chickens were vaccinated against infectious bronchitis (IB) at 12-day-old, Newcastle disease (ND) and infectious bursal disease at 18-days-old. The total mortality and body weight (BW) were calculated at the end of the experiments. Glyphosate residues were determined in serum, liver, spleen, lung, GIT, heart, muscles and kidney using ELISA as mentioned above.

Statistical Analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). Two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Distribution of Glyphosate in Feed and Tissues

The glyphosate residues could be detected in feed, liver, spleen, lung, intestine, heart, muscles and kidney using ELISA in the concentrations of 370, 9.8, 21.1, 24.2, 98.3, 20.4, 5.0 and 16.0 ng/gm, respectively (Table 1).

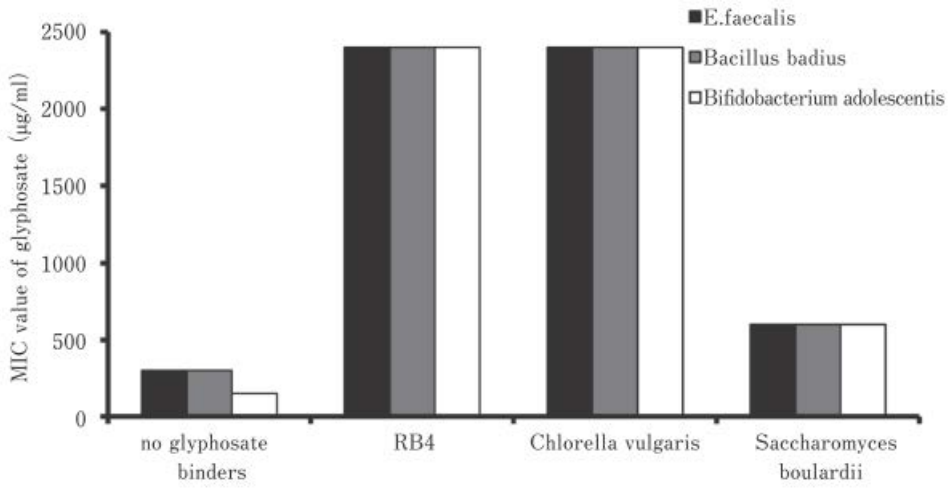
Table 1: Distribution of glyphosate in feed and chickens tissues.

Sample N=30	Glyphosate (ng/gm)		
	Minimum	Maximum	Mean±SD
Feed	190.0	400.0	370.0±92.0
Liver	6.0	13.6	9.8±3.0
Spleen	11.8	25.0	21.1±17.0
Lung	12.0	25.0	24.2±9.0
Intestine	20.0	120.0	98.3±42.0
Heart	17.0	20.0	20.4±0.6
Muscles	3.6	4.9	5.0±0.3
Kidney	0.4	17.6	16.0±13.0

Neutralisation of Glyphosate in vitro

The MIC value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* were 300, 300 and 150 µg/ml, respectively. The RB4 and *Chlorella vulgaris* in concentrations of 1 mg/ml showed the higher neutralization of the antimicrobial effect of glyphosate. The MIC-values of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid or *Chlorella vulgaris* were 2400 µg/ml (Fig. 1). However, the MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence Saccharomyces boulardii was 600 µg/ml (Fig. 1).

Figure 1: Changes in the MIC values of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* using different glyphosate binders.



In vivo Neutralization of Glyphosate Using Humic Acid

In untreated chickens, the glyphosate concentrations in serum, liver, spleen, lung, GIT, heart, muscles and kidney were 2, 14, 21, 24, 101, 20, 6 and 6 ng/gm, respectively, however, in humic acid treated chickens, glyphosate residues were 0.88, 9.78, 11.79, 12.20, 43.6, 17.4, 1.9 and 6. 2 ng/gm, respectively. Supplementation of humic acid caused a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, GIT, heart, muscles and kidney, respectively (Fig. 2). At 30-day-old, there is no significant improvement of body weight and total mortalities between humic acid-treated and untreated chickens (Table 2), the average body weight of both was 1.69 Kg. However at 39-day-old, the average body weight of 2.456 Kg while it was 2.339 Kg in untreated chickens (Table 2).

Figure 2: Effect of humic acid supplementation on glyphosate accumulation in chickens. Glyphosate was measured using ELISA and expressed as ng/gm. Asterisks denote significant decrease of glyphosate in humic acid treated chickens (* $P=0.05$, ** $=P<0.001$).

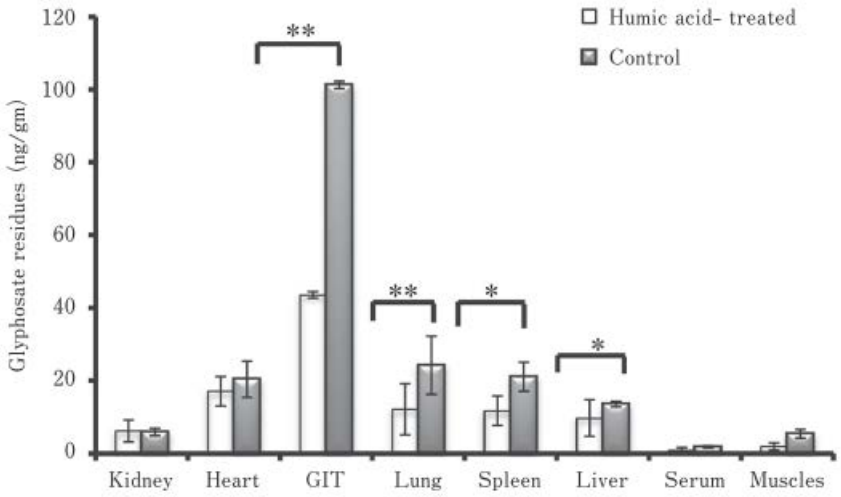


Table 2: Effect of humic acid supplementation on the production parameters.

Parameter	Humic acid-treated chickens	Non-treated chickens
Total number	22500	23100
Slaughtered number at 30-day-old	6509	6509
Slaughtered number at 39-day-old	14581	15573
Body weight at 30-day-old (average/kg)	1.69	1.69
Body weight at 39-day-old (average/kg)	2.453	2.339
Total feed intake (kg)	76530	78690
Food conversion ratio	1.64	1.66

Discussion

Distribution of Glyphosate in Feed and Tissues

Glyphosate residues in food and feed have been on the rise, due to higher rates and frequency of application, which in turn is due to increasing weed resistance (Samsel and Seneff, 2013). In the present study glyphosate residues could be detected in liver, spleen, lung, intestine, heart, muscles, kidney and animal feed (Table 1). The maximum residue levels (MRLs) of glyphosate in soya bean, maize, cereal grains, cotton seed, alfalfa, hay, sorghum straw, wheat and wheat straw were agreed by the United Nations Food and Culture Organization's to be 20, 5.0, 30, 40, 500, 500, 50, 200 and 300 mg/kg (WHO, 1994). Data on the real presence of glyphosate and its metabolite in feed from glyphosate sprayed crops are sparse. A now common practice of crop desiccation through herbicide administration shortly before the harvest assures an increased glyphosate residues in food sources as well (Baig et al., 2003; Ellis et al., 1998). Also, the maximum daily intake (MDI) of glyphosate depends on the ration composition and the percent of each component in the ration. Glyphosate residues concentrate in approximately 80% genetically modified plants grown for food and feed up to 400 ppm, maximal residual levels.

Neutralisation of Glyphosate in vitro

Many studies have reported that glyphosate can be sorbed to humic acids (Piccolo et al., 1996; Banta et al., 2009; Mazzei and Piccolo, 2012). In the present study the humic acid RB4 neutralized the antimicrobial effect of glyphosate in vitro. The MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of RB4 humic acids or Clorella vulgaris were 2.4 mg/ml.

Chlorella has also useful detoxifying properties. The use of oral supplements of *Chlorella pyrenoidosa*

has been reported to significantly reduce dioxin levels in breast milk of 35 nursing women in Japan (Nakano et al., 2007). Also Chlorella supplementation significantly reduced liver toxicity and cadmium-accumulation in cadmium poisoned rats (Shim et al., 2008).

Yeast has been used as general performance promoter in poultry feeds and has been shown to have beneficial effects against mycotoxins exposure (Celyk et al., 2003, Santin et al., 2003, Baptista et al., 2004). The absorbent ability of yeast to mycotoxins could be attributed to the presence of innumerable sites on its surface for physical adsorption of molecules (Shetty and Jespersen 2006). In the present study *Saccharomyces boulardii* showed a low absorbent ability to glyphosate (Fig. 1).

Neutralisation of Glyphosate by Humic Acid Supplementation in vivo

The use of humic acids and their sodium salt for the oral treatment of all animals on food production farms is currently permitted. Supplementing animal feeds with non-nutritive adsorbents as humic acid has proven to substantially reduce mycotoxicosis (Sabater-Vilar et al., 2007) and improved the performance, carcass, GIT and meat quality traits (Ozturk et al., 2011). In our study, the mortality was negligible with no difference between control and humic acid-treated group. Also the humic acid-treated chickens showed no improvement in feed conversion in birds and body weight at 30-day-old (Table 2). Kocabagli et al. (2002) reported an improvement in feed conversion in birds that were given 0.25% humic acid either from 0 to 42 d or during grow-out periods only, between d 21 to 42. A similar conclusion was drawn by Yoruk et al. (2004), who showed a better feed conversion in hens supplemented with 0.1-0.2% humic acid, and it did not affect body weight. On the contrary, Rath et al. (2006) found that humic acid-treated chickens showed a reduction in body weight, and the feed conversion ratio was numerically higher.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication provides information about the levels of parent glyphosate residues in feed and tissues of broiler chicken (including edible tissues such as muscle and liver). This may allow to estimate residue transfer factors from poultry feed to poultry meat. Therefore, the publication is considered relevant. The authors further investigated the impact of a feed supplementation with humic acid on the transfer of glyphosate residues in poultry tissues. It was concluded that the supplementation with humic acid allows to significantly decrease the residues of glyphosate in poultry tissues (-63% in muscle and -28% in liver). Thus, the control group (which received feed without humic acid supplementation) represents a worst case in terms of residues and is more relevant from a regulatory perspective. The highest residues found in chicken muscle and liver were extremely low (ca. 0.005 mg/kg and 0.018 mg/kg, respectively). This is consistent with the results of the submitted poultry feeding studies (which were conducted at dose levels far above the dietary exposure of the broiler chickens in the publication). However, both the experimental procedures and the obtained results are not described with a sufficient level of accuracy and it is difficult to figure out exactly what was done and how the presented results were generated. The sample preparation procedure (with consecutive steps at 100°C and -80°C) is quite unusual and no method validation data are presented. Because of that, the publication is reliable with restrictions.

1. Information on the study

Data point	CA 6.4.2
Report author	Shelver W.L. *et al.*
Report year	2018
Report title	Distribution of chemical residues among fat, skim, curd, whey, and protein fractions in fortified, pasteurized milk
Document No.	ACS Omega 2018, 3, 8697 − 8708
Guidelines followed in study	None stated
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:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

The distribution of 12 environmental contaminants or metabolites with diverse polarities (2,2′,4,4′,5-pentabromodi-phenyl ether; bisphenol A; estrone; glyphosate; β-hexabromocyclo-dodecane; imidacloprid; 2,3′,4,4′,5-pentachlorobiphenyl; 3′-methylsulfone 2,2′,4,5,5′-pentachlorobiphenyl; 1,2,7,8-tetrachlorodibenzo-*p*-dioxin; 2-hydroxy-1,3,7,8-tetrachlorodibenzo-*p*-dioxin; tetrabromo-bisphenol A; and triclocarban) among skim milk, fat, curd, whey, whey retentate, and whey permeate was characterized. Analysis of these compounds along with 15 drugs previously studied provided a robust linear model predicting the distribution between skim and fat and the chemical's lipophilicity (log *P*, $r^2 = 0.71$; log *D*, $r^2 = 0.79$). Similarly, distribution between curd and whey was correlated with lipophilicity (log *P*, $r^2 = 0.63$; log *D*, $r^2 = 0.73$). Phenolic compounds had less predictable distribution patterns based on their lipophilicities. Within the whey fraction, chemicals with greater lipophilicity are associated with whey proteins more than hydrophilic chemicals. The resultant model could help predict the potential distribution of chemical contaminants among milk products in cow milk, if present.

Materials and Methods

Selection of drugs and concentrations

Chemicals selected for study had to be potential environmental contaminants, encompass a wide range of lipophilicities, and be available with radiolabel (^3H or ^{14}C) incorporation. The chemicals selected had a log *P* range of − 3.3 to 7.3. Chemical structures, site of radiolabel, specific activity (SA), and physio-chemical properties are provided in **Table 1**.

To detect potential concentration-dependent distribution, chemical concentrations spanning 3 orders of magnitude (*i.e.*, 20 − 2000 nM) were generally used. The lowest concentration (usually 20 nM) was typically relevant to possible contamination scenarios with sufficient activity to allow radiochemical detection. Higher concentrations were used to determine whether concentration influenced overall xenobiotic distribution. In some instances, concentrations were adjusted because of limited solubility or if the SA of the radiolabeled compound was inadequate for the sensitivity of the analysis (**Table 1**). As a result of adding unlabeled chemical (typically 9:1 parts) for the highest dose, SA was lowered, relative to low concentration.

Table 1: Drug Structures and Physicochemical Properties.

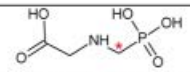
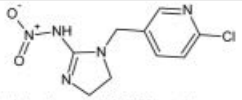
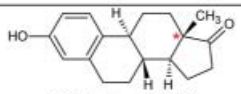
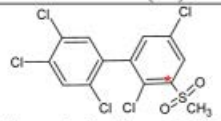
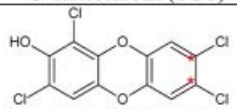
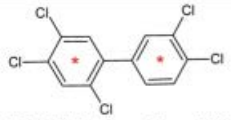
[a] Compound radioactively labeled with a directed label and specified on the structure with a red asterisk. An asterisk within a ring indicates a uniform label on the ring. Exceptions: IMI and *β*-HBCD carbon labels are unknown.

