グリホサートカリウム塩

要旨及び評価結果

（農作物及び畜産物への残留）

検索期間：2010 年 1 月 1 日～2019 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

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1. Information on the study

Data point	CA 6.10.1
Report author	Berg C.J. et al.
Report year	2018
Report title	Glyphosate residue concentrations in honey attributed through geospatial analysis to proximity of large-scale agriculture and transfer off-site by bees
Document No.	PLoS ONE 13(7): e0198876
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

Honey taken directly from 59 bee hives on the Hawaiian island of Kaua‘i was analyzed for glyphosate residue using ELISA techniques. Glyphosate residue was detected (> LOQ) in 27% of honey samples, at concentrations up to 342 ppb, with a mean = 118 ppb, S.E.M. 24 ppb. Of 15 honey samples store-purchased on Kaua‘i, glyphosate was detected in 33%, with a mean concentration of 41 ppb, S.E.M. 14. Glyphosate residue was not detected in two samples from the island of Molokai but was in one of four samples from the island of Hawai‘i. Presence and concentration of glyphosate residues were geospatially mapped with respect to Hawaiian land divisions. Mapping showed higher occurrence of glyphosate that was over LOQ (48%) and concentrations of glyphosate (mean = 125 ppb, S.E.M. 25 ppb; N = 15) in honey from the western, predominantly agricultural, half of Kaua‘i versus the eastern half (4%, mean = 15 ppb; N = 1). Geographic Information System analysis of land use percentage was performed within a circular zone of 1 km radius around each hive. Various land use types within each circular zone were transcribed into polygons and percent land use calculated. Only agriculture land use showed a strong positive correlation with glyphosate concentration. High glyphosate concentrations were also detected when extensive golf courses and/or highways were nearby. This suggests herbicide migration from the site of use into other areas by bees. Best management practices in use for curtailing pesticide migration are not effective and must be carefully re-assessed.

Materials and Methods

Sample collection

Honey samples were collected directly from hives by beekeepers on the island of Kaua‘i in three batches from 2013 through 2016 (**Table 1**). Samples were opportunistically obtained from all accessible parts of the island. Collections were constrained by lack of bee hives in the area or beekeepers’ unwillingness to provide samples. A strict confidentiality agreement was needed to get participation in the study. For some sites, sample batches were collected over time, to increase sample size. The timing was irrespective of seasonality of honey production by the bees. Each sample came from a single unique hive and its location was precisely recorded. Two other batches of honey were obtained from merchants and comprised honey from many hives under control of the manufacturing company.

In the fall of 2013 (Batch 1) two honey samples were collected by beekeepers, by scraping the honey comb with the open mouth of a clean glass mason jars and sealing the jars. These samples were stored at room temperature in a closed box, in a cabinet, until shipment to Microbe Inotech Laboratories, Inc., St. Louis, MO, for analysis of glyphosate concentration.

During the spring of 2015 (Batch 2) 36 samples of honey were collected directly from their unique hives by beekeepers of Kaua‘i, using only the certified pre-cleaned 40 ml amber borosilicate glass vials

provided to collect and store the honey. Vials were immediately sealed under a signed and dated custody seal by the collector and delivered directly to one of the authors (CJB, RK), along with a signed confidentiality statement containing contact information, date of collection, and hive location. Samples were stored at room temperature in a closed box, in a cabinet until shipment for analysis.

In fall of 2016 (Batch #3) 21 samples were collected by beekeepers and delivered to one of the authors (CJB), under the same procedures and stored for shipment as Batch #2.

In the winter of 2016 (Batch #4) 23 samples of honey were purchased from local famers' markets, produce stands, and stores. Honey was decanted into glass vials, sealed, and stored as above. Commercially produced honey is a composite from many hives. Source location was broadly determined from the label or from discussion with merchants. Date of honey collection is unknown. Samples were sent to Abraxis Inc, laboratory for analysis.

Batch #5 comprises three honey samples. Two samples were from the island of Molokai. One was purchased at a store on Molokai and the other was obtained from the beekeeper's bottled supplies. Both samples were a composite from hives at each beekeepers' farm. The farms' hives, which were located on Google Earth Pro™, were widely separated and thus represented different bee foraging sites. The third sample was purchased at a Kauai store and the source locality identified as from the island of Hawaii by its label.

Table 1: Honey collection data and laboratory where glyphosate was analyzed by ELISA.

Batch Number	Sample ID	Date Collected	ELISA analysis location
Hive Samples			
Batch #1	37, 38	Fall 2013	Micro Inotech Lab.
Batch #2	1 to 36	Summer 2015	Surfrider Lab.
Batch #3	39 to 59	Fall 2016	Surfrider Lab.
Merchant Samples			
Batch #4	91 to 23	Winter 2016	Abraxis Lab.
Batch #5	60, 61, 62	Winter 2016	Surfrider Lab.

Sample analysis

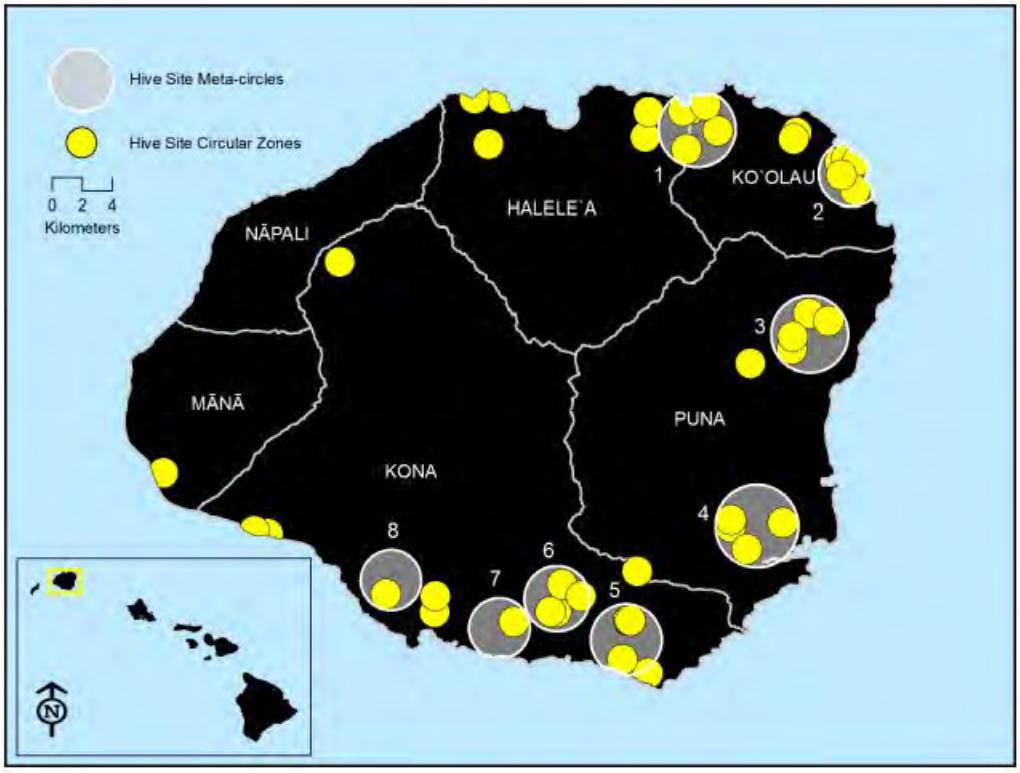
ELISA analysis was performed at each laboratory using the Abraxis method [1]. Abraxis test kit (cat. #500086) and microtiter equipment were used. The sample preparation method for honey followed published procedures [1, 17] (S1 Appendix). Samples were processed and read with a microplate reader Model 4303 [18] from Abraxis Inc. and analyzed using Molecular Devices Soft max pro evaluation program (4-Parameter). Results from Surfrider laboratory analysis were certified correct by Abraxis staff. Limit of quantitation (LOQ) was 15 ng/mL (15 ppb). Samples are stated as having detectable levels of glyphosate only if they are > LOQ.

Abraxis' ELISA methods for analysis of glyphosate have been compared to standard liquid chromatography and tandem mass spectrometry methods but not for honey. Therefore, 14 samples from Batch #2 were analyzed by both methods for validation. The results closely correlated with $R^2 = 0.99$ (S2 Appendix). Only ELISA derived data were used in this study.

Geospatial analysis

Presence and concentration of glyphosate residues were geospatially mapped with respect to general geography of the island and land use. Ancient Hawaiian biogeographical and management land divisions (Moku) (**Figure 1**) [19] were identified using the Google Earth Pro™ (GEP).

Figure 1: Distribution of 1 km radius circular zones (yellow) around hives on island of Kaua‘i. Meta-circles of grouped circular zones are shaded in grey and numbered (N = 8). Moku divisions are indicated by white lines and each Moku is named.



Circular zones

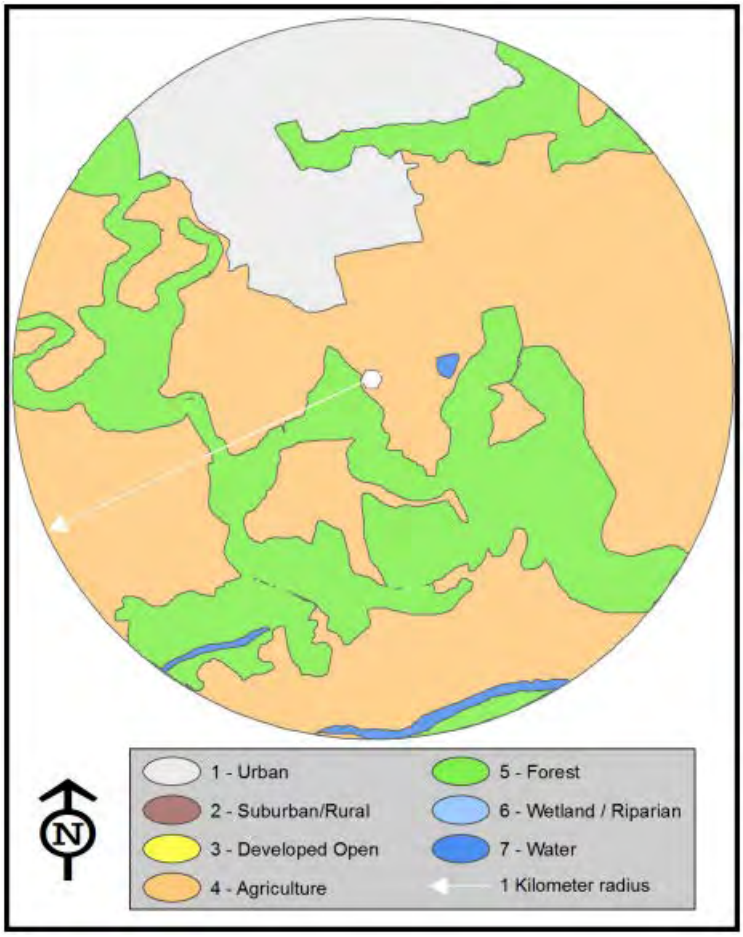
Bees have been reported to forage as far as 9.5 Km from the hive [20,21] with a mean distance closer to 1 km at times subject to patchiness of flowering resources [21]. Depending upon resource availability, the probability of plant visitation decreased non-linearly from the hive and > 85% probability of visitation was at less than 1 km [22]. Beekeepers note that bees forage as close to the hive as possible [23], especially on Kaua‘i where naturally occurring plants and crops bloom year-round. Foraging on Kauai may also be constrained by discrete watersheds, bounded by mountainous ridges.

Based on this information, and to avoid overlapping of foraging sites, a 1 km radius was used to define the bees’ foraging zone around each hive. Geospatial information analysis was applied using the GEP program with Digital Globe™ (DG) images to delineate circular zones 1 km in radius around each hive (**Figure 1**).

The land area within each circular zone was further sub-divided into discrete polygons, based upon land cover designations derived from NOAA C-CAP twenty-one classifications [24] (**Table 2**). Habitat codes were reclassified to seven land use categories.

Individual polygons were delineated in GEP using an optical mouse and area covered was calculated. The land area of each habitat type was then summed to provide a measure of the total land area (m^2) in each land use polygon (**Figure 2**). Each circular zone comprised 314.16 hectares, unless ocean area was excluded. A total of 18,872 hectares of land area were processed using the latest GEP images (years 2013–2014) and knowledge of current land use. Visual ground truthing was performed on sites known to differ from GEP images.

Figure 2: Circular zone around a central hive, drawn with 1 Km radius. Polygons represent different land uses categories. Site #16 provided as an illustration.



The percent of the current land use was calculated for each habitat type represented by the polygons within the hive sites' circular zones. These percentages were then correlated with the concentration of glyphosate residue from the hive in the circular zone. One hive (#48, Mānā Moku) was excluded from polygon land use calculations, as it had been moved among sites within the Moku.

A second independent geospatial analytical method for land use categorization used the NOAA Coastal–Change Analysis Program (C-CAP) [24] and ArcGIS Version 10.5 [25] (S3 Appendix). It derived area (m^2) within the 1 km radius circular zones using a program that automatically identified different types of ground cover (**Table 2**). A comparison of the two methods for accuracy in determining current land use patterns showed GEP preferable, so it was used in this study (S3 Appendix).