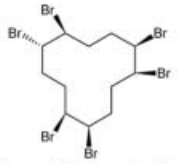
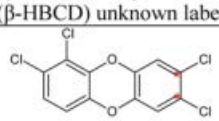
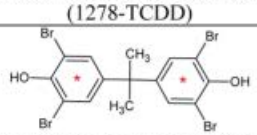
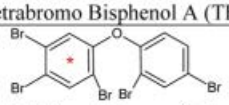
[b] SAs were adjusted depending on dose, as indicated. Values in parentheses are nominal concentrations

for initial fortification.

[c] Average log *P* calculated from literature log *P* values accessed from www.chemspider.com, www.drugbank.ca, www.ebi.ac.uk/chembl/, and pubchem.ncbi.nlm.nih.gov/ on 7/14/2017 using the predicted and experimental values were available.

[d] Values for log *D* at pH 6.8 were calculated using log *P* values from above sources and pK_a's from www.drugbank.ca, www.ebi.ac.uk/chembl/, www.druginfosys.com, pubchem.ncbi.nlm.nih.gov/, Johansson and Anlér [11] accessed on 7/14/2017.

Compound[a]	Class/ Use	M.W. S.A. (nCi/nmol)[b]	log P[c]	pKa[d]	log D[d]
 ^{14}C-Glyphosate (GLY)	Herbicide/ pesticide	169.07 g/mol 50 (20 nM/ 200 nM) 5.0 (2000 nM)	-3.26 ± 1.53	5.89 ± 0.40	-4.24 ± 1.49
 ^{14}C-Imidacloprid (IMI) unknown label	Insecticide/ pesticide	255.66 g/mol 25.3 (20 nM/ 200 nM) 2.5 (2000 nM)	0.39 ± 0.59	5.28	-0.38 ± 0.59
 ^{14}C-Bisphenol A (BPA)	Plasticizer	228.28 g/mol 53.5 (20nM/ 200nM) 6.0 (2000nM)	3.60 ± 0.27		3.60 ± 0.27
 ^{14}C-Estrone (E1)	Hormone	270.37 g/mol 51.3 (20nM/ 200nM) 5.7 (2000nM)	3.62 ± 0.45		3.62 ± 0.45
 ^{14}C-3'-methylsulfone-2,2',4,5,5'-pentachloro biphenyl (3-MeSO$_2$-PCB-101)	PCB Metabolite	404.52 g/mol 53 (20/100/500nM)	4.62		4.62
 ^{14}C-Triclocarban (TCC)	Antibacterial / disinfectant	315.58 g/mol 30 (20 nM/ 200 nM) 3.0 (2000 nM)	5.39 ± 0.45		5.39 ± 0.45
 ^{14}C-2-hydroxy-1,3,7,8-tetrachloro dibenzo-*p*-dioxin (2-OH-1378-TCDD)	TCDD Metabolite	337.97 g/mol 64.6 (20 nM/ 100 nM) 12.8 (500 nM)	6.15 ± 0.32		6.15 ± 0.32
 ^{14}C-2,3',4,4',5-pentachlorobiphenyl (PCB-118)	Coolants/ plasticizers/ hydraulic fluids/ pesticides/ flame retardant	326.43 g/mol 10.3 (50 nM/ 200 nM) 2.5 (2000 nM)	6.78 ± 0.35		6.78 ± 0.35

 ^{14}C-β-hexachlorocyclododecane (β-HBCD) unknown label	Flame Retardant	641.69 g/mol 2 (200/500/2000 nM)	7.22 ± 0.65		7.22 ± 0.65
 ^{14}C-1,2,7,8-tetrachloro dibenzo-*p*-dioxin (1278-TCDD)	Industrial and incineration byproduct	321.97 g/mol 67.8 (20 nM/ 200 nM) 6.8 (2000 nM)	6.22 ± 0.72		6.22 ± 0.72
 ^{14}C-Tetrabromo Bisphenol A (TBBPA)	Flame Retardant	543.87 g/mol 25 (20 nM/ 200 nM) 3.7 (2000 nM)	6.69 ± 0.58		6.69 ± 0.58
 ^{14}C-2,4',4,5,5'-pentabromo diphenyl ether (BDE-99)	Flame Retardant	564.69 g/mol 49 (20 nM) 8.76 (200 nM) 0.98 (2000 nM)	7.31 ± 0.62		7.31 ± 0.62

Chemicals, supplies, and equipments

Raw (unpasteurized, nonhomogenized) cow milk was obtained from the bulk milk tank located at the North Dakota State University (Fargo, ND) Dairy farm within 48 h of milking. Non-radiolabeled chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO), U.S. Pharmacopeia (Rockville, MD), or other common vendors. Radiolabeled E1, GLY, PCB-118, and *β*-HBCD were procured through American Radio-labeled Chemicals, Inc. (ARC, St. Louis, MO). A mixture of the *β*- and *γ*-diastereoisomers of [^{14}C]-HBCD was identified in the ARC product. Flash chromatography on a silica gel column eluted with hexane containing increasing amounts of methylene chloride (0 − 50%) was used to isolate [^{14}C]-*β*-HBCD. [^{14}C]-BPA and [^{14}C]-TCC were purchased from Moravek Inc. (Brea, CA). [^{14}C]-IMI was a gift from Bayer Crop Science (Research Triangle Park, NC). [UL-7,8-ring^{14}C]-1278-TCDD was purchased from ChemSyn Science Laboratories (Lenexa, KS). [^{14}C]-2,2′,4,4′,5-pentabromodi-phenyl ether (BDE-99) was synthesized using published methods [23]. 2-OH-1378-TCDD was prepared in-house from [UL-7,8-ring^{14}C]-1278-TCDD by *in vitro* oxidation with human CYP1A1R Baculosomes (Cypex Ltd., Dundee, UK) and a glucose-6-phosphate dehydrogenase regenerating system according to manufacturer's instructions. [^{14}C]-2,2-bis(4-hydroxy-3,5-dibromophenyl)propane (TBBPA) was synthesized by brominating bis[^{14}C]-phenol A with 4.2 equivalents of bromine in 1:1 methanol/water; bis[^{14}C]-phenol A was prepared in-house from [UL-^{14}C]-phenol (2.0 mCi, 25 mCi/mmol) and acetone according to a published method [24]. 3′-[^{14}C]-MeSO$_2$-PCB-101 was synthesized de novo by Cadogan coupling as described in Haraguchi *et al*. [25] using sodium [^{14}C]-methyl thiolate for label introduction.

Silica gel plates were purchased from Analtech (Newark, DE). Scintillation cocktails were purchased from MP Biochemicals, LLC, (Ecolite; Solon, OH) or PerkinElmer (Waltham, MA; Carbosorb, and Permafluor). Amicon Ultra-15 centrifugal filters were purchased from Millipore (Billerica, MA). An Allegra X-14R centrifuge was obtained from Beckman-Coulter (Brea, CA). Liquid milk product fractions were mixed with scintillation fluid and assayed using a Tri-Carb 1900 liquid scintillation counter (LSC, Packard, Meriden, CT). Solid milk product samples were combusted using a Packard model 307 tissue oxidizer (Meriden, CT), trapped into Carbosorb, diluted with Permafluor, and then assayed by LSC. Sample purity was assessed by TLC and radioassay using a Bioscan AR-2000 Imaging Scanner for TLC (Washington, DC).

Determination of chemical purity and confirmation of test article stability

TLC analyses were used to assess chemical purities before and after the experiments, although for GLY, high-performance liquid chromatography instead of TLC was employed. Initial analyses were used to evaluate dose purity, whereas post-incubation analyses were used to evaluate whether chemical

degradation occurred during milk processing. TLC conditions and results are included in Table S3. GLY radiochemical purity (98.0 ± 0.4%, n = 4) was determined based on Nagatomi *et al*. [26] using a Waters 2695 HPLC, a radiometric detector (Packard LFA 515TR, PerkinElmer, Waltham, MA), and a Dionex IonPac AS 12 column (4 × 200 mm, 9 µ m, Dionex Company, Sunnyvale, CA). The mobile phase was isocratic 0.2% aqueous formic acid/acetonitrile (5/95, v/v), and the flow rate was 1 mL/min.

Milk processing and radiochemical analysis

The milk processing experiments consisted of three sequential phases. Specific details pertaining to preparation of phases are reported in Hakk *et al*. [7] and Shappell *et al*. [8]. Briefly, 12 tubes of raw milk (50 mL) were pasteurized at 63°C for 30 min. Triplicate tubes were fortified with each level of radiolabeled chemicals using three working solutions or with the appropriate solvent for blank milk, as described in **Table 2**. In phase 1, the fortified, pasteurized, whole milk samples were separated into milk fat and skim milk by centrifugation after equilibration; the partitioning of chemical between these phases was then determined by radiochemical detection methods. In phase 2, the skim milk originating from phase 1 was partitioned into curd and whey (enzymatically with rennet) and the distribution of the target chemical between these phases determined by radiochemical detection. In phase 3, the residual whey (15 mL) from phase 2 was separated into a protein-enriched fraction (> 10 kD), retentate (~ 5 mL) and permeate (~ 10 mL) fractions using ultracentrifuge filters. To determine if degradation occurred during processing, milk fat, curd, and whey from the highest dose concentration were extracted and analyzed by TLC side by side with radiolabeled standards with the exception of GLY because no satisfactory TLC method was found. The main difference in the current study compared to the cited research [7-9] was that here the radiolabeled compounds were fortified only once into whole milk and not anew at the beginning of each phase (**Figure 5**), resulting in lower initial chemical concentrations in skim and whey fractions.

Table 2: Compound Associated with Casein or Whey Protein (nmol/mg Protein and Percent Association Based on Whole Milk).

[a] SA of some compounds required different doses, as indicated by bold text. Each fortified level contains three replicates.
[b] These data were derived from phase 2 data and have whey associated drug subtracted, using "0% moisture curd" as described in text.
[c] These data were derived from phase 3 data as described in the text.
[d] Less than limit of quantitation (<LOQ). LOQ for PCB-118 is 1.92 nmol/L and for β-HBCD was 9.87 nmol/L.
[f] Inconsistent with other doses. No explanation.

compound	nominal conc. of whole milk[a] (actual) nM	nmol/mg casein protein[b]	nmol/mg whey protein[c]	conc. in casein/conc. in whey protein	mean % casein association based on whole milk[d]	mean % whey association based on whole milk[d]
GLY	20 (22)	0.08	0.15	0.53	7.92	3.68
	200 (217)	0.71	1.40	0.51		
	2000 (2059)	6.52	14.09	0.46		
IMI	20 (20)	0.21	0.11	1.91	15.43	3.19
	200 (201)	2.20	1.13	1.95		
	2000 (2066)	22.33	12.29	1.82		
BPA	20 (22)	0.44	0.23	1.91	45.76	6.68
	200 (216)	4.54	2.25	2.02		
	2000 (1992)	42.43	20.88	2.03		
E1	20 (20)	0.21	0.10	2.10	17.86	3.52
	200 (200)	1.66	0.96	1.73		
	2000 (1796)	15.89	8.70	1.83		
3-MeSO$_2$-PCB-101	20 (24)	0.06	0.04	1.50	4.25	0.99
	100 (70)	0.18	0.12	1.50		
	500(628)	1.60	1.08	1.48		
TCC	20 (19)	0.05	0.10	0.50	5.92	3.62
	200 (193)	0.54	0.95	0.57		
	2000 (1938)	5.68	9.72	0.58		
2-OH-1378-TCDD	20 (22)	0.16	0.64	0.25	17.85	16.79
	100 (107)	**0.77**	**3.26**	0.24		
	500 (556)	**4.71**	**16.16**	0.29		
PCB-118	**50 (60)**	**0.07**	**<LOQ**[c]		3.42	0.70
	200 (221)	0.35	0.24	1.46		
	2000 (2203)	2.90	2.37	1.22		
β-HBCD	**200 (229)**	0.38	<LOQ[c]		2.95	0.59
	500(656)	1.09	0.59	1.85		
	2000 (2067)	3.21	2.11	1.52		
1278-TCDD	20 (27)	0.11	0.06	1.83	4.14	1.29
	200 (184)	0.46	0.34	1.35		
	2000 (1784)	5.07	3.58	1.42		
TBBPA	20	0.27	0.79	0.34	18.01	22.96
	200 (234)	3.53	9.36	0.38		
	2000 (1815)	21.63	76.47	0.28		
BDE-99	20 (20)	0.12	0.03	4.0	6.66	1.20
	200 (178)	1.05	0.38	2.76[f]		
	2000 (1890)	18.33	3.35	5.47		

Calculation of chemical associated with casein and whey Protein

The percentage of chemical associated with whey proteins was calculated according to Shappell *et al.* [8]. Briefly, the amount of free chemical measured in permeate (calculated by concentration and volume) was subtracted from the total amount of chemical present in retentate. The difference was assumed to be the amount of chemical associated with whey protein. Residual radioactivity on ultrafilters (measured by combustion analysis) was considered nonspecific binding and was subtracted from the fortified whey results; however, radioactivity present in filter washes was included with retentate radioactivity.

Averaged Kjeldahl protein concentrations in curd from Shappell *et al*. [8] and Lupton *et al*. [9] and the resultant 0% moisture curd radioactivity (see below) along with its SA were used to calculate nanomole per milligram casein protein association. Similarly, averaged Kjeldahl protein concentration in retentate from Shappell *et al*. [8] and Lupton *et al*. [9] and the protein associated radioactivity and its SA in retentate was used to calculate nanomole per milligram whey protein association.

Statistical analyses

Standard statistical methods were used to calculate means and variability and make inferences with respect to the significance of differences between means. Linear regression was used to assess dose dependence of the observed drug distribution log ratio of [chemical] milk fat /[chemical] skim milk or 0% moisture [chemical] curd /[chemical] whey. Dose dependency was based on instances when the slope differed ($P < 0.05$) from zero. Because curd is 70% moisture and contains a small quantity of entrained whey, a 0% moisture curd radioactivity value was calculated by subtracting entrained whey-associated radioactivity (calculated based on the percent moisture) from curd. The value representing entrained whey was added back to the whey fraction.

Coefficient of variation with respect to measured partition values across doses was typically much less than 10%, whereas literature values for log *P* for a given chemical could sometimes differ by an order of magnitude or greater. Therefore, distribution data were modeled using mean log P values ± SD for each chemical. Mean log P values were calculated from predicted and measured entries included in Chemspider, DrugBank, ChemBL, and Pubchem databases. For 3′-$MeSO_2$-PCB-101, the log *P* value was derived from using conversion of chlorocyclohexatriene into *p*-chlorophenyl methyl sulfone as a model, which has log differences of 1.76. By using PCB-101 log *P* of 6.38 and subtracting 1.76, the log *P* of 3′-$MeSO_2$-PCB-101was derived as 4.62. Log *D* values were calculated as described by Scherrer and Howard [16] using a pH of 6.8 (reflecting the pH of milk); to obtain a theoretical range of log *D* values for each compound, the range of log P values derived from the above sources was used in conjunction with the range of pK_a values obtained from the same sources; log *D* values were averaged and SDs calculated. Relationships between the log distribution ratios and lipophilicity (log *D* and log *P*) were performed using linear function and included the 99% CI and prediction interval by GraphPad Prism Version 7.03 (Graph-Pad Software, La Jolla, CA).

Results and Discussion

Chemical distribution from whole milk into milk fat and skim milk.

Milk partitioning into lipid was highly reproducible, with typical coefficient of variance (CV) values of ≤ 5%; exception was GLY with CV up to 19% (Tables S5−S16). The high CV of GLY was due to its low partitioning into milk fat (Table S5). Similarly, CV of partitioning into skim milk was ≤ 5%; exceptions were BDE, *β*-HBCD, 3′-$MeSO_2$-PCB-101, PCB, and TCC because of low amounts in the skim milk. Recoveries (sum of total radioactivity in skim milk and milk fat) were > 90%, ranging from ~ 91% (for chemicals with log $D \geq 6.7$) up to 100% for GLY (Figure 1 and Tables S5−S16). Distribution of chemical residues was not dose-dependent over the range of doses used (linear regression slope $P > 0.05$), suggesting that a chemical's distribution between skim milk and milk fat would be constant regardless of the concentration. In the absence of overt physiologic effects such as toxicity or effects on blood flow to the mammary gland, such results suggest that whole milk composition (*i.e.*, across species or breed types) would influence a chemical's presence in milk to a greater extent than the dose received.

For the 12 chemicals tested, distribution into milk fat ranged from < 3% (0.95% for GLY and 2.5% for IMI) to > 80% of the total amount added (3′-$MeSO_2$-PCB-101, TCC, PCB-118, *β*-HBCD, 1278-TCDD, and BDE-99). Intermediate distributions into milk fat occurred for phenolic compounds (BPA, 39%; TBBPA, 46%; 2-OH-1378-TCDD, 54%; and E1, 74%) (Tables S5−S16, **Figure 1**).

As would be anticipated, the data indicated that nonpolar chemicals concentrate into high lipid milk fractions. The concentration ratios in milk fat relative to whole milk for moderately polar phenolic compounds were about 10 (BPA, 8.2; TBBPA, 10.5; 2-OH-1378-TCDD, 11.2; and E1, 15.8) and were ~ 18−20 for highly nonpolar persistent environmental contaminants (BDE-99, *β*-HBCD, 3′-$MeSO_2$-PCB-101, PCB-118, TCC, and 1278-TCDD; Figure 1). Also as expected, polar chemicals partitioned to

a large degree into skim milk, resulting in milk fat/whole milk concentration ratios of < 1 (GLY was 0.2, and IMI was 0.5; Figure 1). For the phenolic compound BPA, substitution of four phenyl hydrogens with bromines to form TBBPA (Table 1) increased lipophilicity (log D = 3.60 vs 6.69) and was reflected by TBBPA's milk fat/whole milk concentration ratio of 10.5 compared to that of 8.2 for BPA (**Figure 1**). Hydroxylation of a molecule decreases its relative lipophilicity with respect to its non-hydroxylated analogue, as is commonly observed during oxidative metabolism. Although 1278-TCDD and 2-OH-1378-TCDD have very similar log *D* values (6.15 and 6.22, respectively) hydroxylation resulted in reduced lipid solubility and a ~ 30% reduction in milk fat distribution. However, the addition of a more polar functional group onto a pentachloro biphenyl molecule to form 3′-$MeSO_2$-PCB-101 did not shift the milk fat distribution pattern when compared to PCB-118. One possible explanation may be due to the change of chlorine substitution pattern.

Figure 1: Chemical distribution and relative concentration ratios from whole milk into skim milk and milk fat fractions. Bars represent percent mean of all concentrations (n = 3 concentrations, 3 replicates per concentration, replicate exceptions are n = 2 replicates each for 1278-TCDD 20 and 200 nM and n = 2 replicates for BDE-99 2000 nM) ± SD of the three dose means based on disintegrations per minute (dpm) of skim milk and milk fat fractions compared to whole milk dpm. Values on graph represent the mean ratio of the drug concentration in the fraction (milk fat or skim milk) to the initial drug concentration in whole milk ± SD of means between doses (n = 3 mean dose ratios). Sum of stack plot represents total chemical recovery. log *D* values given for each compound at bottom of plot.

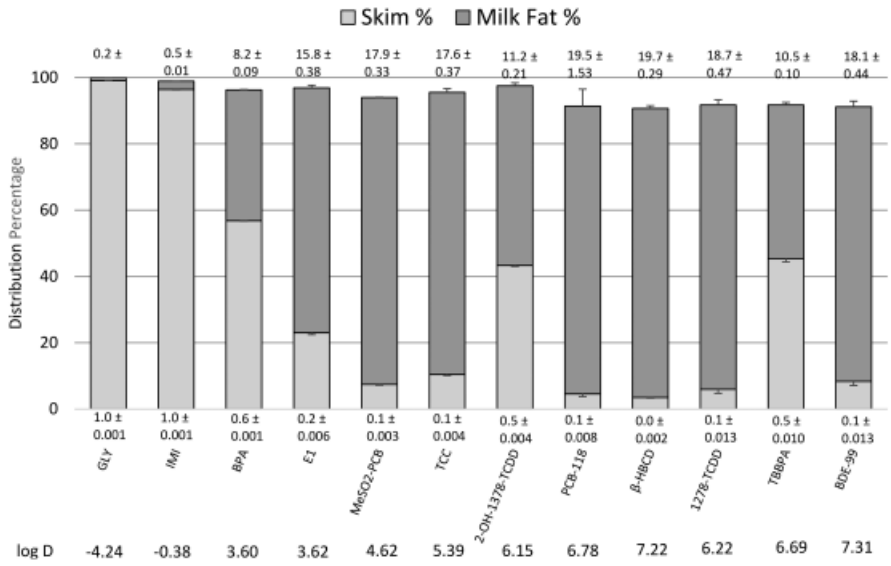


Table S5: GLY Phase 1 Average Distribution Data and Ratios.

Phase 1	Whole			Skim Milk				Milk Fat				Percent Recovery	Obs. Ratio
Mean	Spike DPM	Volume (mL)	Initial nM	Total DPM	Skim Milk Volume (mL)	Final nM	% GLY Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% GLY Dose	Total % based on Whole	[milk fat]/[skim milk]
0nM		48.83			46.51				2.33				
20nM	117,903	48.90	21.72	116,815	46.58	22.59	99.10	948	2.31	3.69	0.80	99.90	0.16
200nM	1,178,074	48.90	217.04	1,167,088	46.52	226.03	99.07	11,398	2.38	43.21	0.97	100.04	0.19
2000nM	1,117,710	48.90	2,059.36	1,107,061	46.48	2,145.80	99.05	11,973	2.42	444.70	1.07	100.12	0.21
St Dev													
0nM		0.08			0.03				0.05				
20nM	1,658.	0.00	0.31	1,951	0.02	0.39	2.58	140	0.02	0.51	0.13	1,658.35	0.02
200nM	5,136	0.01	0.99	10,791	0.02	2.18	1.07	973	0.04	3.22	0.09	5,135.79	0.02
2000nM	11,354	0.01	21.18	4,072	0.08	7.45	1.11	2,330	0.07	73.05	0.20	11,354	0.03
% RSD													
0nM		0.16			0.06				2.07				
20nM	1.41	0.01	1.41	1.67	0.05	1.72	2.60	14.82	0.85	13.95	15.56	2.70	12.15
200nM	0.44	0.02	0.46	0.92	0.05	0.97	1.08	8.54	1.65	7.45	8.86	1.05	8.16
2000nM	1.02	0.03	1.03	0.37	0.17	0.35	1.12	19.46	3.01	16.43	18.54	0.92	16.51

Although literature describing the milk partitioning of the exact compounds studied here has not been found, there are several relevant studies available for comparison. For example, Jensen and Hummel [10] administered 2,4,5-trichlorophenoxy-acetic acid containing 2,3,7,8-TCDD to lactating dairy cows and found that 2,3,7,8-TCDD residues in cream exceeded those in milk by a factor of about 10. Although this is much lower than our reported ratio of ~ 19 for 1,2,7,8-TCDD (**Figure 1**), the difference could originate from the "medium heavy cream" used in the Jensen and Hummel study [10] which would have a fat content < 36%. On the basis of our previous reports by Hakk *et al.* [7] and Lupton *et al.* [9], our milk fat had an average fat content of 82%. Regardless, our data confirmed those of Jensen and Hummel [10] in that the majority of dioxin residues would be associated with milk fat.

Compounds with a log *D* or *P* value of about 6 consistently concentrated in milk fat (or cream as cited in references). Concentrations of dichlorodiphenyltrichloroethane (DDT, Table S4) (log *D* 6.22 and log *P* = 5.92) in raw whole milk (5% lipid), skim milk, and cream (70% lipid) were reported as 7.5, 0.2, and 67.2 ppm, respectively, with a cream/whole milk ratio of 9.0 [12]. Pasteurization produced a slight increase of the cream/whole milk distribution ratio, as pasteurized whole milk contained 6.0 ppm and cream contained 70.2 ppm DDT resulting in a cream/whole milk ratio of 12 [12]. Langlois *et al.* [13] reported the identical ratio of cream/whole milk for DDT in spite of a fat content for cream of only 37%. Relative to the Mann [12] and Langois *et al.* [13] reports, higher milk fat/whole milk concentration ratios were found in this study for compounds having log *P* = ~ 6 (TCC, log *P* = 5.39, ratio 17.6; 1278-TCDD, log *P* = 6.22, ratio 18.7; PCB-118, log *P* = 6.78, ratio 19.5; **Figure 1**), which is also consistent with IVR (log *P* = 6.61, ratio 18.4) as reported by Hakk *et al.* [7]. The exception was 2-OH-1378-TCDD (log *P* = 6.15) which had a milk fat/whole milk concentration ratio of 11.2 in this study (**Figure 1**). These lower concentration ratios reported in the literature versus the current findings may be a reflection of differences in composition of the milk fat prepared here and the cream prepared in the cited reports.

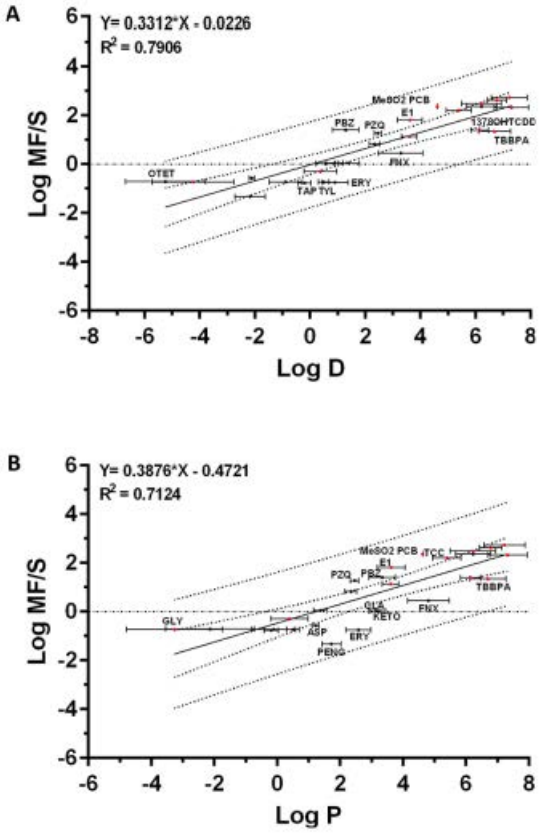
A compound with a log *P* value similar to that of BPA (log *P* = 3.60) is the organophosphate cruformate (log *P* = 3.33, Table S4), which was fed to cows [14]. Similar to BPA, which concentrated eightfold in fat relative to whole milk, cruformate concentrated about fi vefold into cream [14]. If values were adjusted to reflect lipid mass yield (15% of whole milk in their study, 10% in ours) the fivefold concentration would increase to ~ 7.6-fold, in close agreement with the eightfold concentration found for BPA. For fenthion (log *P* = 3.21, Table S4), an organothiophosphate insecticide, the concentration ratio of fat/whole milk was ~ 5, with 80 − 90% of the fenthion found in the fat fractions [15]. In the current work, the E1 (log *P* = 3.62) milk fat/whole milk concentration ratio was ~ 16 and 3′-$MeSO_2$-PCB (log *P* = 4.62, calculated) was ~ 18. Thus, the present results and those of O'Keeffe *et al.* [14, 15] suggested that factors in addition to log *P* also govern chemical disposition in milk.

Similar to the studies done by Hakk *et al*. [7] and Lupton *et al*. [9], GLY and IMI (this study) distributed predominantly into the skim milk; thus, the concentration ratio between skim milk/whole milk was ~ 1, whereas the ratio of milk fat/whole milk was ~ 0.2 (Figure 1). Hakk *et al.* [7] observed similar distributions for compounds with low log *D* values, for example, OTET, PENG, and ERY, as did Lupton et al. [9] for ASP, CIPR, TAP, and TYL despite the diversity of chemical structures.