Table 2: Land use NOAA C-CAP classification descriptions.

This Study	Land use category	C-CAP	Land use classifications	Description of ground cover
		1	Unclassified	
1	Urban	2	High Developed	heavily built-up urban centers as well as large constructed surfaces in suburban and rural areas. Large buildings
		3	Medium Developed	constructed surface mixed with substantial amounts of vegetated surface. Small buildings
2	Suburban/Rural	4	Low Developed	class 3, with the addition of streets and roads with associated trees and grasses
3	Developed Open	5	Developed Open	parks, lawns, athletic fields, golf courses, and natural grasses occurring around airports and industrial sites
4	Agriculture	6	Orchard	herbaceous (cropland) and woody (e.g., orchards, nurseries, and vineyards) cultivated lands
		7	Pasture land	grasses, legumes or grass-legume mixtures planted for livestock grazing or the production of seed or hay crops
		8	Grassland	Grassland: grasses and non-grasses (forbs) that are not fertilized, cut, tilled, or planted regularly
		20	Bare land	bare soil, rock, sand, silt, gravel, or other earthen material with little or no vegetation
5	Forest	9	Deciduous forest	Deciduous Forest areas dominated by single stemmed, woody vegetation
		10	Evergreen forest	67 percent of the trees remain green throughout the year. Both coniferous and broad-leaved
		11	Mixed Forest	areas in which both evergreen and deciduous trees are growing and neither predominate
		12	Scrub/shrubs	woody vegetation: true shrubs,young trees, and trees or shrubs that are small
6	Wetland/ Riparian	13	Palustrine Forested Wetland	non-tidal wetlands dominated by woody vegetation >5m
		14	Palustrine Scrub/Shrub Wetland	non-tidal wetlands dominated by woody vegetation less than or equal to 5 meters
		15	Palustrine Emergent Wetland	non-tidal wetlands dominated by persistent emergents, emergent mosses, or lichens
		16	Estuarine Forest Wetland	tidal wetlands dominated by woody vegetation >5m, salinity >0.5ppt
		17	Estuarine Scrub/Shrub Wetland	tidal wetlands dominated by woody vegetation <5m, salinity >0.5ppt
		18	Estuarine Emergent Wetland	erect, rooted, herbaceous hydrophytes. Perennial plants usually dominate these wetlands
7	Water	19	Unconsolidated Shore	substrates lacking vegetation: beaches, bars, and flats
		21	Open water	open water with less than 25 percent cover of vegetation or soil.

Meta-circles

Analysis was done to determine if non-glyphosate using areas (e.g. containing forest, water, organic farms and residential) could be differentiated from areas of higher glyphosate use, as determined by conversations with the beekeepers. Eight meta-circles were made, comprising multiple 1-km circular zones that were grouped as having the same general land use description (**Table 2**, **Figure 1**) and situated in grouped watersheds. These meta-circles were encircled within a computer-generated circumference (mean 1707 hectares) that fully contained 3 to 9 circular zones of the same land-use practices (ranging from 1256 to 2365 hectares). In total, 41 samples were included within these eight meta-circles.

Large-scale divisions (East-West side of island, Moku)

The island of Kauai is divided by mountainous ranges and orographic rainfall in to two different biogeographical zones [16]. The drier leeward west-side of Kaua‘i comprises the Moku of Kona, Nāpali, and Mānā for approximately 73,710 hectares, 51.3% of the island’s area, while the wetter windward east-side comprises the Moku of Puna, Ko‘olau, and Halele‘a for approximately 70,049 hectares, 48.7% of the island’s area. Moku are identified by geological and biogeographic features [19] (**Figure 1**).

Statistical analysis

Data was analyzed with Microsoft Excel and Access (means, medians, S.D., S.E.M, t-tests, linear and exponential line fits). Analyse-it, a plug-in for Excel, was used for correlations and AICc line fits. TIBCO Spotfire Analyst® was used to produce the Trellis plots and non-parametric Kruskal-Wallis analysis.

Results

Island-wide

ELISA measured glyphosate concentrations in honey taken directly from the hive ranged from < LOQ to 342 ppb (**Table 3**). Sixteen (27.1%) of 59 samples had glyphosate concentrations detected over the

ELISA limit of quantitation (LOQ = 15 ppb).

Calculations of mean concentrations were done in two manners: using all sample ELISA data (N = 59, mean = 33.5 ppb, standard error of the mean, S.E.M. = 9.3) or for only those samples with ELISA values greater than the LOQ (N = 16, mean = 118.3, S.E.M. = 24.0).

Spatial and temporal variations at hive sites

Six separate sites had samples taken from multiple unique hives on those sites. At two of these six sites (Samples # 52, 53; 54, 56, 58), all hives had no glyphosate detected. At three of these six sites (Samples # 18, 59; 8, 14, 20, 21; 34, 35, 36), all hives had glyphosate > LOQ. At one site (Samples # 55, 57), only one hive had detectable glyphosate (Sample # 57) (27 ppb), while the other hive had none detected.

An extremely large feral beehive sampled in 2013 had 92 ppb glyphosate (**Table 3**, Sample # 37). In 2015, this site had four samples taken from widely spaced parts of the hive (Samples 8, 14, 20 & 21). Analysis yielded values ranging from 33 ppb to 342 ppb (mean = 147.7 ppb, S.E.M. = 69.7 ppb).

Two different sites were sampled in 2015 and again in 2016. Each of these two sites had multiple hives. Both sites showed an increase in concentration levels over time (0 ppb to 27 ppb for samples 55 & 57; 25 ppb to 95 ppb for Samples 18 & 59).

Of the store-bought samples (**Table 4** and Table A in S4 Appendix), 33.3% of those from Kaua‘i had glyphosate residue > LOQ (mean = 41 ppb, S.E.M. = 14.2)

East-West side of island

Presence and concentration of glyphosate residues were mapped with respect to ancient Hawaiian biogeographical and management land divisions (Moku) [19]. When all 59 samples were analyzed, there was a higher glyphosate concentration (mean = 61.6 ppb, N = 31, S.E.M. = 16.2) (**Table 5** and Tables B and C in S4 Appendix) in honey from the leeward western half of Kaua‘I versus the windward eastern half (mean = 2.4 ppb, N = 28, S.E.M. = 0.9). Mean values between the western and eastern sides are different (t-test, p = 0.001, df = 57) (Table D in S4 Appendix).

If only glyphosate values > LOQ are used (N = 16), the western Moku had 15 samples, 48.4% of which had glyphosate > LOQ (mean = 125.1 ppb). The eastern Moku had only 1 sample over the LOQ (3.6%). This sample value (15.2 ppb) is just greater than the LOQ.

A Trellis plot was made showing the glyphosate concentration across samples, grouped by side of island and by Moku. When all 59 samples are plotted, there is a clear pattern of the higher glyphosate concentrations in the western Moku vs the eastern Moku (Figure 3). No samples were collected from the remote western Moku of Napali.

Moku

Moku differed greatly in the and mean concentration of glyphosate in honey (Table B in S4 Appendix). Puna and Ko’olau Moku had no samples >LOQ and Halele’a had only one > LOQ. No samples were collected from remote Napali and only one sample from Mana. Concentrations from the west side Kona Moku were different from the three east-side Moku ($p < 0.003$) (Table E in S4 Appendix).

Since it is not known if these samples are from a normally distributed population, a non-parametric Kruskal-Wallis test was performed. This test confirmed the above parametric tests that glyphosate distributions were different depending upon the side of the island and the Moku (p = 0.0008 and 0.004, respectively) (Table F in S4 Appendix).

Source location of honey purchased from merchants on Kaua‘i was obtained from the label and discussions with vendors. Percentage of samples with glyphosate residue > LOQ and mean concentrations of glyphosate differed among Moku sampled (**Table 6** and Table A in S4 Appendix). Area with the greatest percentage of samples with glyphosate was in the agricultural district of Kona on the west side of the island. This is the same trend seen as with the hive samples (**Figure 3**).

Table 3: Glyphosate concentration and percent of land use (by category) within the circular zones surrounding the hives.

Google Earth Polygon Land Use Classification								[Glyphosate]
Sample #	% Urban	% Suburbs	% Open	% Ag	% Forest	% Wetland	% Water	ppb
1	71.4%	1.1%	6.6%	3.1%	0.0%	17.6%	0.2%	< LOQ
2	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
3	0.0%	13.5%	0.0%	70.9%	15.5%	0.0%	0.0%	
4	31.1%	0.0%	9.0%	30.0%	29.7%	0.0%	0.3%	
5	22.6%	0.0%	13.3%	21.0%	42.8%	0.0%	0.3%	< LOQ
6	19.8%	0.0%	3.3%	76.9%	0.0%	0.0%	0.0%	80
7	0.0%	10.4%	66.5%	3.2%	19.8%	0.0%	0.0%	
8	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	61
9	0.0%	46.6%	23.1%	1.1%	29.2%	0.1%	0.0%	
10	0.0%	6.5%	87.5%	4.4%	0.0%	0.0%	1.6%	< LOQ
11	0.0%	0.0%	4.2%	69.7%	26.1%	0.0%	0.0%	
12	0.0%	0.0%	48.2%	19.8%	32.0%	0.0%	0.0%	15
13	0.0%	30.9%	26.6%	8.6%	34.0%	0.0%	0.0%	
14	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	342
15	15.58%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	
16	15.3%	0.0%	0.0%	53.8%	30.1%	0.0%	0.9%	
17	0.0%	4.0%	1.8%	0.0%	94.2%	0.0%	0.0%	
18	25.4%	0.0%	74.1%	0.1%	0.0%	0.0%	0.3%	25
19	52.9%	0.0%	44.6%	2.4%	0.0%	0.0%	0.0%	< LOQ
20	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	155
21	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	33
22	0.0%	45.8%	3.0%	33.9%	13.3%	0.0%	4.1%	
23	0.0%	50.2%	14.8%	0.4%	34.6%	0.0%	0.0%	
24	0.0%	1.5%	11.2%	64.3%	23.0%	0.0%	0.0%	
25	6.8%	10.1%	57.1%	2.3%	23.5%	0.0%	0.3%	
26	0.0%	1.0%	6.7%	68.6%	23.7%	0.0%	0.0%	
27	18.9%	0.0%	50.5%	0.0%	29.4%	0.0%	1.2%	
28	0.0%	0.0%	47.5%	11.5%	41.0%	0.0%	0.0%	< LOQ
29	0.0%	14.4%	0.5%	75.0%	10.1%	0.0%	0.0%	
30	0.0%	7.0%	30.2%	8.7%	54.1%	0.0%	0.0%	
31	0.0%	30.1%	0.0%	0.0%	67.6%	2.4%	0.0%	
32	0.0%	30.2%	8.3%	1.8%	57.7%	0.0%	2.0%	
33	22.2%	5.4%	61.4%	0.0%	7.8%	3.2%	0.0%	
34	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	187
35	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	178
36	0.0%	11.9%	1.5%	71.9%	7.4%	7.1%	0.2%	172
37	5.5%	1.8%	0.2%	90.5%	0.0%	0.0%	1.9%	92
38	36.2%	0.0%	2.8%	61.0%	0.0%	0.0%	0.0%	78
39	20.7%	4.2%	44.3%	3.5%	27.4%	0.0%	0.0%	
40	0.0%	49.0%	0.0%	12.9%	38.1%	0.0%	0.0%	< LOQ
41	17.1%	0.3%	0.0%	58.9%	23.8%	0.0%	0.0%	60
42	0.0%	0.0%	1.4%	67.9%	30.7%	0.0%	0.0%	
43	0.9%	1.2%	81.8%	4.7%	11.4%	0.0%	0.0%	
44	0.0%	28.2%	0.0%	25.3%	44.2%	0.0%	2.3%	< LOQ
45	0.0%	3.2%	0.0%	0.0%	95.5%	0.0%	1.3%	
46	6.8%	56.2%	0.0%	0.0%	37.0%	0.0%	0.0%	< LOQ
47	0.0%	0.0%	0.0%	19.5%	80.5%	0.0%	0.0%	
48	19.7%	0.0%	10.2%	16.3%	50.2%	0.0%	3.6%	292
49	20.0%	49.6%	0.0%	0.0%	29.0%	0.0%	1.3%	
50	0.0%	1.5%	0.0%	50.4%	48.1%	0.0%	0.0%	
51	0.0%	16.4%	0.0%	0.0%	83.6%	0.0%	0.0%	
52	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
53	19.0%	4.2%	2.2%	51.6%	21.4%	0.0%	1.6%	
54	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
55	18.0%	4.2%	2.2%	42.6%	31.2%	0.0%	1.8%	
56	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
57	15.6%	13.8%	1.4%	43.6%	23.1%	0.0%	2.6%	27
58	19.0%	4.0%	2.2%	47.3%	24.1%	0.0%	3.4%	
59	25.4%	0.0%	74.1%	0.2%	0.0%	0.0%	0.3%	95

Table 4: Concentration and percentage of glyphosate detected in store-bought honey. Samples originated from three Hawaiian islands and international blends. Samples categorized as Organic or Non-Organic.