Using literature values of log *P* and pK_a for each chemical (**Tables 1**, S1, and S2), mean and standard deviation (SD) log *D* values were calculated for ionizable compounds [16]. Relationships between log *D* or log *P* values and log [milk fat]/[skim milk] distributions, including 99% confidence interval (CI) and prediction interval, are shown in **Figure 2A** (log *D*) and **2B** (log *P*). There are apparent uncertainties with respect to log *D* or log *P* for many of the studied compounds (**Figure 2A,B**). In general, distribution uncertainties with regard to log *D* or log *P* are much greater than the error associated with measurements of milk fat or skim partitioning. By combining the log [milk fat]/[skim milk] data of the current set with results obtained from those of Hakk *et al*. [7] and Lupton *et al*. [9], the linear regression with log *D* had a regression coefficient of 0.79 and with log *P*, the resulting linear regression had an $r^2 = 0.71$ (**Figure 2A,B**). The slightly better regression using log *D* data reinforces the conclusions of Hakk *et al.* [7] and Lupton *et al.* [9] that log D was a better predictor of the distribution between milk fat and skim milk than log *P*. Nevertheless, **Figure 2A** indicates that based on the 99% CI for log *D*, numerous outliers were present when all 27 compounds were modeled. Outliers with respect to the 99% CI for the log *D* plot (**Figure 2A**) included ERY, FNX, TAP, TBBPA, 2-OH-1378-TCDD, and TYL, compounds which distributed more toward skim than predicted. 2-OH-1378-TCDD likely would fall within the 99% CI based on the SD of the calculated log *D*. Conversely, E1, 3′-$MeSO_2$-PCB-101, OTET, PBZ, and PZQ distributed more toward milk fat than predicted. Overall, the greatest limitation to predicting the behavior of any one chemical contaminant in milk seems to be the uncertainty associated with literature log *P* and pK_a values used to calculate log *D* values in the model derivation.

Slopes of the linear log *D* and log *P* models were not 1, but 0.33 and 0.39, respectively (**Figure 2**). There was no reason to expect a 1:1 relationship between log *D* or *P* values of a chemical and its distribution between milk fat and skim milk. The lower slopes do indicate modeled chemicals that typically distribute to a greater extent into skim milk than merely reflected by their log *D* or *P* values. Distribution data were not affected by the presence of degradates because none were detected by thin-layer chromatography (TLC) (Table S3). The model slopes highlight the differences between the simple, ideal, octanol/water partition system and the complex milk matrix which consists of water, lipid, protein, sugar, minerals, and micelles. We hypothesize that the presence of these additional milk components could account for the enhanced distribution into skim milk. For instance, milk proteins (casein, *β*-lactoglobulin, and lactalbumin) enhanced the solubilization of DDT in water [17].

Figure 2: Regression analyses of log[chemical]$_{milk\ fat}$ /[chemical]$_{skim\ milk}$ (log *F/S*) vs log *D* and log *P* (pH 6.8). Plot A is the regression analysis of log *F/S* vs log *D*. Plot B is the regression analysis of log *F/S* vs log *P*. Error bars on the log *D* and log *P* for the chemicals reflect the variability of values reported in the literature. Compounds outside the 99% CI but within 99% of the prediction interval are labeled. Regressions are based on data from 27 chemicals. Red dots are chemicals of the current study, whereas black dots are chemicals published in Hakk *et al.* [7] and Lupton *et al.* [9].



Chemical distribution from skim milk into curd and whey.

Recoveries of radioactivity across tested chemicals were ≥ 95% (sum of whey and curd), with the highest mean recovery (106.5%) occurring for *β*-HBCD and the lowest recovery occurring for PCB-118 (90.7%). The CVs for within dose replicates in whey and curd were generally < 4% for the majority of chemicals tested; however, the CVs for the most lipophilic persistent organic pollutants, that is, 1278-TCDD, BDE-99, *β*-HBCD, 3′-MeSO$_2$-PCB-101, and PCB-118, were considerably higher, exceeding 3% for whey (range 3.9 − 16.0%) and 4% for curd (range 4.3 − 10.0%; **Figure 3** and Tables S17−S28). Higher CVs for these lipophilic chemicals in whey are to be expected, especially at lower concentrations, because of the small percentage of each compound that distributed into whey. Chemical distributions were generally not dose-dependent for 0% moisture curd/whey ratios across the starting concentrations present in skim milk, although a dose dependency was apparent for BDE-99 ($p < 0.05$). An ~ 8% increase in association with the curd fraction was measured with BDE-99 with each 10-fold increase in dose, that is, from 73% to 80% to 92%, respectively. Initial concentrations in skim milk were 1.7, 13, and 204 nM (Table S28).

For the 12 compounds tested in the current study, chemicals retained in the curd fraction ranged from approximately 16.5% for GLY to 86% for *β*-HBCD when related to residual chemical in the skim milk of phase 1 (Tables S17 − S28). Distribution into curd was largely proportional to a chemical's lipophilicity. Of the most lipophilic compounds tested, ~ 80% of chemical was distributed into curd (1278-TCDD, BDE-99, *β*-HBCD, and PCB-118). Compounds having moderate lipophilicity, that is, TBBPA, 3′-MeSO$_2$-PCB-101, 2-OH-1378-TCDD, and TCC, were more evenly distributed into both curd (40 − 60%) and whey (35 − 60%). Highly polar compounds had the lowest affinity for curd, for example, GLY (16.5%) followed by IMI (23.7%; **Figure 3**, Tables S17−S28).

Figure 3: Drug distribution and relative concentration ratios from skim milk into whey and curd fractions. Bars represent percent mean of all concentrations (n = 3 concentrations; n = 3 replicates per concentration, replicate exceptions are n = 2 replicates each for PCB-118 50 and 200 nM, n = 2 replicates each for β-HBCD 200 and 500 nM, n = 2 replicates each for 1278-TCDD 20 and 200 nM, and n = 2 replicates for BDE-99 20 nM) ± SD of all three dose mean percentages based on dpm of whey and curd (at 70% moisture) fractions compared to forti fi ed skim milk dpm. Numerical values on the graph represent the mean ratio (n = 3) of the drug concentration in the fraction (curd or whey) to the initial drug concentration in skim milk ± SD. BDE-99 distribution was dose-dependent ($P < 0.05$). Sum of stacked plots represents total, unadjusted drug recovery values.

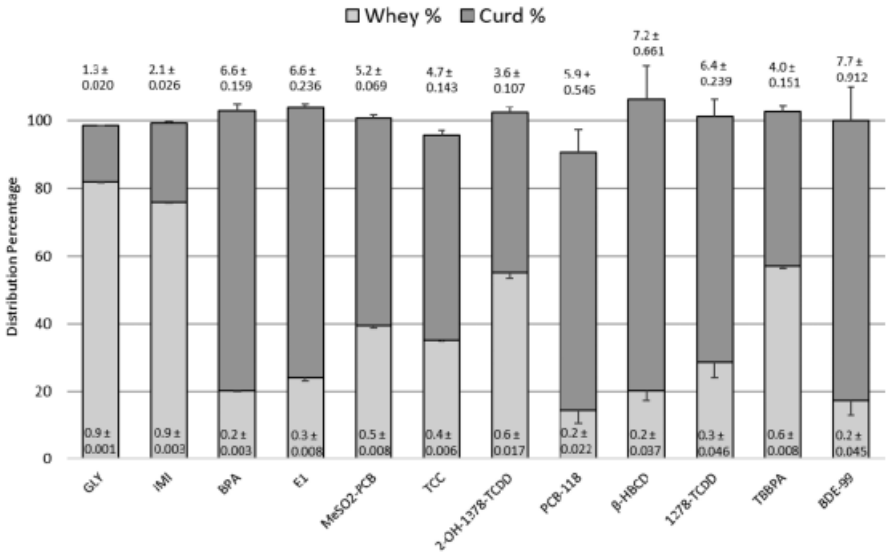


Table S17: GLY Phase 2 Average Distribution Data and Ratios.

Phase 2	Skim Milk			Whey Fraction					Curd Fraction					Percent Recovery	Obs. Ratio	Adj. Ratio
Average	Initial DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% GLY Dose	% GLY Dose (Corrected)	Total DPM	Mass (g)	Final nM (nmole/kg)	% GLY Dose	% GLY Dose (0% moisture)	Percent Recovery based on Skim Milk	$[GLY]_{curd}/[GLY]_{whey}$	$[GLY]_{0\%Curd}/[GLY]_{whey}$
0nM		45.33			39.59					6.01						
20nM	114,206	45.54	22.59	93,858	40.05	21.11	82.20	90.69	18,869	5.76	29.51	16.52	8.03	98.72	1.40	2.63
200nM	1,139,999	45.44	226.03	936,093	39.85	211.60	82.12	90.65	189,618	5.88	290.74	16.63	8.10	98.75	1.37	2.45
2000nM	1,081,531	45.41	2,145.80	885,328	39.83	2,002.63	81.86	90.37	177,067	5.87	2,715.70	16.37	7.86	98.23	1.36	2.38
St Dev																
0nM		0.08			0.37					0.29						
20nM	1,882	0.04	0.39	907	0.16	0.28	1.41	1.55	189	0.15	0.49	0.19	0.05	1.59	0.04	0.30
200nM	10,494	0.02	2.18	3,990	0.12	0.45	0.95	1.04	728	0.13	7.32	0.10	0.02	1.05	0.04	0.21
2000nM	3,918	0.07	7.45	2,693	0.02	5.56	0.06	0.07	4,651	0.09	38.25	0.45	0.44	0.50	0.02	0.07
% RSD																
0nM		0.18			0.94					4.74						
20nM	1.65	0.08	1.72	0.97	0.40	1.33	1.71	1.70	1.00	2.54	1.67	1.13	0.65	1.61	3.01	11.24
200nM	0.92	0.05	0.97	0.43	0.31	0.21	1.16	1.15	0.38	2.23	2.52	0.61	0.30	1.07	2.56	8.45
2000nM	0.36	0.15	0.35	0.30	0.05	0.28	0.08	0.08	2.63	1.52	1.41	2.74	5.59	0.51	1.54	2.94

When curd data (normally 70% moisture) were expressed on a dry matter basis, the concentration ratios of 0% moisture curd to whey (Tables S17−S28) were > 100 for the most lipophilic compounds, that is, 1278-TCDD (115), BDE-99 (327), β-HBCD (152), and PCB-118 (136), and for two of the phenolics, BPA (111) and E1 (104). Other phenolic compounds, that is, TBBPA and 2-OH-1378-TCDD, had much lower concentration ratios of 32 and 18, respectively, whereas 3′-$MeSO_2$-PCB-101 (56) and TCC (46) were also lower than the most lipophilic compounds. The 0% moisture curd/whey concentration ratios

for the most polar compounds ranged from 9.2 for IMI to 2.5 for GLY (Tables S17−S28).

Results for TBBPA were unexpected based on its structural similarity to BPA. The fire-retardant TBBPA is identical in the base structure to the plasticizer BPA with the exception that the 4-ortho hydrogens, with respect to the phenolic hydroxyls, are replaced by bromines. Bromination of the ortho-protons enhanced lipophilicity (log *P*) of TBBPA compared to BPA. In the 0% moisture curd/whey, however, the concentration ratio decreased from 111 for BPA to 32 for TBBPA (Tables S17−S28). Based solely on lipophilicity (log *P*), the curd/whey concentration ratio would have been expected to increase for TBBPA relative to BPA. One possibility for the lower concentration ratio for TBBPA is that the much larger atomic radius of bromine (compared to hydrogen) resulted in steric hindrances for potential casein − TBBPA interactions.

Hydroxylation and methylsulfonation of chemicals altered distribution patterns in milk. Aromatic hydroxylation decreased lipophilicity slightly and thus increased distribution into skim milk for phase 1 and into whey for phase 2. For example, 2-OH-1378-TCDD had a greater distribution into the whey (~ 30% greater) compared to 1278-TCDD. Comparison of PCB-118 and 3′-MeSO$_2$-PCB-101 also indicated that a methyl sulfone group decreased lipophilicity (log *D* 6.38 vs 4.62, respectively) and increased (> 25%) distribution into whey. Despite a different chlorine substitution pattern between this pair of chemicals, the presence or absence of a methyl sulfone functional group likely plays a more important role in determining the effect on curd versus whey distribution. The full nature of this partitioning difference is undoubtedly based on more than hydrophobic interaction, for example, possible chemical/protein interactions or sequestration.

Published reports related to the partitioning of chemicals tested in this study into whey and curd are scant, but structures and characteristics of chemicals cited for comparison are provided in Table S4. For example, concentrations of the aromatic, chlorinated insecticide DDT (log *D* = 6.22 and log *P* = 5.92) were greater in cheddar cheese than in whey after milk processing, with cheese and whey concentrations of 47 and 0.5 ppm, respectively [12]. Similarly, Swiss-type cheese made from milk produced by dairy cows fed DDT contained ~ 8 times the original DDT concentration of whole milk, though DDT was not reported in whey [13]. In other studies, however, DDT was unstable during processing and 27 − 53% of the starting DDT degraded to DDE and DDD [18] during the manufacturing of cheese. While DDT was not identified in whey at the dipping stage, it was measured in the whey pressed from curd [19]. Whey produced during the processing of raw whole milk had levels of DDE and DDD that increased twofold when measured at acidification, and concentrations were the same in the cheese product [19]. Similar concentrations of DDT were reported for whole milk and cheddar or Monterey cheese, indicating some net loss of DDT, as total cheese mass would be less than the original milk mass. No changes in DDT concentration were observed during storage.