Category			Samples N	> LOQ %	> LOQ Mean ppb
Location					
	Hawaii	Island:			
		Kaua'i	15	33.3	41.0
		Hawai'i	4	25.0	16.4
		Molokai	2	0	NA
	International		5	40.0	51.5
Type					
	Organic		5	20.0	30.6
	Non-Organic		21	33.3	42.0

Table 5: Glyphosate concentration by side of island and the six Moku. All 59 sample values used. Napali Moku had no samples ("ns").

Moku	Glyphosate Mean ppb	Median	S.D.	S.E.M.	Count
Windward:					
Koolau	0	0	0	0	10
Puna	2.5	0	5.1	1.7	9
Halelea	4.1	0	6.3	2.1	9
Totals	2.41	0	4.9	0.92	28
Leeward:					
Kona	53.9	11.7	80.9	14.8	30
Mana	292.2	292.2	na	na	1
Napali	ns	na	na	na	ns
Totals	61.61	13	90.3	16.2	31

Figure 3: Glyphosate concentrations across samples by side of island and within each Moku. Mean glyphosate (ppb) is shown by the horizontal line for each Moku. Side of the island and Moku names are listed at the top of the plot. Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles.

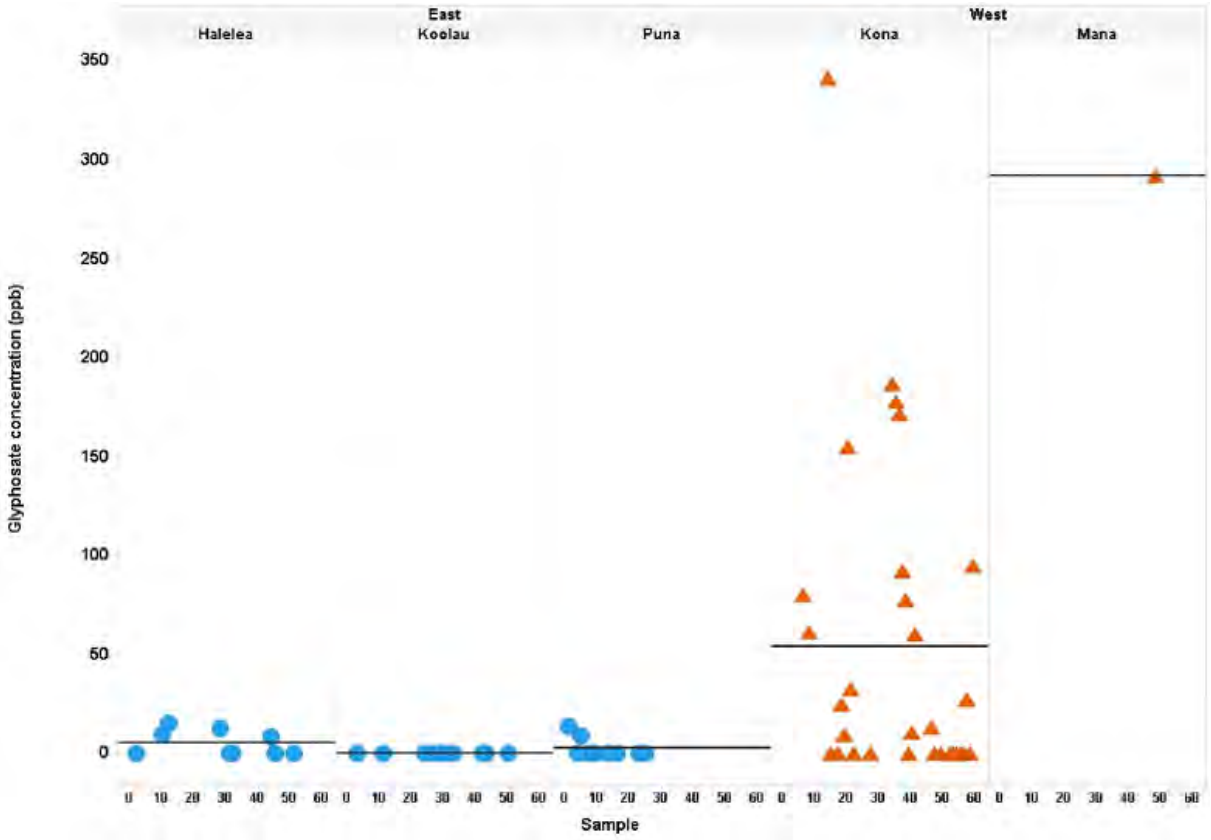


Table 6: Prevalence and concentration of glyphosate in Kauai honey from store-bought samples.

Moku	All samples N	> LOQ N	> LOQ % total	> LOQ mean	> LOQ SEM
Puna	6	1	16.7	15.0	na
Koolau	5	1	20.0	61.8	na
Kona	4	3	75.0	43.1	22.2

Circular zones and land use polygons

Land use within an area of 1 Km radius around each of the hives was determined using Google Earth Pro™ (GEP) (N = 59 hives from Kaua'i). These circular zones were divided into single land use polygons and the total meter^2 coverage for each of the seven land types was calculated. The percent of the total allocated to each of the seven land use types of each site was summarized with the glyphosate concentration found in the samples from that site (**Table 3**).

AICc analysis was performed to determine correlations between presence of glyphosate in honey and various land uses. Non-zero glyphosate data (N = 23) was used for these analyses. The exponential model for land use and glyphosate was chosen, as it has the highest correlation and strongest AICc values, compared with other line fits (Table G in S4 Appendix). Agriculture land use in the immediate 1 km radius vicinity of the hive showed the highest positive correlation with glyphosate concentration (**Table 7**, R^2 = 0.594) and the strongest AICc compared with the other land use categories. Open, Suburbs, Urban, and Forest land use all showed weak negative correlations (negative Parameter Estimates) between land use and glyphosate concentration. Wetland and Water land use showed very weak positive correlations. The negative correlations (e.g. Forest) is due to these land use types not being independent variables; rather, they are multicollinear (Figure A in S4 Appendix).

Concentration of glyphosate in honey was plotted versus the percent land use in agriculture. Samples with non-zero glyphosate were used (N = 23). **Figure 4** shows that the higher glyphosate concentrations are correlated with sites that have high percent agriculture land use (> 60% agriculture).

The hives in the western Moku (orange triangles) have a strong correlation with higher glyphosate when there is higher percent land use as agriculture. Hives in the eastern Moku (blue circles) had very low glyphosate, even with 60% to 80% of the area in agriculture (**Table 3**).

Table 7: Correlation of glyphosate concentration (ppb) in honey samples and the percent land use.

Land Use	R^2	AICc	SE of fit (RMSE)	Parameter estimate	95% CI	95% CI	SE	p-value	Exponential Equation
Agriculture	0.594	-8.664	0.784	2.552	1.594	3.511	0.461	0.000	$Y = 12.58 * 12.84^X$
Forest	0.326	2.967	1.010	-3.977	-6.572	-1.383	1.247	0.004	$Y = 65.24 * 0.01874^X$
Open	0.123	9.030	1.152	-1.465	-3.242	0.311	0.854	0.101	$Y = 50.03 * 0.231^X$
Suburbs	0.086	9.973	1.176	-2.276	-5.638	1.087	1.617	0.174	$Y = 46.98 * 0.1027^X$
Urban	0.049	10.897	1.200	-1.422	-4.274	1.430	1.371	0.311	$Y = 47.01 * 0.2412^X$
Wetlands	0.017	11.660	1.220	3.659	-9.110	16.427	6.140	0.558	$Y = 36.23 * 38.8^X$
Water	0.011	11.796	1.224	13.180	-44.017	70.377	27.504	0.637	$Y = 34.83 * 5.296e+05^X$

Meta-circles

In order to expand land use to watersheds or larger areas, meta-circle analysis was done on eight clusters of circular zones situated all around the island (**Figure 1**). They comprise similar environments. Discussions with beekeepers were used to develop a general description of each meta-circle (**Table 8**) as to predominant land use and glyphosate use.

The percent of each of the seven types of land use was calculated for each circular zone in each meta-circle (**Table 3** and Table H in S4 Appendix). Then the mean percent of each type of land use was calculated for each meta-circle. The highest percent land use was used to describe the meta-circle, if that land use type was at least 70%. If it was less than 70%, then a composite was used; the second highest type of land use was added to the highest land use type. This process was repeated until the composite land use designation comprised at least 70% of the meta-circle. This composite description is shown in

Table 8, in the column "Composite land use type".

The mean concentration of glyphosate in honey was calculated using all samples within each meta-circle (N = 48 samples total). The percentage of samples which had glyphosate > LOQ was also calculated (N = 16 total). Only three meta-circles had significant glyphosate residues and all were in areas on the western side of Kaua'i. The two meta-circles with the most glyphosate, Ag. 1 and Ag. 2, were in areas of large scale agriculture use. The Koloa meta-circle had some agricultural use and contained the circular zones with large amounts of golf courses and or highway present, as discussed below.

A Trellis plot was made to show glyphosate concentration across samples, grouped by meta-circle (**Figure 5**). Within each meta-circle, samples are plotted versus the percentage of agriculture for that sample. There is a clear pattern of the higher glyphosate concentrations for samples in the western meta-circles (orange) vs samples in the eastern meta-circles (blue). The samples with glyphosate > LOQ (triangles) are also all in the western meta-circles, while the eastern meta-circles all have glyphosate < LOQ (circles) (**Figure 5**).

Figure 4: Glyphosate concentration versus the percent land use in agriculture (N = 23). Samples from the western Moku are shown as orange triangles and eastern Moku as blue circles. Exponential fit is $Y = 12.6\ e^{12.8X}$, $R^2 = 0.594$.

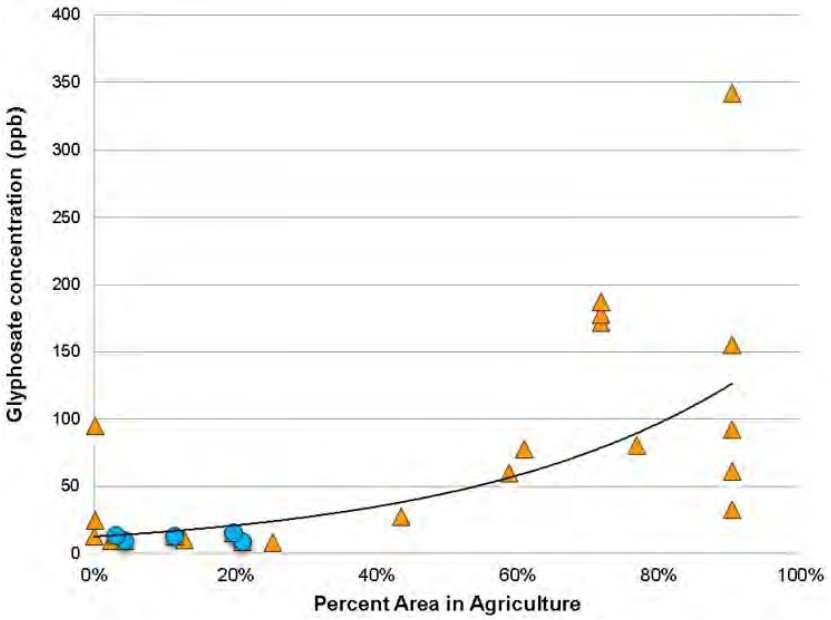
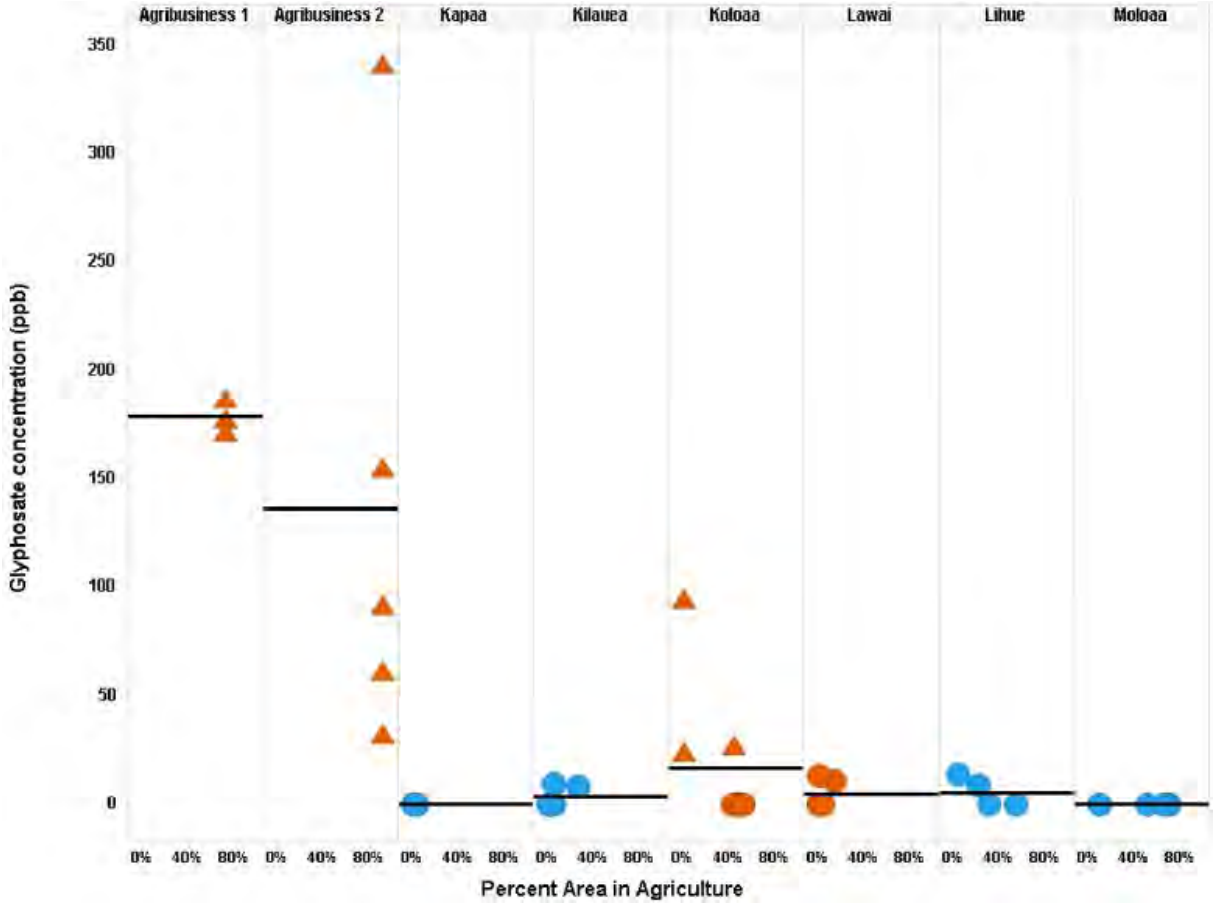


Table 8: Meta-circle composition, mean glyphosate concentration, and percent prevalence. Meta-circle # corresponds to Figure 1.

Meta-circle #	Meta-circle	Number of Circular Zones	General Description	Composite % land use	Composite land use type	Mean ppb	% > LOQ
1	Kīlauea	5	Rural, Suburbs	72.0%	Open, Forest	< LOQ	0%
2	Moloa'a	6	Organic farming	89.2%	Agriculture, Forest	< LOQ	0%
3	Kapa'a	4	Suburbs	82.0%	Open, Forest, Suburbs	< LOQ	0%
4	Līhu'e	4	Urban, open, agriculture	87.7%	Urban, Agriculture, Forest	< LOQ	0%
5	Kōloa	9	Suburbs, golf, resort	74.7%	Agriculture, urban, Forest	16.3	33%
6	Lāwa'i	5	Suburbs	82.9%	Forest, Suburbs, Open	< LOQ	0%
7	Ag. 1	3	Large scale agriculture	71.9%	Agriculture	179.0	100%
8	Ag. 2	5	Large scale agriculture	90.5%	Agriculture	136.6	100%

Figure 5: Glyphosate concentrations across samples within each meta-circle. Mean glyphosate (ppb) is shown by the horizontal line for each meta-circle. Meta-circle names are listed at the top of the plot. Samples from the western Moku are shown as orange and eastern Moku as blue. Samples with glyphosate > LOQ are shown as triangles, while those < LOQ are as circles.



Golf courses and highways

A smaller specific land use, golf course, was identified from GEP images, but was subsumed in the "Developed Open" C-CAP category (**Table 2**). There were only eight circular zones which encompassed golf course(s) and all had glyphosate residues in honey (**Table 9A**). Percent area in golf course varied from 1.2% to 16.2%. Three of those samples (samples #34, 35, 36) were from different hives on the same farm and were also associated with high percent (> 70%) agricultural land use. Two hives with the highest percent land use as golf course (samples # 18 and #59) were from the same residence with very low agricultural land use.

Major highways were identified as another small specific land use. These were subsumed under the Urban and Suburban/Rural categories (**Table 2**). Portions of highways were contained within 76% of the circular zones (Table I in S4 Appendix). Those in the top 10% of cumulative length of highway (> 4.6 km) had three samples with glyphosate > LOQ (25 to 95 ppb) (**Table 9B**). Frequent spraying of golf courses and highways may explain the presence of glyphosate (> LOQ) in samples # 18, 57, and 59.

Table 9: (A) 8 samples with highest % area Golf; (B) 6 samples with highest km highway present.

A				
Sample #	Glyphosate ppb	% Ag	% Golf	Km Highway
34	187	71.9%	1.2%	1.1
35	178	71.9%	1.2%	1.1
36	172	71.9%	1.2%	1.1
19	10	2.4%	1.6%	3.4
1	14	3.1%	4.8%	2.0
28	13	11.5%	13.7%	2.0
18	25	0.1%	16.2%	4.7
59	95	0.2%	16.2%	4.7
B				
55	0	42.6%	0.0%	4.6
57	27	43.6%	0.0%	4.6
59	95	0.2%	16.2%	4.7
18	25	0.1%	16.2%	4.7
52	0	51.6%	0.0%	4.7
53	0	51.6%	0.0%	4.7

Discussion

The presence of glyphosate residue in honey samples taken directly from the hive has been shown to correlate with areas that geospatial analysis has identified as comprised mainly of large-scale mono-crop agriculture. This suggests both a source and a pathway whereby pesticides migrate from site of use into other areas. Glyphosate residue >LOQ was found in 27.1% of the hives and 33.3% of store bought honey from Kauai, lower than the 59% in store bought honey from around the world [1]. With hive-collected honey, geospatial analysis was able to further identify: which side of the island (west), which Moku (Kona and Mana), which areas (agriculture meta-circles), and most specifically which land use (agriculture) had the greatest prevalence and greatest concentration of glyphosate in honey.

Purchased samples from the other Hawaiian islands had lower mean concentrations and a smaller percentage contaminated than those from Kauai. The mean concentration of glyphosate from international samples purchased on Kauai was 51.5 ppb, similar to the 64 ppb in Rubio [1]. Samples from Brazil and a sample from a blend of USA and Argentina approximated values reported earlier, while the blend from Brazil, Mexico and Uruguay did not [1].

One of five Kauai purchased samples (20%) labeled organic had glyphosate residues > LOQ (mean 30.6 ppb) compared to 45% (mean 50 ppb) reported elsewhere [1]. This supports supposition of some migration of pesticides from areas of application to organic farms. The twenty-one Kauai samples not labeled as organic had both a higher occurrence (33.3%) and higher mean concentration (42.0 ppb) of glyphosate than the organic labeled samples, suggesting application of glyphosate near the hives. Honey from traditional agriculture sites around the world had 62% with glyphosate > LOQ and mean 66 ppb [1], expressing widespread use of glyphosate in agriculture.

The actual process of how Kauai bees obtained, carried and processed glyphosate is not known and was not addressed in this study, but is discussed elsewhere [13,14]. As honey was obtained directly from the hive using clean vials, this eliminated the possibility of contamination occurring during processing. Each sample was unique to a single hive, not blended from various sites. A survey of beekeepers confirmed that their hives did not get sprayed with glyphosate. Uptake could have occurred if the bees themselves got sprayed while foraging, if flowers frequented by the bees contained glyphosate from either direct spraying or aerial drift, or if water that the bees drank on plants or on the ground was contaminated in some way. In all cases, contamination could have occurred at a distance from the hive. Geospatial analysis mallowed the determination that within a 1 km radius of the hive, glyphosate contamination was most closely associated with large scale agriculture. The proximity of golf courses and highways were also associated, but to a lesser degree. General land use changes and landscape composition may have indirect detrimental effect on bee fitness, although the association between pesticide and landscape composition was not investigated.

The presence of both restricted use pesticides and glyphosate in bee pollen and honey, even at very low

levels, identifies an important pathway whereby pesticides migrate from site of application to the hive and into the human food supply [12–14, 26]. Geospatial analysis can help honey producers estimate spatial pesticide exposure risks inherent in intensive agriculture. When bees are used for commercial large-scale crop pollination, hive placement can be optimized so that the bee colonies are not seriously impacted by pesticides that the bees must endure while foraging [26–27]. Linking spatial and temporal dynamics of flowering crops, agri-environmental schemes, and pesticide applications would lead to better understanding of environmental risk assessment, management of pollination services, and protecting biodiversity [26–28].

Supporting information

Supporting information with is available online:

S1 Appendix. Abraxis technical bulletin.
https://doi.org/10.1371/journal.pone.0198876.s001

S2 Appendix. ELISA verification with mass spectrometry.
https://doi.org/10.1371/journal.pone.0198876.s002

S3 Appendix. Geospatial analytical method comparison.
https://doi.org/10.1371/journal.pone.0198876.s003

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey.
https://doi.org/10.1371/journal.pone.0198876.s004

This information is summarised at the end of this document.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication provides residue levels for glyphosate in honey produced in Hawaii (majority of samples) but also Argentina, Brazil, Canada, Mexico, Uruguay and USA (mainland). It is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. As honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated indirectly by comparison with an LC-MS/MS method. A total of 14 honey samples were analysed with the two methods and the results were shown to be similar. The publication, however, does not provide validation data for the LC-MS/MS method (recovery rates from fortified samples).

The study showed a higher detection rate of glyphosate than in the EU-monitoring for 2016-2017. Besides the different origin of the samples, this may also be due to the use of different analytical methods with different LOQs. In line with the EU-monitoring the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.342 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

S1 Appendix. Abraxis Technical Bulletin

Glyphosate in Honey and Corn Syrup Sample Preparation

1. *Intended Use*

   For the detection of Glyphosate in honey and corn syrup.

2. *Sensitivity*

   0.015 ppm in matrix

3. *Materials and Reagents Required*

   Analytical balance
   Microcentrifuge tubes
   4 mL glass vials with Teflon-lined caps
   Disposable pipettes
   Micropipettes with disposable plastic tips
   Vortex mixer
   Microcentrifuge
   Timer
   Plate shaker or Micro-well plate holder with insert retainer for vortex mixer
   1 N Hydrochloric Acid (HCl)
   Glyphosate sample diluent
   Abraxis Glyphosate Plate ELISA Kit

4. *Notes and Precautions*

   This procedure is intended for use with honey and corn syrup (light and dark). Other matrices should be thoroughly validated before use with this procedure.

   Hydrochloric Acid must be handled with care. Wear appropriate protective clothing (gloves, glasses, etc.). Avoid contact with skin and mucous membranes. If contact occurs, wash with copious amounts of water and seek appropriate medical attention.

   Due to the viscous nature of the prepared samples, the microtiter plate should be placed on a plate shaker or vortex mixer fitted with a micro-well plate holder adapter for the incubations with the antibody and conjugate solutions. This will allow for the appropriate mixing of all reagents in the microtiter wells.

5. *Sample Preparation Procedure*

   5.1 Weigh 0.5 g of sample into an appropriately labeled microcentrifuge tube.

   5.2 Add 0.5 mL of 1 N HCl. Vortex for 2 minutes.

   5.3 Add 3.96 mL of Glyphosate Diluent to a clean, appropriately labeled 4 mL glass vial. Add 40 µL of the acid-treated sample (from step 5.2) to the Glyphosate Diluent in the vial (1:100 sample dilution). Vortex. This will then be analyzed as sample, see Derivatization of Standards, Control, and Samples in the Reagent Preparation section of the Glyphosate Plate ELISA Kit user's guide.

6. *Evaluation of Results*

   The Glyphosate concentration in the samples is determined by multiplying the ELISA results by a factor of 200.

   Samples showing a concentration lower than standard 1 (0.075 ppb) should be reported as containing < 15 ppb of Glyphosate. Samples showing a higher concentration than standard 5 (4.0 ppb) can be reported as containing > 800 ppb of Glyphosate or diluted further and re-analyzed to obtain an accurate quantitative result.

7. *Performance Data Recovery*

   Honey samples were spiked with various amounts of Glyphosate, prepared as described above,

and then derivatized and assayed using the Glyphosate Plate Assay. Average recovery was 113%.

Corn syrup samples (light and dark) were spiked with various amounts of Glyphosate, prepared as described above, and then derivatized and assayed using the Glyphosate Plate Assay. Average recovery was 104%.

S2 Appendix. ELISA verification with mass spectrometry

To verify ELISA techniques for measuring glyphosate in a honey matrix (LOQ of 15 ng/g, 15 ppb) honey remaining in 14 vials from the Batch 2 samples analysed with ELISA were sent to Quality Services International GmbH (QSI), (Bremen, Germany) for analysis of herbicide residue by gas chromatography/mass spectrometry (GC-MS/MS) and/or liquid chromatography mass spectrometry (LC-MS/MS) methods (QSI method # 88505) with a LOQ of 0.01 mg/kg (10 ppb) (Table A). All concentrations derived from ELISA were used in analysis, however QSI did not report readings for levels < 10 ppb, so a value of zero was assigned for data analysis.