Lipophilic compounds in this study concentrated in the curd to a greater extent than whey, but the lipophilic pesticide lindane (log *P* = 3.99), a cyclo-chlorinated structure with similarities to *β*-HBCD, did not concentrate in cheese or yogurt (produced from curd) made from contaminated raw milk [20]. The authors attributed the lack of concentration to heat treatment during pasteurization which resulted in phenolic metabolite formation. Pasteurization resulted in a 65 − 73% reduction in lindane, with more losses during refrigeration of yogurt (1.4 − 8% over 3 days) and cheeses (36.7% in Ras cheese during 6 months in storage). Although the effects of pasteurization and storage were not investigated in the current study, similar losses in *β*-HBCD might occur. Contrary to Abou-Arab [20], Langlois *et al*. [13] found that lindane concentration in curd (4.3 ppm) was approximately 12 times that measured in whey (0.34 ppm). In a second study, Langlois *et al*. [21] determined that curd concentrations of endrin (log *D* and *P* = 4.9) were about eight times those in whole milk (5.48 vs 0.7 ppm), whereas whey concentration was only 0.06 ppm (curd/whey concentration ratio = 91). Surprisingly, heptachlor (log *P* = 5.46), with higher lipophilicity than endrin, was present in whey (0.17 ppm) at approximately 1/20 the concentration measured in curd (3.77 ppm) (curd/whey concentration ratio = 22) [21]. Cruformate (log *P* = 3.33), which has a log *P* similar to BPA (3.60) and E1 (3.62), had a dose-dependent distribution. At a starting milk concentration of 0.07 ppm, cruformate was 22 times more concentrated in curd than that in whey (0.43 vs 0.02 ppm, respectively), but with a starting milk concentration of 0.16 ppm, the curd/whey concentration ratio was 31 (0.92 and 0.03 ppm, respectively) similar to that of BPA (29) and E1 (24) [14].

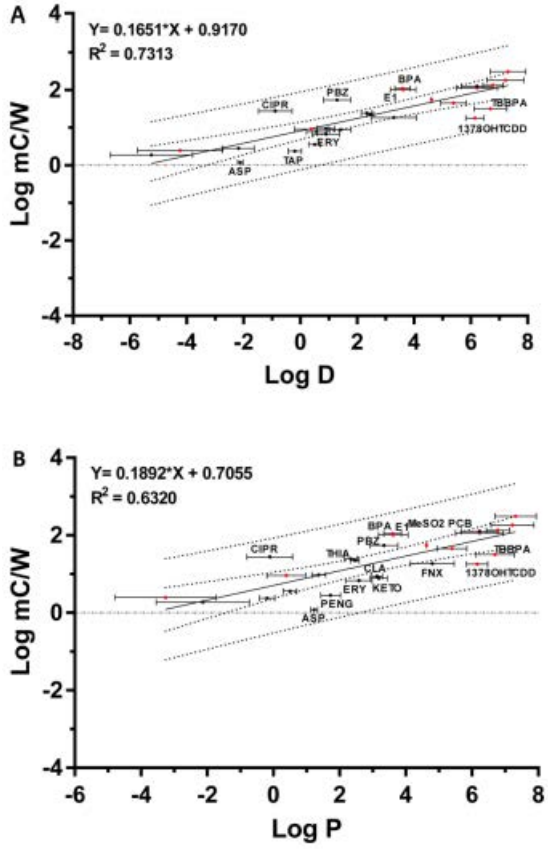
Hydrophilic compounds distributed more evenly between curd and whey. For example, the curd/whey

concentration ratio for GLY (log $D = -4.24$) was 1.4 and for IMI (log $D = -0.38$) was 2.4, similar to SDMX (ratio 3.2), PENG (ratio 1.2), OTET (ratio 1.4), ERY (ratio 2.4), and KETO (ratio 2.4) as previously reported [8]. Given the diversity of chemical structures tested, the log *D* value of hydrophilic compounds does provide some predictive measure for curd and whey distribution. Similarly, TAP and TYL possessed fairly low curd/whey concentration ratios, that is, 1.3 and 1.5, respectively [9].

Figure 4A (log *D*) and **4B** (log *P*) shows the relationships between log D or log P values and log[0% moisture curd]/[whey] concentration ratios, including 99% CI and prediction interval. By combining the log[0% moisture curd]/[whey] data of the current set with those of Shappell et al. [8] and Lupton *et al.* [9] the regression with log D had an $r^2 = 0.73$, whereas the log *P* regression had an $r^2 = 0.63$ (**Figure 4A,B**). The higher regression coe ffi cient obtained using log D data reinforces the previous conclusion that log *D* is a better predictor of the distribution between curd and whey than log *P* [8,9].

On the basis of the 99% CIs for the log *P* regression, numerous outliers were present when all 27 compounds were modeled. Outliers for the curve fit on a log *D* basis (Figure 4A) included ASP, ERY, 2-OH-1378-TCDD, TAP, and TBBPA compounds which distributed more toward whey than predicted. Conversely, BPA, CIPR, E1, and PBZ distributed more toward curd than predicted. In the log *P* model (Figure 4B), four additional chemicals (CLA, KETO, FNX, and PENG) fell outside of the 99% CIs.

Figure 4: Regression analyses of log[chemical]$_{0\%\text{moisture curd}}$ /[chemical]$_{\text{whey}}$ (log *mC/W*) vs log *D* and log *P* (pH 6.8). Plot A is the regression analysis of log *mC/W* vs log *D*. Plot B is the regression analysis of log *mC/W* vs log *P*. Error bars on the log *D* and log *P* for the chemicals reflect the variability of values reported in the literature. Compounds in between the 99% CI and 99% of the prediction interval are labeled. Red dots are chemicals of the current study, whereas black dots are chemicals published in Shappell *et al.* [8] and Lupton *et al.* [9]. Regressions are based on data from 27 chemicals.



Chemical distribution from whey into retentate and permeate.

In order to assess the percent of drug associated with the whey proteins, ultra filtration in conjunction with centrifugation was performed (phase 3, **Figure 5**). The expected volume of retentate was 33% of the applied sample volume based on centrifugation time and speed, with the actual measured mean for

all compounds being 37 ± 3.3%. Mean recovery of radioactivity across all compounds was 100 ± 4.5%. Mean non-specific binding of compounds to filters ranged from 0.2% for GLY to 22.5% for E1. Compounds with > 3% filter binding include PCB-118 (6.4%), BDE-99 (7.1%), β-HBCD (8.2%), BPA (13.4%), and E1 (22.5%) (Tables S29 − S40). Although compounds with high log *D* values could be expected to be "sticky" in the aqueous medium, four compounds with high log *D* values [TCC (log *D* = 5.39), 2-OH-1378-TCDD (log *D* = 6.15), 1278-TCDD (log *D* = 6.22), and TBBPA (log *D* = 6.69)] had filter binding of ≤ 2.4%.

The associations of the 12 xenobiotics with whey protein, as determined by the percentage of compound measured in the retentate, revealed three groupings (**Figure 6**). The first was represented by GLY and IMI that have negative log *D* values (− 4.24 and − 0.38, respectively), where there was essentially no association with the whey protein (< 5%) occurred (Tables S29 and S30). The second grouping was composed of BPA, E1, 3′-MeSO$_2$-PCB-101, 2-OH-1378-TCDD, and 1278-TCDD, which had moderate associations with whey protein, ranging from 33 to 76% (Tables S31−S33, S35, and S38). Similar to our findings of ~ 64% association of E1 with whey protein, Wolford and Argoudelis [22] reported 48 and 53% of 17 β-estradiol and E1, respectively, associated with whey protein. The third grouping was composed of those compounds that were almost totally associated with retentate whey proteins (84 − 98%, one outlier of 107% for PCB-118 due extremely low starting radiocarbon in the whey). Chemicals in this grouping included BDE-99, β-HBCD, PCB-118, TBBPA, and TCC (Tables S34, S36, S37, S39, and S40). If present in whey, these compounds would concentrate in whey-derived protein products.

The percent of whole milk dose associated with either casein or whey proteins is reported in **Table 2**. About 25% of TBBPA and 2-OH-1378-TCDD from whole milk distributed to whey, yet ~ 90 and 70% (TBBPA and 2-OH-1378-TCDD, respectively) of that were associated with whey protein.

Figure 5: Scheme of milk partitioning processes that yielded cream and milk fat from whole milk (phase 1) curd and whey from skim milk (phase 2) and retentate and permeate from whey (phase 3).

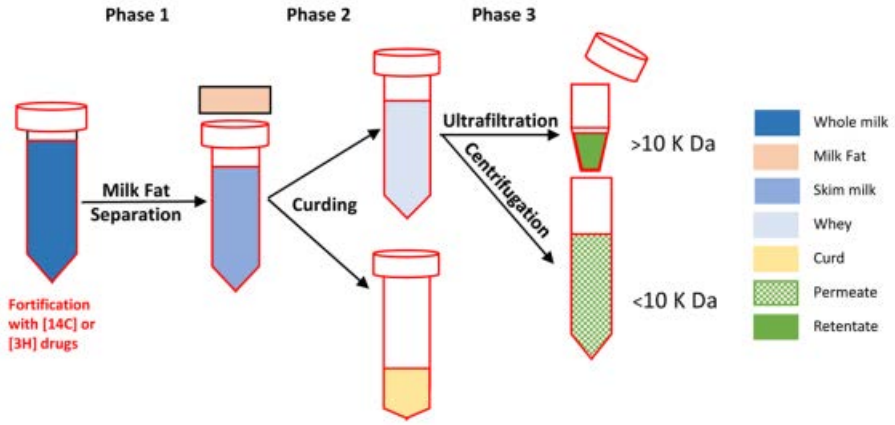


Figure 6: Drug distribution from whey into permeate, retentate, and filter fractions. Bars represent percent mean of all concentrations (n = 3 concentrations, concentration exceptions are PCB-118 and *β*-HBCD n = 2 concentrations; n = 3 replicates per concentration, replicate exceptions n = 2 replicates for TCC 20 nM, n = 2 replicates each for 1278-TCDD 200 and 2000 nM, n = 2 replicates for BDE-99 200 nM) ± SD of all three dose mean percentages based on dpm of permeate and retentate fractions compared to fortified whey dpm. Horizontal lines on each bar represent the actual retentate and permeate volume percentage after centrifugation. Sum of stacked plots represents total, unadjusted drug recovery values).

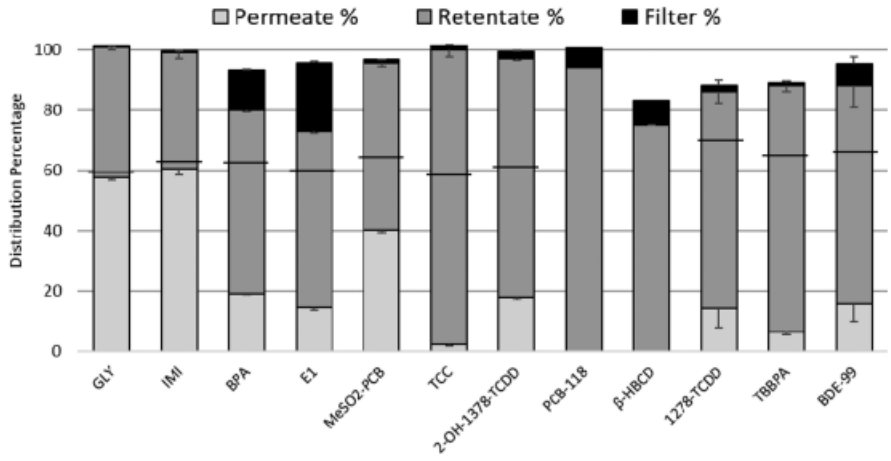


Table S29: GLY Phase 3 Average Distribution Data and Ratios.

Phase 3	Whey			Retentate Fraction				Permeate Fraction				Filter		Total Dose Recovery	Protein Association
Mean	Initial DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% GLY Dose	Total DPM	Volume (mL)	Final nM	% GLY Dose	Total DPM	% GLY Dose	Percent Recovery based on Whey	% Associated
0nM		14.86			5.82				9.12						
20nM	34,827	14.86	21.11	15,225	6.16	22.25	43.73	19,722	8.78	20.24	56.62	57	0.16	101.12	4.6
200nM	349,549	14.88	211.60	148,612	6.00	223.16	42.52	202,487	8.96	203.51	57.93	490	0.14	101.20	4.4
2000nM	330,961	14.89	2,002.63	142,417	6.02	2,132.92	43.03	192,264	8.96	1,934.18	58.09	484	0.15	101.91	4.7
St Dev															
0nM		0.01			0.17				0.17						
20nM	458	0.01	0.28	231	0.10	0.06	1.19	371	0.10	0.23	0.33	11	0.03	0.85	0.6
200nM	864	0.01	0.45	4,001	0.14	1.20	1.24	3,503	0.14	1.02	0.91	26	0.01	0.71	0.3
2000nM	1,160	0.01	5.56	1,910	0.08	6.83	0.73	1,738	0.09	5.50	0.33	26	0.01	0.44	0.2
% RSD															
0nM		0.0			2.85				1.85						
20nM	1.31	0.1	1.33	1.52	1.70	0.25	2.72	1.88	1.13	1.13	0.58	18.57	18.79	0.84	12.4
200nM	0.25	0.04	0.21	2.69	2.26	0.54	2.92	1.73	1.59	0.50	1.57	5.37	5.52	0.71	6.7
2000nM	0.35	0.08	0.28	1.34	1.29	0.32	1.69	0.90	1.00	0.28	0.57	5.37	5.61	0.44	4.8

Chemical concentration based on protein mass for casein and whey proteins

Using 0% moisture curd data from phase 2, the amount of chemical associated with caseins was calculated based on proteins present in curd and largely result from agglutination of casein (**Table 2**). Similarly, using phase 3 data, the amount of chemical associated with whey proteins can be calculated (**Table 2**). Chemical saturation of casein or whey protein was not observed because the mass of chemical per milligram protein increased as the concentration increased. In some instances, the initial expected fortification concentrations in whole milk differed from measured concentrations, as seen with 3′-MeSO$_2$-PCB-101 and *β*-HBCD. Whey protein association values for the lowest dose of BDE-99 are questionable because the starting skim milk contained < 2 nM and whey 0.3 nM. However, confidence in casein/whey protein association results is enhanced by the agreement found across doses (**Table 2**),

exception was BDE-99, where ratios ranged from 2.8 to 5.5.