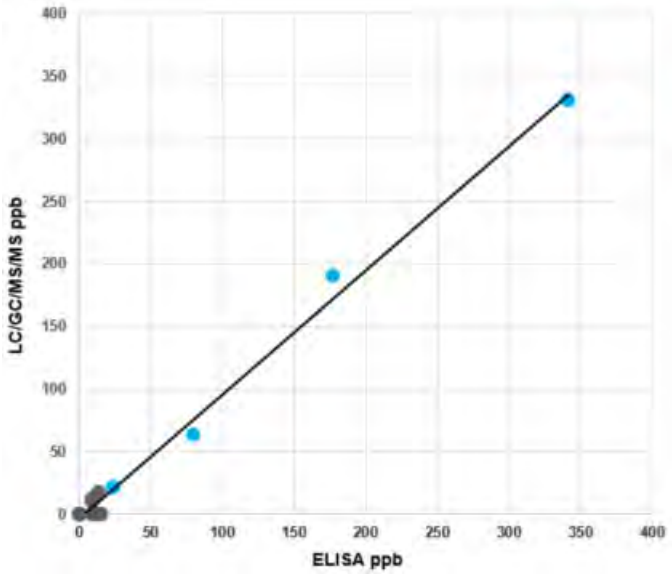
Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	Glyphosate ppb	Glyphosate ppb
Sample #	ELISA	QSI
1	13.6	17
5	8.8	10
6	**80.2**	**63**
7	0	0
10	9.2	0
12	15.2	0
14	**341.6**	**330**
16	0	0
18	**24.6**	**21**
19	9.6	12
25	0	0
27	0	0
28	12.6	0
35	**178.0**	**190**
LOQ=	15	10

Results for all 14 samples analysed by both methods correlated well (Figure A). Standard error of y for each x-value is 8.6 ppb. Only 4 samples had both the ELISA and LC/GC/MS/MS values over their respective LOQ, but the correlation coefficient remained high (Figure A).

Although sample size was small, a correlation coefficient $R^2 = 0.99$ supports the ELISA tests for accuracy, in addition to the use of blank and standards within each test run [1]. Comparison of ELISA techniques for monitoring glyphosate with chromatography-mass spectrometry have consistently found high correlations between the techniques in tests of various matrices, e.g. water [2,3], animal urine and animal tissues [4]. The use of Abraxis methods of ELISA determination of glyphosate in honey is well substantiated.

Figure A: Correlation of glyphosate concentration in honey split-samples using ELISA and LC-GC-MS/MS techniques. Linear fits: $Y = 0.99\ x - 3.1$, $R^2 = 0.993$ (N = 14; black and blue circles); $Y = 0.99\ x + 6.1$, $R^2 = 0.994$ (N = 4; blue circles).



S3 Appendix. Geospatial analytical method comparison

Development, application and comparison of two means for quantifying current land use practices within 1 km radius of a bee hive.

Geospatial analysis was performed in ArcGIS 10.5 on two separate Habitat datasets

1. Coastal Change Analysis Program (C-CAP) High Resolution Land Cover (1-4 meter resolution). Derived from high resolution imagery and analyzed according to the Coastal Change Analysis Program (C-CAP) protocol to determine land cover.
2. Vector polygons digitized in Google Earth Pro™ (GEP) using Digital Globe™ (DG) images (2013-2014) of 30-50 cm resolution as a base layer.

Coastal Change Analysis Program (C-CAP)

C-CAP analysis was conducted in January of 2016. Data downloaded was produced at a 1-4 meter resolution and utilized 35 full or partial WorldView2 multispectral scenes and the 2005 high-resolution Kauai C-CAP data set. The imagery and base classification were included in a multi-step semi-automated change detection process to extract land cover features in the 2010 imagery. Habitat within this dataset is classified into one of 21 different habitat classifications using a 2.5 meter cell size.

In order to extract out raster cells within the 2 kilometer boundary (1 Km radius) per hive site, the data set was masked using a vector dataset. This dataset was created by plotting each of the 38 hive sites in ArcGIS using their UTM location. Locations were converted into a point shapefile and then buffered by 1 km to create the 2 kilometer boundary polygon. Individual polygons were dissolved into one record to create the Mask to extract out pixels of the CCAP raster. Masking a raster using a vector is similar to the "Clip" geoprocessing routine done between two vector datasets. A vector representing an outline of the island was used to further mask the raster, removing pixels that were beyond the coastline, seemingly representing ocean (Figure A, B).

In order to quantify the percentage of habitat within each hive site boundary area (buffer a.k.a. circular zone), the raster pixels were converted into a polygon feature class (vector) for vector geoprocessing. This polygon conversion resulted in 26,176 records/polygons, representing 26,176 cells within the original Raster dataset residing in the hive site boundary area. The "Intersect" geoprocessing tool was

used next to assign to each record the corresponding hive site number it fell within. Habitat codes were reclassified, reducing the number of habitats considered by the analysis to seven land use categories. These were used in identifying the candidate habitats bees are believed to be foraging. Using the "Dissolve" geoprocessing tool, the habitat polygons were dissolved by Hive Site and reclassified Habitat Code, and the results stored in a geodatabase so that the area for each habitat could be reported using the Shape Area field. Totals for the amount of habitat polygon cells residing within each hive site boundary were then summed and the percentage for each habitat within the boundary calculated.

Figure A: ArcGIS 10.5 geoprocessing tools: Clip, Dissolve and Intersect.

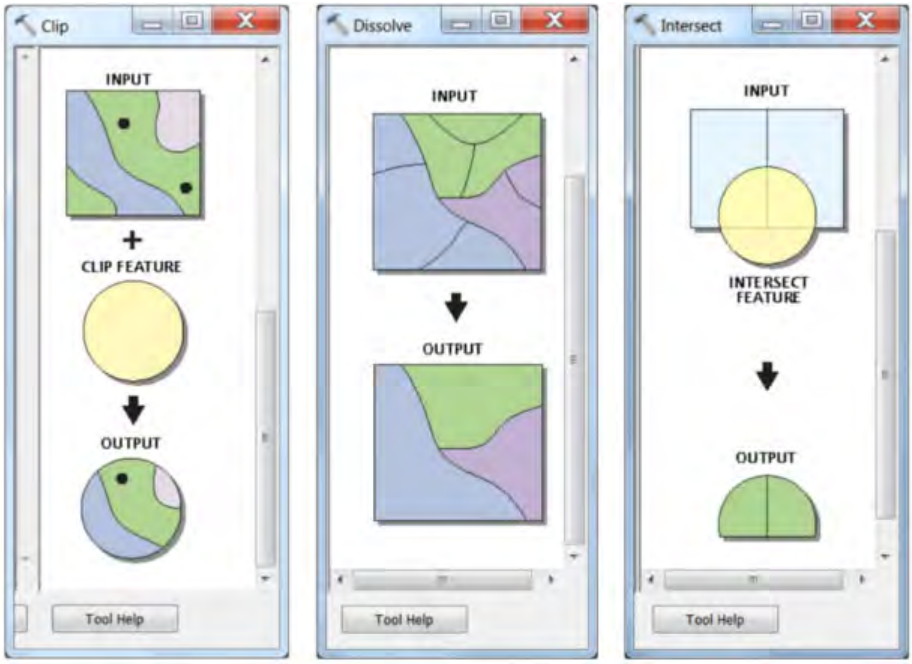
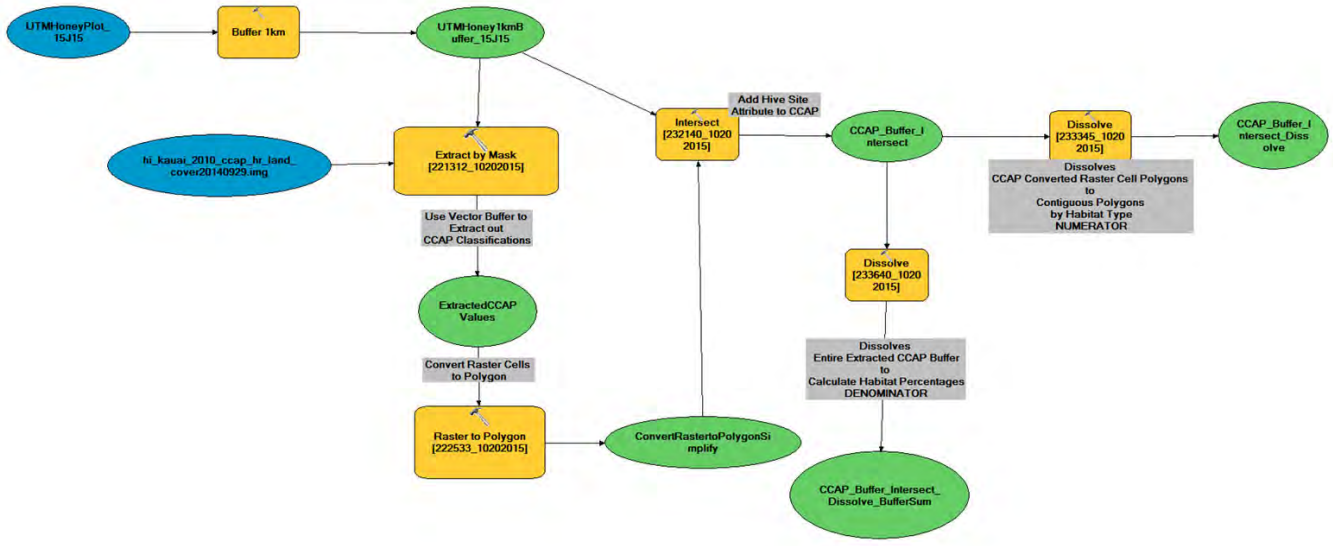


Figure B: Schematic of geoprocessing tools used to improve calculations of polygon areas for the C-CAP dataset.



Google Earth Pro™ (GEP)

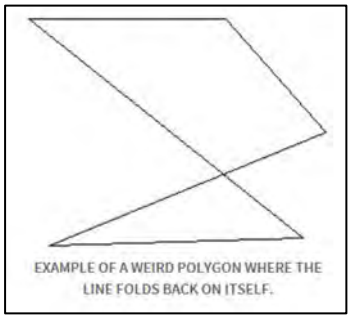
Digitizing in a Geographic Information System is the process of converting geographic data from a hard-

copy or scanned image into a vector dataset by tracing features; features are captured in coordinates and stored as either a point, line or polygon vector dataset. For this analysis, "heads up digitizing" in GEP was used to create discrete habitat polygons based on the reclassified habitats in the C-CAP analysis. Polygons created in GEP were stored as a KML/KMZ file, imported into ArcGIS 10.5 and converted into a feature class residing in a geodatabase so that areas of each habitat polygon could be calculated in square meters.

Upon importing the polygons from Google Earth, numerous topological errors were discovered in the polygons themselves, the most pervasive being knots, loops and slivers. These occur when "…the digitizer has an unsteady hand and moves the cursor or puck in such a way that the line being digitized ends up with extra vertices and/or nodes". Knots and loops result when a line forming a boundary of a polygon folds back on itself, creating small polygon like geometry known as "weird polygons".

Polygon features are enclosed areas created from a series of vertices that are connected with a continuous line traveling in one direction whereby the starting and ending point are coincident (Fig C). Because the depiction of the polygon begins with a start point and travels in one direction, the resulting geometry of the polygon means the GIS can interpret what area is 'right' as opposed to 'left' of the boundary, as well as what area is enclosed by the boundary of the entire polygon; when a knot or loop occurs, the topology of the polygon actually becomes confounded due to the extra node between them. As a result, right and left sides of the boundary violates the topological relationship of the polygon itself, preventing performance of common geoprocessing tasks (clip, intersect and dissolve).

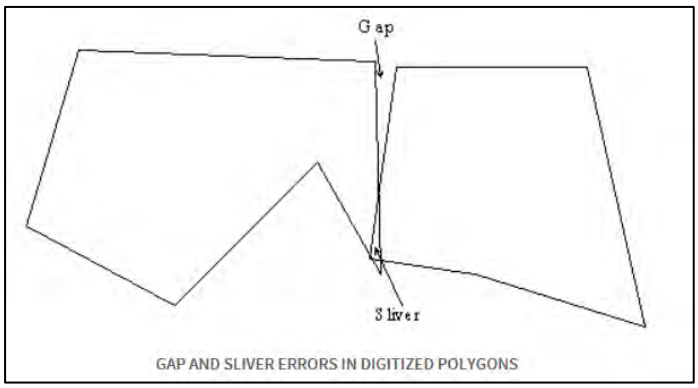
Figure C from https://www.gislounge.com/digitizing-errors-in-gis/



EXAMPLE OF A WEIRD POLYGON WHERE THE LINE FOLDS BACK ON ITSELF.

Another confounding topological error involves slivers. "Slivers are gaps in a digitized polygon layer where the adjoining polygons have gaps between them or where the two adjacent polygons overlap in error". This can inadvertently lead to areas among the polygons to have conflicting attributes as to what habitat the slivers represent (Fig D).