For the majority of chemicals tested (BDE-99, BPA, E1, *β*-HBCD, IMI, 3′-MeSO$_2$-PCB-101, PCB-118, and 1278-TCDD), the association with caseins was greater than that for whey proteins (ratio > 1, **Table 2**). The importance of methodology is evident when comparing ourfindings to those of Wolford and Argoudelis [22] that used equilibrium dialysis with E1 and the slightly more hydrophilic compound E2. They reported that E1 and E2 were largely (> 84%) bound to protein when incubated in skim milk, and > 50% of the bound estrogens was associated with whey proteins. These data are in contrast to our findings for E1, in which the association (nmol/mg protein) ratio was approximately 2 for casein/whey.

The difference between the results of the two studies was most likely the precipitation of curd caseins in the present work versus the presence of soluble caseins used for dialysis by Wolford and Argoudelis [22] (1979).

Other chemicals that preferentially associated with caseins relative to whey protein (ratio > 1) include THIA (2.5), IVR (2.0) [8], TYL (1.4), CIPR (2.0), and PZQ (1.5) [9]. Although the current work used a majority of chemicals with log *D* greater than 3.4, our previous reports described only one such chemical (IVR). The casein/whey protein association ratio of IVR was more similar to BPA (2.0), E1 (1.9), and IMI (1.9) (**Table 2**).

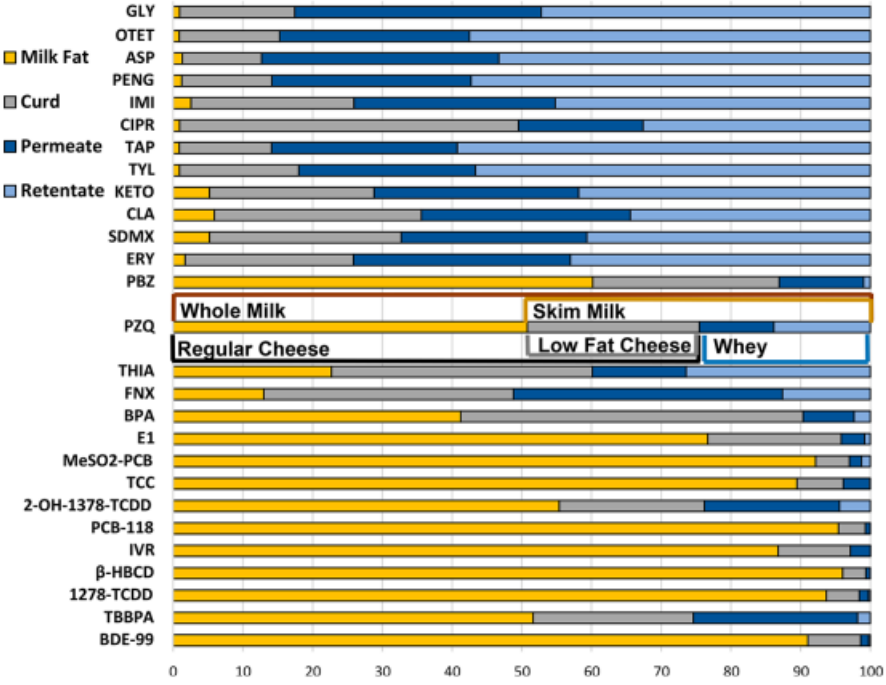
In spite of higher distribution of GLY into whey than curd (**Figure 3**), there was in fact very little preferential retention of GLY associated with whey protein (Figure 6). Similarly, TCC, 2-OH-1378-TCDD, and TBBPA also had casein/whey protein ratios <1. Although most of the total TCC dose was partitioned with milk fat (mean 85%), the remainder distributed almost equally between whey and 0% moisture curd (57% curd, Table S22). TCC remaining in the whey was concentrated almost exclusively in the retentate (98%) during ultracentrifugation (Table S34). The log *D* values of 2-OH-1378-TCDD (6.15) and TBBPA (6.69) did not predict the respective mean casein/whey protein ratios of 0.26 and 0.33. Both chemicals also distributed to a lesser extent than predicted into milk fat. The common feature of both compounds is a hydroxyl moiety between two halogens (chlorines for 2-OH-1378-TCDD and bromines for TBBPA).

Previously studied chemicals that had higher association for whey proteins versus caseins were PENG (casein/whey ratio = 0.2), ERY (0.5), KETO (0.4), SDMX (0.8) [8]; TAP (0.5), CLA (0.4), and FNX (0.25) [9]. Although the distribution between lipid and aqueous phases was markedly dependent on the property of proteins, namely lipophilicity, small-molecule binding to proteins seems to be more dependent on specific functional groups within the protein. Identifying the specific functional groups and binding domains that can associate with studied chemicals within a plethora of whey and casein proteins lies outside the scope of the present research.

Relation to consumer products

To determine how the distributions of these compounds, if detected in whole milk, related to consumer products, the percent distributions intomilk fat, curd, retentate, and permeate were calculated in relation to the starting concentration in whole milk. **Figure 7** includes the experimentally derived percentages of each compound in high-fat products which would include butter, cream, and cheese; low-fat products would include skim milk, low fat cheese, yogurt, and low-fat derived whey protein products such as whey protein powders and baby formulas. Comparable to compounds previously tested [8,9], higher log *D* compounds (i.e., E1, 3 ′ -MeSO 2 -PCB-101, TCC, PCB-118, *β*-HBCD, 1278-TCDD, and BDE-99) generally distributed to high-fat products such as butter and cream. High-fat products that contain protein (*i.e.*, cheese) will concentrate both mid- to high-range log *D* molecules such as BPA, 2-OH-1378-TCDD, and TBBPA along with the higher log *D* compounds. Two compounds with low log *D*'s, that is, GLY and IMI, will primarily distribute into aqueous products, such as skim milk and whey.

Figure 7: Normalized percentages of chemicals calculated from whole milk to be in the milk end-products of milk fat, curd, permeate, and retentate based on data generated from the current studies as well as those reported in Hakk *et al.* [7], Shappell *et al.* [8], and Lupton *et al.* [9]. The PZQ bar has additional information on which milk end products comprise whole milk, skim milk, curd, low-fat curd, and whey, as a guide to where drug may partition during commercial milk processing. For percentage of chemical associated with whey protein see supplemental information tables S29 − S40).



Determining where a compound would concentrate in consumer products will also depend on the processing steps involved and what specific end product is being manufactured. For example, whole milk processed into skim milk and cream would generally have compounds with high log *D* values concentrated in butter and cream, whereas compounds with low log *D* values will be in skim milk. Compounds with mid-range log *D* values will be split between the higher fat products and skim milk. However, if whole milk is processed directly into cheese, then the mid-range and high-range log D value compounds will mainly concentrate in the cheese.

Conclusions

The partitioning of 12 environmental contaminants or metabolites into milk fractions was assessed. Partitioning between milk fat and skim milk and between 0% moisture curd and whey was usually governed by the compound's lipophilicity. If a chemical was found in whey, the more nonpolar the compound the more likely it would be found in whey protein products. Phenolic compounds were the main chemicals that fell outside of the 99% CIs of the models' regression analyses. These models provide a tool using log *D* as the primary chemical property to predict the distribution of chemicals into various milk products.

Supporting information

Supporting information with (Tables S1−S40) is available online:

http://pubs.acs.org/doi/suppl/10.1021/acsomega.8b00762/suppl_file/ao8b00762_si_001.pdf.

Only the tables relevant to glyphosate (S5, S17, S29) are shown in this summary.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The purpose of the described work was to investigate the partitioning of 12 environmental chemicals of diverse polarities into various milk fractions. One of the tested chemicals was glyphosate. The experiments were conducted with radio-labelled test materials which were fortified to raw (unpasteurized, non-homogenized) cow milk (3 fortification levels were investigated for each compound). Thereafter, the milk was processed into skim milk, milk fat, curd, whey, whey retentate and whey permeate. A linear model predicting the distribution of chemicals between skim milk and milk fat based on their lipophilicity was established. The distribution between curd and whey was also correlated with lipophilicity. Phenolic compounds had less predictable distribution patterns based on their lipophilicities.

During processing of whole milk to skim milk and milk fat, glyphosate partitioned essentially to skim milk (> 99%). Only about 1% of the glyphosate fortified to whole milk was recovered in milk fat. Following curding of the skim milk, most glyphosate remained in the whey fraction (> 80%). The associations of glyphosate with whey protein (calculated by subtracting the amount present in permeate from the amount present in retentate) was very low (< 5%). As expected due to its hydrophilicity, glyphosate primarily distributes into aqueous products, such as skim milk and whey. The distribution pattern between the various milk fractions was similar for the various amounts of glyphosate fortified to whole milk (range of ca. 0.004 mg/L to 0.348 mg/L).

Although the distribution of residues between skim milk and milk fat is not a data requirement for hydrophilic compounds like glyphosate, this information is considered relevant to risk assessment. Overall, the publication is deemed reliable. Normally, the distribution of residues between skim milk and milk fat should be investigated with raw milk containing incurred residues (in the context of metabolism or feeding studies) and not by (artificially) fortifying raw milk. However, due to the very low transfer of glyphosate-derived residues in milk, the approach used in the publication seems to be the best option to determine the distribution of parent glyphosate residues between skim milk and milk fat.

1. Information on the study

Data point	CA 6.10.1
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.	Food Additives & Contaminants: Part A, 2019, Vol. 36, No. 3, 434-446
Guidelines followed in study	None stated
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:	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.

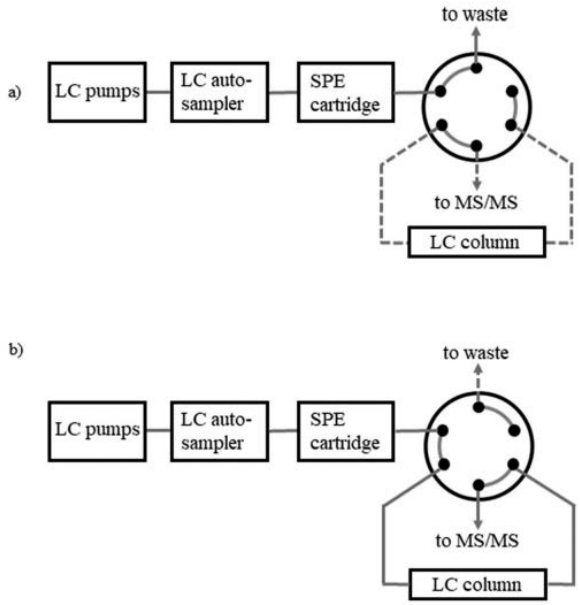


Table 1: LC gradient elution program and six-port switching valve position.

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.

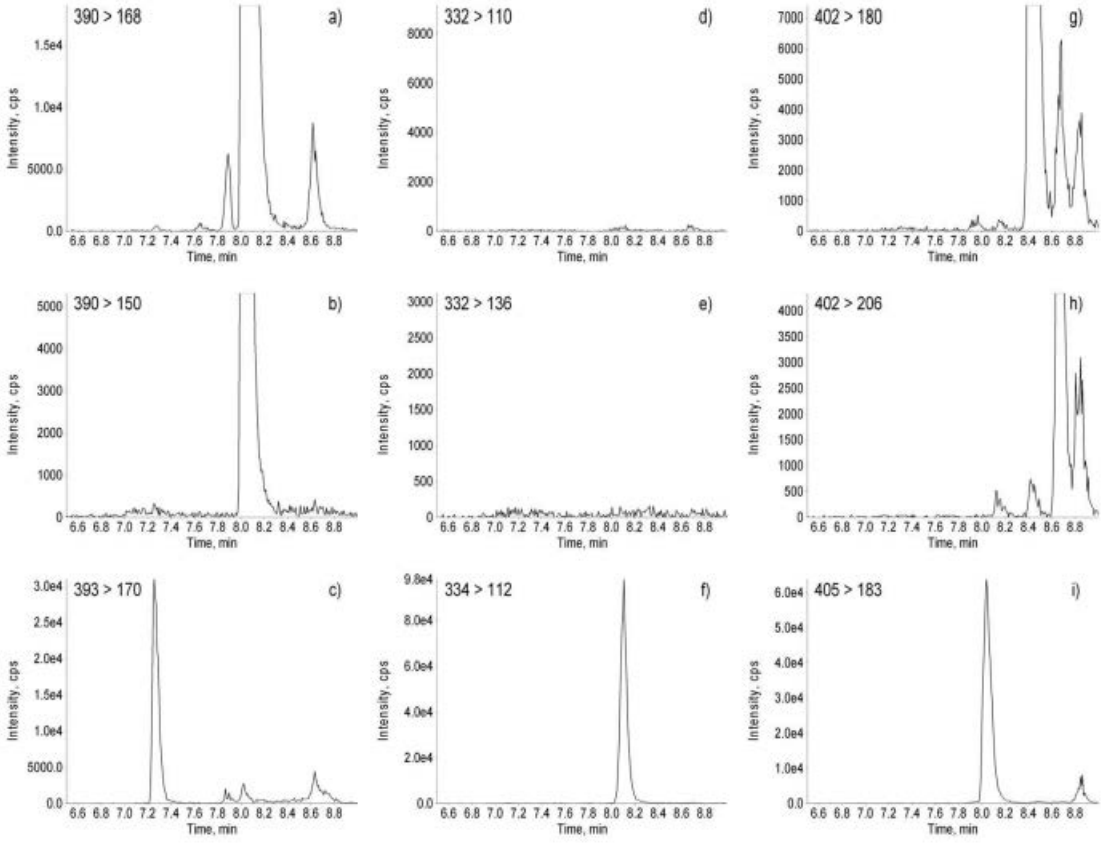
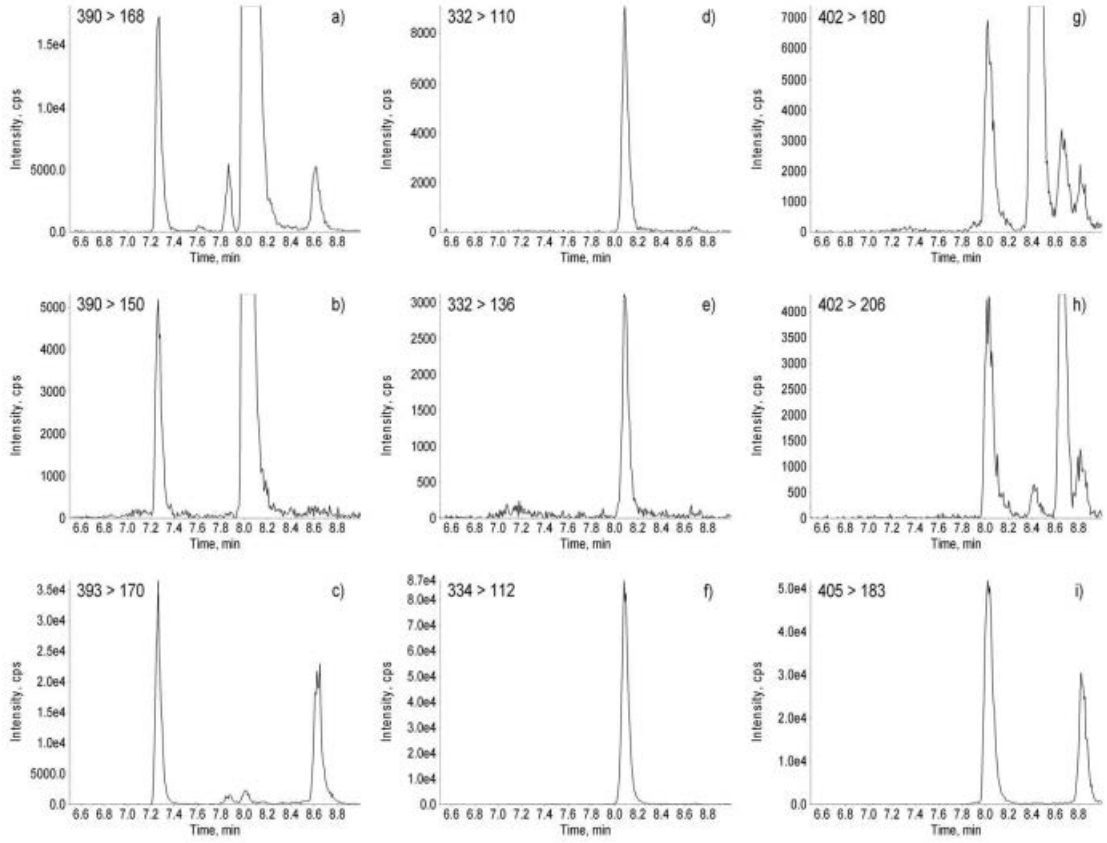


Figure 3: Reconstructed ion chromatograms for blank honey fortified with 5 µg/kg each of glyphosate, AMPA, and glufosinate as well as 25 µg/kg of each isotopically labelled internal standard. The quantitation and confirmatory MRM transitions (respectively) are: (a)+(b) glyphosate; (d)+(e) AMPA; and (g)+(h) 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 standardization 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 ionization 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.*

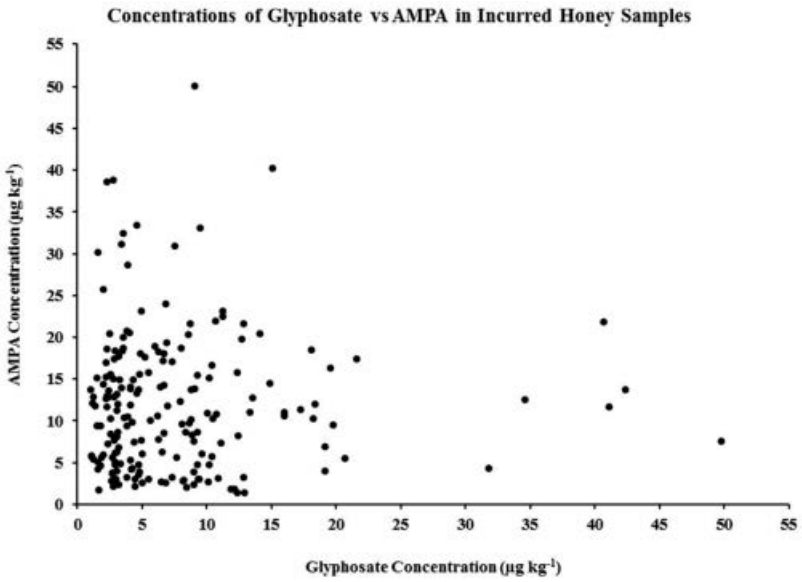
Compound	# of Detections	Median (µg kg^{-1})	90th Percentile (µg kg^{-1})	95th Percentile (µg kg^{-1})	Maximum (µg kg^{-1})
glyphosate	197	4.9	14.2	19.2	49.8
AMPA	198	10.3	20.8	28.7	50.1
glufosinate	125**	1.4	6.1	9.9	33.0

*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.

Conclusions

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.

1. Information on the study

Data point	CA 6.4.2
Report author	Von Soosten D. *et al.*
Report year	2016
Report title	Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows
Document No.	J. Dairy Sci. 99 :5318–5324
Guidelines followed in study	None stated
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:	Yes/Reliable

2. Full summary of the study according to OECD format

Executive Summary

From 6 balance experiments with total collection of feces and urine, samples were obtained to investigate the excretion pathways of glyphosate (GLY) in lactating dairy cows. Each experiment lasted for 26 d. The first 21 d served for adaptation to the diet, and during the remaining 5 d collection of total feces and urine was conducted. Dry matter intake and milk yield were recorded daily and milk and feed samples were taken during the sampling periods. In 2 of the 6 experiments, at the sampling period for feces and urine, duodenal contents were collected for 5 d. Cows were equipped with cannulas at the dorsal sac of the rumen and the proximal duodenum. Duodenal contents were collected every 2 h over 5 consecutive days. The daily duodenal dry matter flow was measured by using chromium oxide as a volume marker. All samples (feed, feces, urine, milk and duodenal contents were analyzed for GLY and aminomethylphosphonic acid (AMPA). Overall, across the 6 experiments (n = 32) the range of GLY intake was 0.08 to 6.67 mg/d. The main proportion (61 ± 11%; ± SD) of consumed GLY was excreted with feces; whereas excretion by urine was 8 ± 3% of GLY intake. Elimination via milk was negligible. The GLY concentrations above the limit of quantification were not detected in any of the milk samples. A potential ruminal degradation of GLY to AMPA was derived from daily duodenal GLY flow. The apparent ruminal disappearance of GLY intake was 36 and 6%. In conclusion, the results of the present study indicate that the gastrointestinal absorption of GLY is of minor importance and fecal excretion represents the major excretion pathway. A degradation of GLY to AMPA by rumen microbes or a possible retention in the body has to be taken into account.

Materials and Methods

Six balance experiments with collection of total urine and feces were conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Brunswick, Germany. The experiments were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany

Animals, Feeding, and Design of the Experiments

Overall, the 6 experiments included 32 lactating dairy cows of the German Holstein breed. For experiments 1 to 6, we used 5, 6, 5, 4, 6, and 6 animals per experiment, respectively. The animals were, on average, 90 DIM and in their second to fifth lactation. All cows were fitted with rumen and duodenum cannulas and were housed in a tiestall barn. Milking took place twice daily at 05:30 and 15:30 h. The animals were fed at the milking times. In all experiments the diet was based on maize silage (single forage component) and concentrates in different proportions (Table 1). The composition

of the concentrates as well as the GLY and AMPA concentrations in the concentrates are shown in Table 2. Each balance experiment lasted 26 d. The first 21 d were allowed for equilibration to the experimental diet and the remaining 5 d were the sampling period. In experiments 1 and 2, the quantitative collection of urine and feces was followed by the quantification of daily duodenal dry matter flow (DMF) for 5 consecutive days

Table 1: Forage-to-concentrate ratio of the diet during the experiments.

Experiment	Maize silage (%)	Concentrate (%)
1	60	40
2	60	40
3	70	30
4	55	45
5	70	30
6	70	30

Measurements and Sample Collection

During the sampling period, DMI and milk yield were recorded in each individual animal daily. Feed samples for maize silage were taken twice and concentrate samples once during the sampling period. Milk samples were taken once at morning and evening milking in the sampling periods. Total collection of feces and urine was conducted over 5 consecutive days. Cows were equipped with urine devices for separated drain of urine. The device was manufactured of artificial leather and was fitted and agglutinated around the vulva and pins. A polyvinylchloride tube drained the urine into a canister. The feces were collected in a stainless steel tub, which was positioned below a perforated floor at the end of the tiestall. The urine canister and feces tub were emptied once per day at the same time. Urine and feces were weighed and homogenized. Two percent of the daily fecal amounts were sampled and given into a pooled sample over the 5 consecutive days. A urine sample of 100 mL was taken from total urine volume each day. Urine and feces samples were stored at −20°C until analysis.

In experiments 1 and 2 a chromium oxide (Cr_2O_3) marker (19.8% Cr_2O_3, 79.1% wheat flour and 0.67% aluminum sulfate) was introduced into the rumen via the rumen cannula and was used as a marker for quantitative measurement of the daily duodenal DMF. The administration of Cr_2O_3 was started 11 d before collection of duodenal chyme. Two portions of 50 g of Cr_2O_3 were administered every 12 h. During the duodenal chyme sampling period and 1 d before, 4 portions of 25 g of Cr_2O_3 were given every 6 h. Samples of duodenal contents were taken every 2 h during the 5 consecutive days of sampling. At each sampling, 100 mL of duodenal contents were collected and pooled over 24 h. The samples were stored at −20°C. The individual animal DMI was recorded and samples of the feedstuffs were retained according to the same pattern during the total collection period of urine and feces. Body weight was recorded before the start and after the end of an experiment.

Analyses

Samples of maize silage were dried at 60°C for 72 h. Duodenal contents and feces samples were freeze-dried for determination of DM. The feedstuffs and duodenal and fecal samples were ground through a 1-mm sieve. Aliquots of the morning and evening milk samples were pooled according to their proportion of total daily milk yield. The urine samples were thawed and pooled over the 5 consecutive sampling days according to their daily proportion of the total urine amount over the sampling period. In the daily duodenal samples the chromium concentrations were measured using an inductively coupled plasma optical emission spectrometer (Quantima, GBC Scientific Equipment Pty Ltd., Victoria, Australia) after sample preparation according to Williams et al. (1962). The chromium concentration was used to calculate the daily duodenal DMF. According to the daily duodenal DMF on the 5 sampling days, one aliquot pooled sample was generated per cow per 5 sampling days.

All samples were analyzed for GLY and AMPA in accredited laboratories. Feed and milk samples were analyzed by Wessling GmbH (Altenberge, Germany) and feces, urine, and duodenal chyme by

Medizinisches Labor Bremen (Bremen, Germany). In milk and feed samples GLY and AMPA were extracted with formic acid (0.1%) and methylene chloride. Derivatization was conducted with fluorenylmethoxycarbonyl chloride. After solid phase extraction, GLY and AMPA were determined by using LC-MS/MS.

In feces, urine, and duodenal chime, GLY and AMPA were extracted with water. Derivatization was conducted with trifluoroacetic anhydride and trifluoroethanol. Glyphosate and AMPA were determined by GC-MS/MS.

For all GLY and AMPA analyses an internal standard containing 1,2–$^{13}C_2$ ^{15}N GLY (1 µg/mL) and ^{13}C ^{15}N AMPA (1 µg/mL) was used. The limit of detection (LOD) and the limit of quantification (LOQ) for each substance was calculated form the signal-to-noise ratios. This ratio was 3 for the LOD and 10 for the LOQ. For both GLY and AMPA in feed samples, the LOQ and LOD was 0.02 and 0.007 mg/kg, respectively. For all other matrices the LOQ was 0.01 mg/kg and the LOD was 0.003 mg/kg. The recoveries for GLY and AMPA analyses in feed and milk samples were 70 to 120% using an internal standard concentration of 0.625 mg/kg for feed analyses and 0.25 mg/kg for milk analyses. Recoveries for GLY in feces, urine, and duodenal content were 80 to 90, 98 to 101, and 96 to 102%, respectively. Recoveries for AMPA analyses in feces, urine, and duodenal content were 80 to 95, 80 to 101, and 94 to 106%, respectively. For determination of the recoveries in feces, urine, and duodenal content the internal standard concentration was 0.1, 0.001, and 5 mg/kg, respectively.

Calculations

Apparent GLY and AMPA retention was calculated with the following equation:

Apparent GLY/AMPA retention (mg/d) = GLY/AMPA intake (mg/d) – fecal excretion of GLY/AMPA (mg/d) – urinary excretion of GLY/AMPA (mg/d) – milk excretion of GLY/AMPA (mg/d).

Daily duodenal DMF and duodenal GLY/AMPA flow were calculated as follows:

DMF (kg/d) = [chromium application (mg/d)/duodenal chromium concentration (mg/g of DM)]/1000, and

Daily duodenal GLY/AMPA flow (mg/d) = DMF (kg/d) × duodenal GLY/AMPA concentration (mg/kg DM).

Ruminal disappearance of GLY and AMPA was calculated with the following equation:

Ruminal disappearance of GLY/AMPA (mg/d) = GLY/AMPA intake (mg/d) – duodenal GLY/AMPA flow (mg/d).

Results

The determined GLY and AMPA concentrations of the individual sample matrices differed between the experiments. Only in experiment 4 could GLY be detected in the maize silage (0.035 mg/kg of DM). Maize silage in all other experiments contained GLY lower than the LOQ; AMPA was not detected in any of the maize silages. The GLY concentration in the concentrates ranged from 0.02 to 0.95 mg/kg of DM and AMPA concentrations ranged from a value lower than the LOQ to 0.65 mg/kg of DM. Therefore, the concentrates were the main source for exposure of GLY and AMPA in all experiments (Table 2). In urine and feces GLY concentrations ranged from 0.20 to 75.1 µg/L and 0.01 to 0.88 mg/kg of DM, respectively.

Table 2: Composition and concentrations of glyphosate and aminomethylphosphonic acid in the concentrates of the different experiments.