Figure D from https://www.gislounge.com/digitizing-errors-in-gis/



GAP AND SLIVER ERRORS IN DIGITIZED POLYGONS

Manual digitizing habitat polygons is time consuming and tedious. For this analysis, and to reduce anticipated issues related to slivers, it was decided early on in the digitizing process that the largest habitat within a circular zone could be left un-transcribed and the void filled utilizing geoprocessing tools in ArcGIS. Unanticipated topological inconsistencies related to knots and loops however prevented

these geoprocessing tools to be run and thus required that topology of all individual polygons to be inspected and corrected.

"Topology in GIS is generally defined as the spatial relationships between adjacent or neighboring features" Planar topology requires that intersections for lines and polygons in a digital data layer is enforced and that no two lines or polygons cross. This process involves removing twisted or self-intersecting polygons (i.e. knots and loops) so as to ensure that the "inside" of the polygon is on the correct side of the boundary. It also includes removing overlaps (i.e. slivers) found by intersecting each polygon with all other polygons.

Tools from ET Geowizards 11.3 were used to correct planar topology, rigorously testing and correcting for topological correctness and verifying the spatial relationships between neighboring polygons. Eight circular zone sites were chosen to validate the hand-drawn polygon designations and to determine if the process would improve calculations of polygon areas. Overestimation of the initial polygons varied by only 2.5% (n=51, t-test no significant difference in paired data p=.875)

Once the topology of the GEP dataset was reconciled, the "Intersect" geoprocessing tool was used to fill voids and assign a habitat code. The dataset was then "clipped" using an "island" polygon to remove those portions of the circular zone that extended past the coastline. Since there were multiple polygons representing a given habitat within a circular zone, the "dissolve" tool was used to consolidate records so that percent habitat calculations could be completed for each circular zone.

Total area of each habitat type for each 1 km hive site circular zone was summed and the percentage calculated (Table 2 in text). Each circular zone comprised approximately 314.16 hectares, unless ocean surface area was removed. A total of 18,872 hectares of land area was classified for the vector polygon dataset. Visual ground truthing was performed to ensure images in the GEP imagery matched images on the ground.

Comparing results between the C-CAP and GEP Datasets

C-CAP high-resolution land cover for 2010, produced at 2.4 m resolution, was applied to the 38 sites from the 2013 and 2015 sampling and compared to the same data grouped and processed using GEP polygons. For Agriculture and Urban land-cover categories, the two methods produced similar mean values, were not significantly different (t-test), and were well correlated (Table A). For Forest, Open and Water land-cover, the mean values were significantly different.

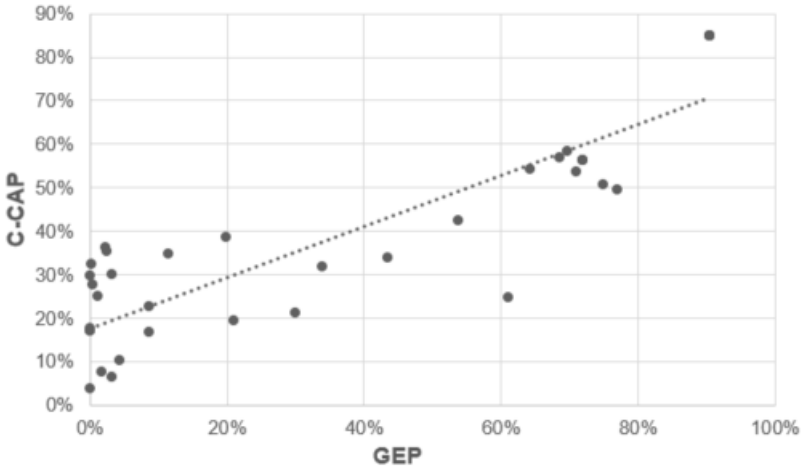
Table A: Glyphosate concentrations in honey matrix using either ELISA techniques or LC-GC-MS/MS techniques. Bold face numbers exceeded both techniques' LOQ and were plotted separately.

	C-CAP Mean	GEP Mean	t-test	correlation
% Ag	39.4%	36.9%	0.423	0.880
% Forest	36.4%	22.4%	0.000	0.836
% Open	12.4%	18.7%	0.078	0.584
% Urban	9.9%	9.6%	0.898	0.832
% Wetland	1.2%	1.2%	0.990	-0.034
% Water	1.0%	0.6%	0.041	0.532

The percentage coverage for Agriculture calculated with the C-CAP method was plotted versus the percentage coverage for Agriculture calculated with the GEP.

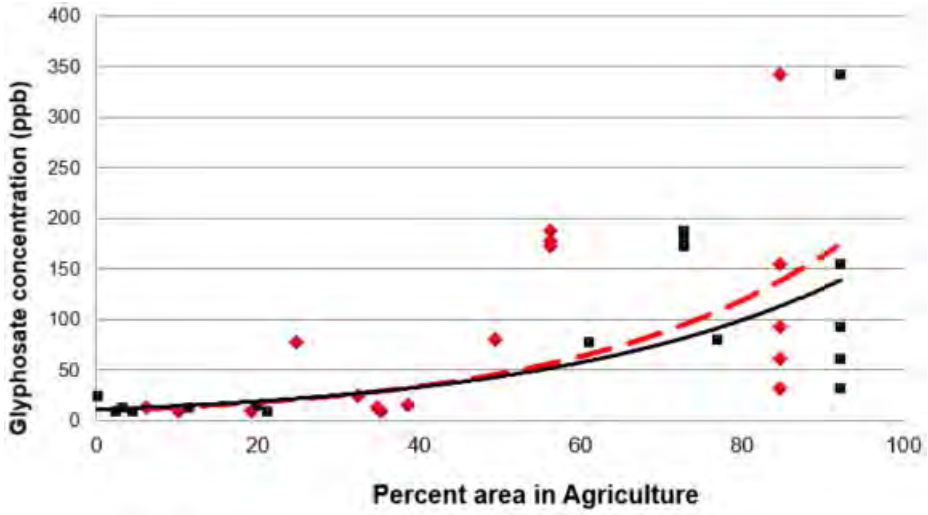
The plot illustrates the difference between GIS analyses of the two datasets and the general under-representation by C-CAP (Figure E).

Figure E: Correlation of % Agriculture in areas surrounding hive sites using C-CAP versus GEP analysis. Linear fit: Y = 0.586 x + 0.177, R^2 = 0.775.



When glyphosate concentrations are plotted against percent acreage in agriculture using the two methods (Fig F), the general trends as expressed by exponential curves are very similar, but the GEP polygon method produces a stronger correlation (R^2 = 0.71, AICc = -9.794).

Figure F: Correlation of % Agriculture and Glyphosate concentration surrounding hive sites using C-CAP and GEP analysis. Excel Analyse It software exponential fits produced Y = 9.648 $e^{0.23121x}$, R^2 = 0.48, AICc = 0.173 (C-CAP, red diamonds; dash line) and Y = 11.02 $e^{0.1628x}$, R^2 = 0.71, AICc = -9.794 (GEP Polygons, black squares; solid line).



There are many factors that would explain the differences in the land use designation and the choice of GEP polygons as the most accurate method for determining land use contemporary with honey production. These include:

- Cell size is 2.4 m for C-CAP vs Digital Globe has a 30-50 cm range. A smaller cell size allows for finer delineation and identification of objects.
- Date the image was accessed: 2010 for C-CAP but 2013-2014 for GEP with ground-truthing in areas in question.
- C-CAP would designate a ground cover as forest, but GEP showed it to be an orchard.
- C-CAP would identify open fields as "Open", but GEP showed that cattle are on it, so it is “Agriculture”.
- C-CAP does not recognize little streams or ponds but GEP resolution does.
- C-CAP sees "Forest" but Google Earth shows "Riparian"
- C-CAP see “Urban” but finer detail allows designation as “Rural/Suburban”

Conclusion

Although manually digitizing GEP polygon delineations is more tedious and time consuming, for the above stated reasons and the stronger correlation of the GEP derived curve, only the GEP polygon delineation method was used for final analysis of the relationship between land use and glyphosate concentration

S4 Appendix. Glyphosate data from Kauai hives and store-bought honey

Table A: Store-bought honey; sources and glyphosate concentration.

Sample Origin				Sample #	Glyphosate ppb
Hawaii	**Island:**	**Moku**	**Area**		
	Kauai	Kona	Waimea Valley	5	15.2
		Kona	Koloa	9	0
		Kona	Kalaheo	11	87
		Kona	Poipu	19	27.2
		Koolau	Waipake	3	5
		Koolau	North/Northeast Kauai	4	6.4
		Koolau	North Shore Kauai	6	60.8
		Koolau	Kilauea	8	0
		Koolau	Kilauea	12	11.2
		Puna	Puhi	1	15
		Puna	Hanamaulu	2	6.2
		Puna	Kapa'a	7	0
		Puna	Kapa'a	10	7
		Puna	Puhi	20	10.4
		Puna	Hanamaulu	21	6.4
	Hawaii Island		Hawaii Island	15	12
			Kealakekua, Big Island	16	7.4
			Kealakekua, Hawaii Island	60	16.4
			Big Island and Oahu	18	8
	Molokai		Molokai	61	0
			Molokai	62	0
Country		Product of Brazil and Canada		17	0
		Product of Brazil and Canada		22	30.6
		Product of Brazil and Canada		14	8.2
		Product of Mexico, Brazil and Uruguay		13	0
		Product of USA and Argentina		23	72.4

Table B: Kauai hive samples categorized by side of island and Moku with glyphosate concentration.

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
WINDWARD							
	Halele'a	2	0				
		10	9.2				
		12	15.2				
		28	12.6				
		31	0				
		32	0				
		44	8.2				
		45	0				

Side of island	Moku	Sample #	Glyphosate ppb	Count	Median	Mean	SD
		51	0	9	0	5.0	6.3
	Ko'olau	3	0				
		11	0				
		24	0				
		26	0				
		29	0				
		30	0				
		33	0				
		42	0				
		43	0				
		50	0	10	0	0	0
	Puna	1	13.6				
		4	0				
		5	8.8				
		7	0				
		9	0				
		13	0				
		16	0				
		23	0				
		25	0	9	0	2.5	5.1
LEEWARD							
	Kona	6	80.2				
		8	61.4				
		14	341.6				
		15	0				
		17	0				
		18	24.6				
		19	9.6				
		20	155.2				
		21	32.6				
		22	0				
		27	0				
		34	187.2				
		35	178				
		36	171.8				
		37	92.2				
		38	77.6				
		39	0				
		40	10.4				
		41	60				
		46	13				
		47	0				
		49	0				
		52	0				
		53	0				
		54	0				
		55	0				
		56	0				
		57	27.4				
		58	0				
		59	95	30	11.7	53.9	80.9
	Mana	48	292.2	1	292.2	292.2	na
	Napali	None	None	None			

Table C: Summary statistics of glyphosate with Kauai hive samples categorized by side of island.

Windward	Count	28
	Median	0
	Mean	2.41
	SD	4.87
Leeward	Count	31
	Median	13
	Mean	61.61
	SD	90.34

Table D: t-test comparing glyphosate from Windward (Eastern) and Leeward (Western) sides of Kauai. Data from Table B1.

Windward-Leeward:	
t-test probability	0.001
degrees of freedom	57

Table E: t-test comparing glyphosate between Moku pairs. Mana Moku had only one sample, thus could not be compared.

Moku differences	**t-test p**
Kona -Koolau	0.001
Kona - Puna	0.002
Kona - Halelea	0.003
Koolau -Halelea	0.043
Puna -Koolau	0.180
Puna - Halelea	0.361

Table F: Kruskal-Wallis analysis of impact of side of island and Moku on glyphosate concentration.

Y (numerical)	**X (categories)**	**H-stat**	**DF**	**N**	**p-value**
Glyphosate	Side	11.3	1	58	0.00077
Glyphosate	Moku	13.3	3	58	0.0041

Table G: AICc analysis of fits for glyphosate concentration vs. % Agriculture.

	Exp.	**Power**	**Linear**	**Log**	**Polynomial**
R2	0.594	0.174	0.417	0.155	0.429
AICc	-8.664	7.662	194.88	195.232	197.055

Table H: Sample #'s included within Meta-circles and their glyphosate concentrations.

Meta-circle #	**Meta-circle Name**	**Sample #**	**Glyphosate ppb**	**Glyphosate ppb Mean**
1	Kilauea	10	9	
		32	0	
		33	0	
		43	0	
		44	8	3.5
2	Moloaa	11	0	
		24	0	
		26	0	
		30	0	
		42	0	
		50	0	0.0

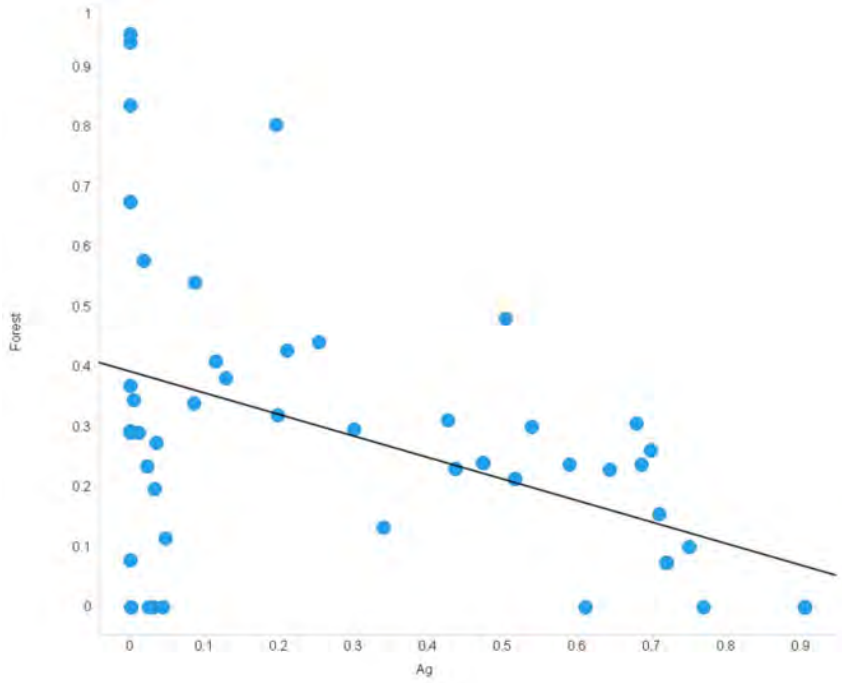
Meta-circle #	Meta-circle Name	Sample #	Glyphosate ppb	Glyphosate ppb Mean
3	Kapaa	7	0	
		9	0	
		23	0	
		25	0	0.0
4	Lihue	1	14	
		4	0	
		5	9	
		16	0	5.6
5	Koloa	15	0	
		18	25	
		52	0	
		53	0	
		54	0	
		55	0	
		56	0	
		57	27	
		59	95	16.3
6	Lawai	27	0	
		39	0	
		40	10	
		46	13	
		49	0	4.7
7	Agribusiness 1	34	187	
		35	178	
		36	172	179.0
8	Agribusiness 2	8	61	
		14	342	
		20	155	
		21	33	
		37	92	136.6

Table I: Samples by Side and Moku with % Agriculture, % Golf, Hiway Km, and Glyphosate concentrations.

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
1	East	Puna	13.6	3.1%	4.8%	2.00
2	East	Halelea	0	0.0%	0.0%	2.39
3	East	Koolau	0	70.9%	0.0%	1.59
4	East	Puna	0	30.0%	0.0%	2.02
5	East	Puna	8.8	21.0%	0.0%	1.74
6	West	Kona	80.2	76.9%	0.0%	2.03
7	East	Puna	0	3.2%	0.0%	0.00
8	West	Kona	61.4	90.5%	0.0%	1.65
9	East	Puna	0	1.1%	0.0%	0.00
10	East	Halelea	9.2	4.4%	0.0%	2.04
11	East	Koolau	0	69.7%	0.0%	0.00
12	East	Halelea	15.2	19.8%	0.0%	0.00
13	East	Puna	0	8.6%	0.0%	0.00
14	West	Kona	341.6	90.5%	0.0%	1.65
15	West	Kona	0	43.6%	0.0%	4.53
16	East	Puna	0	53.8%	0.0%	0.00
17	West	Kona	0	0.0%	0.0%	0.00
18	West	Kona	24.6	0.1%	16.2%	4.66
19	West	Kona	9.6	2.4%	1.6%	3.36
20	West	Kona	155.2	90.5%	0.0%	1.65
21	West	Kona	32.6	90.5%	0.0%	1.65
22	West	Kona	0	33.9%	0.0%	1.44
23	East	Puna	0	0.4%	0.0%	0.00
24	East	Koolau	0	64.3%	0.0%	1.66
25	East	Puna	0	2.3%	0.0%	0.00
26	East	Koolau	0	68.6%	0.0%	1.10
27	West	Kona	0	0.0%	0.0%	2.29
28	East	Halelea	12.6	11.5%	13.7%	2.04
29	East	Koolau	0	75.0%	0.0%	2.03
30	East	Koolau	0	8.7%	0.0%	2.06
31	East	Halelea	0	0.0%	0.0%	2.36
32	East	Halelea	0	1.8%	0.0%	2.63
33	East	Koolau	0	0.0%	0.0%	1.98
34	West	Kona	187.2	71.9%	1.2%	1.08
35	West	Kona	178	71.9%	1.2%	1.08
36	West	Kona	171.8	71.9%	1.2%	1.08
37	West	Kona	92.2	90.5%	0.0%	1.65
38	West	Kona	77.6	61.0%	0.0%	2.18
39	West	Kona	0	3.5%	0.0%	0.32
40	West	Kona	10.4	12.9%	0.0%	0.52
41	West	Kona	60	58.9%	0.0%	0.00
42	East	Koolau	0	67.9%	0.0%	0.00
43	East	Koolau	0	4.7%	0.0%	1.51
44	East	Halelea	8.2	25.3%	0.0%	0.00
45	East	Halelea	0	0.0%	0.0%	0.00
46	West	Kona	13	0.0%	0.0%	1.20
47	West	Kona	0	19.5%	0.0%	0.00
48	West	Mana	292.2	16.3%	0.0%	0.00
49	West	Kona	0	0.0%	0.0%	2.10
50	East	Koolau	0	50.4%	0.0%	2.26
51	East	Halelea	0	0.0%	0.0%	2.25
52	West	Kona	0	51.6%	0.0%	4.73
53	West	Kona	0	51.6%	0.0%	4.73
54	West	Kona	0	47.3%	0.0%	4.35

Sample #	Side	Moku	Glyphosate ppb	% Agriculture	% Golf	Hiway Km
55	West	Kona	0	42.6%	0.0%	4.58
56	West	Kona	0	47.3%	0.0%	4.35
57	West	Kona	27.4	43.6%	0.0%	4.60
58	West	Kona	0	47.3%	0.0%	4.35
59	West	Kona	95	0.2%	16.2%	4.66

Fig A: Multicollinearity amongst land use types. Samples are plotted with their % Forest vs % Agriculture. $Y = 0.39 - 0.36*X$, $R^2 = 0.23$



1. Information on the study

Data point	KCA 6.10.1
Report author	Chiesa L.M. et al.
Report year	2019
Report title	Detection of glyphosate and its metabolites in food of animal origin based on ion-chromatography-high resolution mass spectrometry (IC-HRMS)
Document No.	Food Additives & Contaminants: Part A, 2019, Vol. 36, No. 4, 592-600
Guidelines followed in study	SANTE/11813/2017
Deviations from current test guideline	None
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

Glyphosate and glufosinate are broad spectrum herbicides, widely used in agriculture and in inhabited or industrialised areas, and aminomethylphosphonic acid is a degradation product of glyphosate. In 2015, the International Agency for Research on Cancer reported that glyphosate is a probable carcinogenic. In 2017, however, a scientific opinion of the European Chemicals Agency concluded that glyphosate is not proven to be carcinogenic, mutagenic or to have negative effects on reproduction. Nevertheless, aminomethylphosphonic acid was not considered. Due to their chemical-physical characteristics, these molecules present difficulties that have not yet allowed routine monitoring to be carried out. For these reasons, we developed and validated a simple and versatile liquid extraction, before IC-HRMS analysis, of three different complex matrices: honey, bass fish and bovine muscle. Among the satisfactory validation parameters, the LOQs in the range of 4.30 – 9.26 ng/g demonstrated high method sensitivity, compared to the few works present in literature. Finally, the method was applied to real commercial samples, which showed no traces of the selected pesticides.

Materials and Methods

Chemicals and reagents

Glyphosate, glufosinate ammonium, aminomethyl-phosphonic acid (AMPA) and the internal standard Glyphosate-2-^{13}C,^{15}N were purchased from Merck (Sigma–Aldrich, Merck KGaA, Darmstadt, Germany). All solvents used were of LC-MS or analytical grade. Formic acid (98–100%) was obtained from Riedel-de Haën (Sigma–Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions

Stock standard solutions (1 mg/mL) for each standard were prepared in water and kept at −20°C. Working solutions containing each of the studied analytes at a concentration of 100 ng/mL were prepared daily in methanol containing 1% of formic acid, as suggested by EU Reference Laboratory for pesticides (Anastassiades et al. 2016). Each working solution was maintained at 4°C during the method validation procedures. Plastic flasks and stoppers were used due to the fact that these pesticides tend to interact with glass surfaces.

Sample collection

Three different food matrices of animal origin were selected for the method validation: honey, fish (bass) and bovine muscle. Five commercial samples of each matrix were homogenized to create a pool to be used for the validation. After homogenization, the samples were stored at −20° C until analyses.

Ten real Italian commercial samples each of organic honey, bass and bovine muscle were also collected from different supermarkets of Milan for the application of the method.

Sample extraction

The extraction procedure was very simple and identical for the three different matrices. Homogenized samples (1 ± 0.05 g) of honey, or minced fish or bovine muscle were weighed into 15 mL polypropylene centrifuge tubes. Samples were spiked with the internal standard: 0.2 µg/g for honey and 0.4 µg/g for fish and bovine muscle samples. Three mL of methanol was added followed by 7 mL of acidified deionised water (1% formic acid). The samples were mixed for 1 min using a vortex and then sonicated for 15 min. After centrifugation (2500 × g, 4°C for 10 min), 1 mL of the supernatant was filtered through a mixed cellulose syringe filter (0.45 µm) directly into a plastic 2 mL vial, ready for determination by IC-MS/MS.

IC-HRMS orbitrap analyses

The analyses were performed by an Ionic Chromatography (IC) Dionex ICS-5000+ system (Sunnyvale, CA, USA) made up of Dual Pump (DP), a Conductivity Detector (EG), a Detector/Chromatography Module (DC) and an Autosampler (AS-AP). The ion chromatography separation column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm, 4 µm particle size) maintained at 30°C. The eluent flow rate was 0.30 mL/min with a gradient from 15 mM KOH (aq), held for 8 min, increased to 55 mM KOH (aq) at 20 min, held in these conditions for 4 min and back to 15 mM KOH (aq) at 24.1 min, with a cycle time of 30 min. The KOH eluent was neutralized using a Dionex AERS 500, 2 mm electrolytically regenerated suppressor (Thermo Scientific). The injection volume was 50 µL.

The detector was a Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330°C and 280°C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas were set at 35 and 15 arbitrary units, with S lens RF level of 60.

Instrument calibration was done every analytical session with a direct infusion of an LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan acquisition (FS) was combined with an Independent Data Acquisition mode (DIA), providing the MS2 spectra for confirmatory response, based on an inclusion list. The resolving power of FS was set at 70,000 Full Width at Half Maximum (FWHM). On the basis of our compound list, a scan range of m/z 50–250 was chosen; the automatic gain control (AGC) was set at 1×10^{-6} and the maximum injection time was 100 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5×10^{-4}, with an auto maximum injection time. The precursor ions are filtered by the quadrupole which operates at an isolation window of 1 m/z. Fragmentation of precursors was optimised as three-stepped normalized collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was based on the retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment. The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm glyphosate and its metabolites are reported in **Table 1**. Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HRMS system, determine the exact mass of the compounds, record and elaborate data.

Table 1: Main information (formula, retention time (tr), precursors, main products and polarity) about AMPA, glyphosate, glufosinate and the relative internal standard (IS) analysed by IC-HRMS.

Compound IC-HRMS	Formula	t_r (min)	Precursor (m/z)	Main products (m/z)			Polarity
AMPA	CH6NO3P	14.25	110.00125	62.96417	78.95904	80.97468	(-)
Glyphosate	C3H8NO5P	23.87	168.00673	62.96417	124.01687	149.99612	(-)
Glufosinate	C5H12NO4P	14.14	180.04312	85.02955	94.99042	136.05329	(-)
IS: Glyphosate-2-13C, 15N	[13]C2C[15]NH8O5P	23.85	171.01048	62.96415	80.97471	152.99988	(-)

Validation parameters

Validation was carried out following the European Commission (2017) SANTE/2017 Guidance document on method validation & quality control procedures for pesticide residues analysis in food & feed. The selectivity of the method was evaluated by injecting extracted blank honey, fish and bovine muscle samples. The absence of signal above a signal-to-noise ratio of 3 at the expected retention times of the target compounds was the parameter used to show the absence of interferences.

The matrix-matched calibration curves were obtained by spiking 1 g of the three different matrices with an appropriate volume of the standard working solution to cover the concentration range from 5 to 100 ng/g (five calibration points: 5, 10, 20, 50 and 100 ng/g). The limit of quantification (LOQ) of the methods was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤ 20% (European Commission. 2017. SANTE/11813/2017). The repeatability, evaluated as a coefficient of variation, CV %, was calculated by analysing six replicates at two fortification level (10 and 50 ng/g). Recoveries were calculated by comparing the concentrations of the extracted compounds, spiked before extraction, with those spiked at the end of the extraction procedure at two fortification level (10 and 50 ng/g) for all compounds. The matrix effect was also evaluated using the Matuszewski *et al.* (2003) approach by comparing the corresponding peak areas for standards, spiked after extraction into the extracts, to the peak areas obtained in neat solution standards, expressed as percentage.