	Experiment					
Component (%, unless noted)	1	2	3	4	5	6
Soybean meal	20	20	25	15		
Rape seed meal			13			
Barley grain	22	22		14.7		
Wheat grain	22	22	36.5	29		
Wheat gluten					10	10
Maize grain	18	18		29	35	35
Sugar beet pulp, dried	15	15	18.3	8.4	48	48
Urea	2	2	2.5	1	3	3
Calcium carbonate/dicalcium phosphate			2.5	1.5	1.5	1.5
Sodium chloride			0.2	0.2	0.2	0.2
Mineral and vitamin-mix	1	1	2.0	1.2	2.3	2.3
Glyphosate (mg/kg of DM)	0.95	0.48	0.82	0.08	0.02	0.03
AMPA[1] (mg/kg of DM)	0.65	0.43	0.46	<LOQ[2]	<LOQ	0.02

[1]Aminomethylphosphonic acid (degradation product of glyphosate).
[2]<LOQ = AMPA concentrations in the samples were lower than the limit of quantification (LOQ).

In experiment 1 the highest GLY intake (6.7 mg/d) was observed. The lowest GLY intake (0.08 mg/d) was found in experiment 6 (Table 3). In accordance to the GLY intake, the excretion of feces (4.3 mg/d) and urine (0.44 mg/d) were highest in experiment 1. In experiment 5 the excretion of feces and urine were lowest, at 0.02 and 0.08 mg/d, respectively. In all milk samples the GLY concentration was below the LOQ.

Table 3: Intake and fecal and renal excretion of glyphosate in animals during the sampling period (means ± SD).

Experiment (animals)	Intake in feed (mg/d)	Excretion in feces (mg/d)	Excretion in urine (mg/d)
Experiment 1 (n = 5)	6.67 ± 0.02	4.34 ± 0.35	0.44 ± 0.08
Experiment 2 (n = 6)	3.36 ± 0.01	2.04 ± 0.24	0.26 ± 0.10
Experiment 3 (n = 5)	3.36 ± 0.00	1.69 ± 0.41	0.12 ± 0.02
Experiment 4 (n = 4)	0.53 ± 0.02	0.39 ± 0.05	0.04 ± 0.01
Experiment 5 (n = 6)	0.15 ± 0.21	0.02 ± 0.03	0.08 ± 0.10
Experiment 6 (n = 6)	0.08 ± 0.07	0.04 ± 0.06	0.14 ± 0.14
Experiment 1–6 (n = 32)	2.36 ± 2.61	1.42 ± 1.67	0.18 ± 0.15

The results for AMPA intake and excretion are presented in Table 4. The AMPA intake was on a lower level compared with GLY intake. The highest AMPA intake was observed in experiment 1 (4.57 mg/d) and the lowest in experiment 5 (lower than the LOQ; Table 4). Considerable excreted amounts of AMPA with feces and urine were only observed in experiments 1, 2, and 3. The excretion with feces was lower than the LOQ in experiments 4, 5, and 6. In the same experiments the excretion with urine (0.01 mg/d) was marginal; AMPA concentrations in milk were below the LOQ in all experiments.

Table 4: Intake and fecal and renal excretion of AMPA[1] in animals during the sampling period (means ± SD).

Experiment (animals)	Intake in feed (mg/d)	Excretion in feces (mg/d)	Excretion in urine (mg/d)
Experiment 1 (n = 5)	4.57 ± 0.21	2.25 ± 0.23	0.41 ± 0.05
Experiment 2 (n = 6)	3.03 ± 0.05	1.51 ± 0.21	0.36 ± 0.09
Experiment 3 (n = 5)	1.89 ± 0.00	0.83 ± 0.19	0.15 ± 0.03
Experiment 4 (n = 4)	<LOQ[2]	<LOQ	<LOQ
Experiment 5 (n = 6)	<LOQ	<LOQ	<LOQ
Experiment 6 (n = 6)	0.05 ± 0.04	<LOQ	<LOQ
Experiment 1–6 (n = 32)	1.59 ± 1.77	0.77 ± 0.89	0.16 ± 0.18

[1]AMPA = aminomethylphosphonic acid.

[2]<LOQ = AMPA concentrations in feed, feces or urine samples were lower than the limit of quantification (LOQ), and therefore the intake as well as the excretion with feces and urine was considered as zero.

The duodenal flows of GLY and AMPA in experiments 1 and 2 (measurement subsequent to total collection of feces and urine) are shown in Table 5. The intakes of GLY and AMPA during the duodenal sampling period were different in the 2 experiments, with highest intakes in experiment 1. However, the duodenal flows of GLY and AMPA were in a similar range. In both experiments an apparent ruminal disappearance occurred for both substances; 2.27 mg/d disappeared in experiment 1 and 0.19 mg/d of GLY disappeared in the rumen in experiment 2.

Table 5: Glyphosate and AMPA[1] intake and flow at the duodenum (means ± SD) during times of duodenal sampling followed after the balance experiment 1 and 2.

Experiment (animals)	Intake in feed (mg/d)	Flow at the duodenum (mg/d)	Apparent ruminal disappearance (mg/d)
Glyphosate			
Experiment 1 (n = 5)	6.24 ± 1.61	3.97 ± 0.83	2.27 ± 1.66
Experiment 2 (n = 6)	3.38 ± 0.14	3.20 ± 0.48	0.19 ± 0.34
AMPA			
Experiment 1 (n = 5)	3.54 ± 0.81	2.16 ± 0.57	1.38 ± 1.00
Experiment 2 (n = 6)	2.66 ± 0.11	2.32 ± 0.22	0.34 ± 0.18

[1]AMPA = aminomethylphosphonic acid.

Fecal, renal, and mammary excretion of GLY and the apparent retention of GLY, expressed as percentage of intake, are presented in Table 6. Due to very low intakes of GLY in experiments 5 and 6 these variables were not calculable. Overall, the ratio of GLY intake to excretion of GLY via feces or urine remained independent from the level of GLY intake and averaged 61 ± 11% (fecal; mean ± SD) and 8 ± 3% (renal). The mammary excretion was 0% and the apparent retention 31 ± 13%.

Table 6: Fecal, renal, and mammary glyphosate excretion as well as apparent retention expressed as proportion of glyphosate intake (means ± SD).

Experiment (animals)	Fecal (% of intake)	Renal (% of intake)	Mammary (% of intake)	Apparent retention (% of intake)
Experiment 1 (n = 5)	65 ± 5	7 ± 1	<LOQ[1]	28 ± 5
Experiment 2 (n = 6)	61 ± 7	8 ± 1	<LOQ	31 ± 10
Experiment 3 (n = 5)	50 ± 12	4 ± 1	<LOQ	46 ± 13
Experiment 4 (n = 4)	73 ± 10	8 ± 1	<LOQ	19 ± 10
Experiment 5 (n = 6)	NC[2]	NC	NC	NC
Experiment 6 (n = 6)	NC	NC	NC	NC
Experiment 1–4 (n = 20)	61 ± 11	8 ± 3	<LOQ	31 ± 13

[1]<LOQ = GLY concentrations in milk samples were lower than the limit of quantification (LOQ) and therefore the transfer into milk was considered as zero.

[2]NC = not calculated due to very low glyphosate intake in experiment 5 and 6 (<0.15 mg/d).

For AMPA the fecal, renal, and mammary excretion as well as apparent retention were not calculable

for experiments 4, 5, and 6. In experiments 1, 2, and 3, the average fecal and renal excretion were 48 ± 8 and 10 ± 3%, respectively. The mammary excretion was 0% and the apparent retention was 42 ± 9% (Table 7).

Table 7: Fecal, renal, and mammary AMPA[1] excretion as well as apparent retention expressed as proportion of AMPA intake (means).

Experiment (animals)	Fecal (% of intake)	Renal (% of intake)	Mammary (% of intake)	Apparent retention (% of intake)
Experiment 1 (n = 5)	50 ± 6	9 ± 1	0	42 ± 6
Experiment 2 (n = 6)	50 ± 6	12 ± 3	0	38 ± 9
Experiment 3 (n = 5)	44 ± 10	8 ± 2	0	48 ± 11
Experiment 4 (n = 4)	NC[2]	NC	NC	NC
Experiment 5 (n = 6)	NC	NC	NC	NC
Experiment 6 (n = 6)	NC	NC	NC	NC
Experiment 1–3 (n = 16)	48 ± 8	10 ± 3	0	42 ± 9

[1]AMPA = aminomethylphosphonic acid.

[2]NC = not calculated due to very low AMPA intake in experiment 4, 5, and 6 (<0.06 mg/d).

Discussion

The data from the present study represent the first results on GLY balance data in lactating cows and are therefore of high scientific relevance. In the present study a broad range of GLY exposition (0.08–6.7 mg/d) of the cows was measured. On average, cows were exposed daily to 4 µg of GLY/kg of BW. The maximum exposure of the cows was observed in experiment 1 (11 µg of GLY/kg of BW), and the minimum exposure was in experiment 6 (0.1 µg of GLY/kg of BW). The highest GLY contamination was observed in the concentrates of experiments 1 to 4. The average proportion of soybean meal in these concentrates was 22% and the average GLY concentration was 0.58 mg/kg. If the greatest extent of GLY originated from soybean meal, the concentration of GLY in this ingredient should be 4.5 times higher than in the complete concentrate; this would result in values of approximately 3 mg/kg for soybean meal. This value is in the range of GLY concentrations (0.4–8.8 mg/kg) observed in genetically modified soybeans (Bøhn et al., 2014) and leads to the assumption that GLY in the present investigation originated mainly from soybean meal.

In the present study 61 ± 11% of the ingested GLY was excreted in feces and passed the gastrointestinal tract of the dairy cows unmetabolized. The excretion with urine (8 ± 3% of daily intake) was the second important excretion pathway. In studies with rats, the elimination of ingested GLY with urine was approximately 30% (Brewster et al., 1991; Chan and Mahler, 1992). The difference in urinary elimination of GLY between species might be explained by a possible higher gastrointestinal degradation of GLY to AMPA in dairy cows compared with rats. Gerlach et al. (2014) observed GLY concentrations of approximately 5 to 20 µg/L in urine samples of dairy cows. However, in Gerlach et al. (2014), neither the urine volume nor the GLY intake was measured and GLY excretion was not determined quantitatively. The GLY concentrations in urine of the present study ranged between values lower than the LOQ and 75.1 µg/L and suggest a representative range for conventional feeding conditions in dairy cows. A dietary intake lower than 10 mg/d as measured in the present study did not result in GLY excretion via milk. In all milk samples the GLY concentrations were below the LOQ. Under the conditions of the present study milk was no excretion pathway for GLY, but these results should be verified by further investigations, especially with higher daily GLY intakes.

For the remaining 31 ± 13% of GLY that was not excreted with feces and urine, degradation by rumen microbes could be relevant. For experiments 1 and 2, with the highest GLY intake per day, the duodenal flow of GLY was 36 and 6% lower compared with the daily intake, respectively. These results suggest that GLY might have been degraded in the rumen. Jacob et al. (1988) and Heitkamp et al. (1992) described microbes originating from soil (Pseudomonas sp. strain LBr) with the ability to degrade GLY to AMPA. In contrast to dairy cows, metabolism of GLY in rats was 7% (Anadón et al., 2009) or less than 1% (Brewster et al., 1991). The relevance of rumen microbes for degradation of GLY has to be clarified in further studies.

The fact that a high proportion of GLY (61 ± 11%) passes the rumen and intestine unmetabolized is important regarding potential effects of GLY on microbes in the gastrointestinal tract. In recently

published studies, a relationship of GLY to the development of chronic visceral botulism in dairy cows was hypothesized (Krüger et al., 2013; Gerlach et al., 2014). For ruminants, only a few studies are available regarding the effects of GLY on microbial community and ruminal fermentation parameters. Riede et al. (2014) found no effects of GLY on ruminal fermentation and microbial community in vitro. These results agreed with results of a study in wethers by Hüther et al. (2005), which showed no effects of GLY on pH value and concentration of VFA in rumen fluid. Further research is necessary to clarify whether the unmetabolized GLY in the gastrointestinal tract may affect rumen microbes.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication describes a series of 6 experiments in which dairy cows (n = 4-6 per experiment) were fed with glyphosate-treated feed for 26 days and where the excretion of parent glyphosate and AMPA residues via feces, urine and milk was investigated during the last 5 days of the experiments (i.e. at a time when steady state can be assumed). The intake of parent glyphosate residues ranged between < 0.001 mg/kg bw/day (experiments 4, 5 and 6) and 0.011 mg/kg bw/day (experiment 1) while the intake of AMPA residues ranged between < 0.001 mg/kg bw/day (experiments 4, 5 and 6) and about 0.008 mg/kg bw/day (experiment 1). These intake levels are far below the dose levels investigated in the goat metabolism studies and cow feeding studies submitted in the dossier (since the applicable guidelines require that the dose levels be higher) but are likely to reflect "typical" intake levels of dietary cows. In the experiments it was found that 50-73% of ingested glyphosate was excreted in feces and 4-8% in urine. Similarly, 44-50% of ingested AMPA was excreted in feces and 8-12% in urine (these figures assume that no glyphosate is metabolized to AMPA in the cows). These results are consistent with the results of the submitted goat metabolism studies which show that 47-78% of the administered radioactivity is excreted via feces and 4.7-23% via urine. The residues of parent glyphosate and AMPA in milk were below the limit of quantification of 0.01 mg/kg, which is consistent with the results of the GLP cow feeding studies submitted in the dossier. Although the residue analytical method and residue analyses are not reported with a high level of detail, the results are considered reliable since the general principle of the described analytical procedures is well known and the validity of the residue determination was obviously demonstrated by suitable fortification trials. The publication, therefore, is considered relevant and reliable.

1. Information on the study

Data point	CA 6.9
Report author	Zoller O. et al.
Report year	2018
Report title	Glyphosate residues in Swiss market foods: monitoring and risk evaluation
Document No.	Food Additives & Contaminants: Part B, 2018, Vol. 11, No. 2, 83-91
Guidelines followed in study	None stated
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:	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 optimized 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 recognized 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.