Results and Discussion

Extraction procedure

During the preliminary phase, the QuPPe extraction method proposed by EU Reference Laboratories for Residues of pesticides (Anastassiades *et al.*, 2016) was followed, with good results for glyphosate and AMPA but found not suitable for glufosinate in our matrices after the IC-HRMS analysis. In particular, we observed a different and opposite extraction and chromatographic behaviour of the molecules (in particular for AMPA and glufosinate) on the basis of the different solvents used and injected during the ion chromatography separation. Moreover, the final dilution 1/10 suggested by the Anastassiades *et al.* (2016) or by others (Adams *et al.*, 2017) did not improve chromatographic problems when AMPA or glufosinate was hardly detectable.

So we decided to modify the method starting from a smaller amount of matrix (1 g instead of 5 g) to decrease interferences, investigating also the influences of the different tested extraction solvents compatible with our instrumentation: water, methanol and the related acidified solutions with 1% of formic acid. In particular, using only water (**Figure 1a**) or only methanol (**Figure 1b**) as extraction solvent we had poor results for AMPA, but very satisfactory chromatographic peaks for glyphosate and glufosinate. By using 1% of formic acid in water (**Figure 1c**) we had an improvement of AMPA signal, but it was not yet satisfactory, while with 1% of formic acid in methanol (**Figure 1d**) we observed the reverse situation, good chromatography for AMPA but not for glufosinate, which eluted with a too-jagged and wide peak. So after different trials, changing the percentage of formic acid and the composition of the methanol and acidified water mixture we reached the best compromise with 30% of methanol and 70% of acidified (1% formic acid) water as extraction solution. **Figure 2** reports the extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes after method optimisation.

Figure 1: Extracted parent ion chromatograms from full-scan IC-HRMS analysis of AMPA, glyphosate and glufosinate and influences of the different tested extraction solvents: water (a), methanol (b), 1% formic acid in water (c) and 1% formic acid in methanol (d).

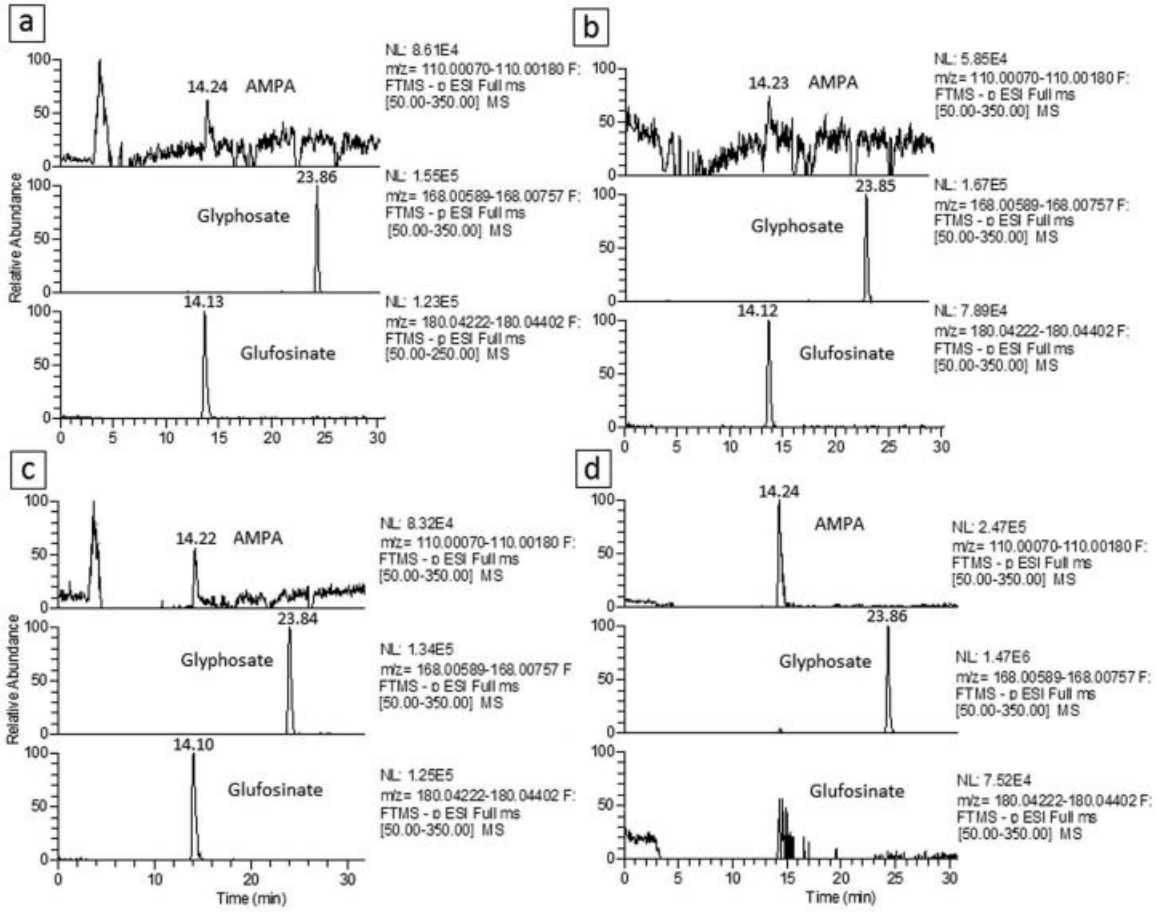
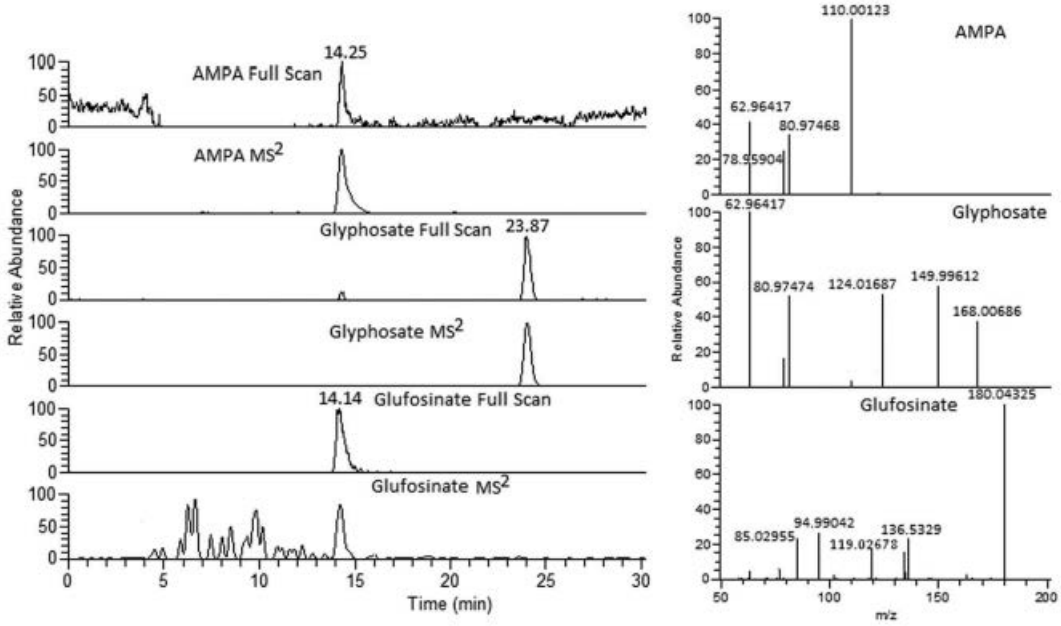


Figure 2: Extracted parent ion chromatograms from full-scan IC-HRMS analysis and from data-independent acquisition mode with the relative fragmentation mass spectra of the three selected analytes (concentration of 10 ng/g) after method optimisation.



IC-HRMS validation parameters

All instrument validation parameters are reported in **Table 2**. The method applied to the three different matrices (honey, bass fish, bovine muscle) showed high specificity, without any interference close to the retention time where the investigated compounds were expected to elute. The good selectivity of the method was demonstrated with a S/N ratio higher than 3 in presence of analytes at the lowest detectable concentrations. All identification criteria passed including retention time stability compared to the standard solution. The mean recoveries ranged between 75 and 112%, indicating the efficiency of the extraction protocol. Matrix validation curves demonstrated a good linearity over the working range with a good fit ($R^2 > 0.99$) for all compounds. Repeatability was calculated using one-way analysis of variance (ANOVA); the CVs were substantially lower than 20%, satisfying the criteria required by the European Commission (2017).

Table 2: Validation parameters about glyphosate, glufosinate and AMPA in the three different matrices analysed by IC-HRMS.

	LOQ (ng g^{-1})	Matrix effects %	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Matrix calibration curve	Linearity R^2
HONEY						
AMPA	9.26	84	11, 4	91, 95	y = 0.15747x-0.514838	0.9996
Glyphosate	4.30	94	7, 7	99, 100	y = 0.295172x+0.858303	0.9975
Glufosinate	5.05	84	13, 12	100,101	y = 0.442863x+0.00858678	0.9957
FISH MUSCLE						
AMPA	5.38	95	4, 2	96, 95	y = 0.0897754x-0.231747	0.9951
Glyphosate	5.08	93	8, 8	80, 93	y = 0.072573x+0.112081	0.9985
Glufosinate	4.36	96	7, 6	112, 96	y = 0.127407x-0.176602	0.9976
BOVINE MUSCLE						
AMPA	6.44	99	12, 9	75, 79	y = 0.091067x-0.113588	0.9925
Glyphosate	6.47	107	13, 10	75, 80	y = 0.063115x+0.0920263	0.9922
Glufosinate	6.25	106	5, 2	76, 80	y = 0.120407x-0.107902	0.9962

*The two concentration levels were 10 and 50 ng g^{-1}.

Regarding the LOQs in the range from 4.30 to 9.26 ng/g, our satisfactory results showed high method sensitivity for glyphosate and its metabolites, when compared to the few reports present in the literature. In fact, Picò *et al.* (2007) reported LOQ of 0.05 mg/kg for glyphosate and AMPA in plant products, such as rice, wheat, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey, etc. using High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; Krüger *et al.* (2014) reported validation parameters spiking at 100 µg/g of glyphosate in animal and human residues through ELISA followed by GC-MS/MS analysis. The overview of approximate LOQs reported by Anastassiades *et al.* (2016) in the range of 0.01–0.02 mg/kg obtained by the QuPPE extraction followed by LC-MS/MS analysis or those of Chamkasem *et al.* (2016) in the range of 4–26 ng/g using LC-MS/MS system are a little higher than our results. In **Table 3** we report all the LOQs and other information on the different studies presented in the literature about glyphosate, AMPA and glufosinate in food of animal origin. Matrix effects were modest in the three different matrices with a percentage variation lower than the 20% (from 84% to 107%) recommended by the European Commission (2017).

Based on our results the use of high-resolution mass spectrometry and hyphenation with ion chromatography has been demonstrated to be very effective for the analysis of these challenging analytes in very complex matrices of animal origin. Particularly, as stated by Rajski *et al.* (2018) in their analogous study on anionic pesticides in fruits and vegetables, the high ion-exchange capacity, the efficiency, the diameter reductions and the characteristic chemistry of bonded functional groups of IC columns are a major factor for the separation and identification of the highly polar pesticides, scarcely retained in reversed-phase LC, avoiding moreover any derivatisation step. The high-resolution mass spectrometry allowed obtaining low background matrix signals, improving the sensitivity in terms of LODs and efficient trapping and stability of low m/z ions, improving selectivity. The high MS resolving power and mass accuracy down to 1 ppm, combined with the rapid scan speed, also provide high specificity (Chiesa *et al.* 2018). The possibility to do retrospective analyses is an added value.

Table 3: Literature about glyphosate in products of animal origin.

Authors	Matrix	Analyte	LOQ	Analytical Instrumentation
Wang, Jaw, and Chen	Fish	Glyphosate	/	Beckman LS 1000C liquid scintillation counter.
Alferness and Iwata (1994)	Muscle, kidney, liver and fat beef, eggs and milk	Glyphosate, AMPA	0.01 μg g^{-1}	Capillary Gas Chromatography with Mass-Selective Detection
Picò et al. (2007)	Plant products, vegetables, fruits and tea, pig and chicken muscles, aquatic products, chestnut, honey	Glyphosate, AMPA	0.05 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Bo et al. (2007)	Vegetables, fruits, cereals, pig and chicken muscles, aquatic products, honey	Glyphosate, AMPA	0.05 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Krüger et al. (2014)	Liver, kidney, lung, spleen, muscle and intestine of cow	Glyphosate	100 μg g^{-1}	ELISA- Gas Chromatography-Mass Spectroscopy
Chamkasem et al. (2016)	Milk	Glyphosate, Glufosinate, and AMPA	100 μg g^{-1}	Liquid chromatography/ tandem mass spectrometry
Anastassiades et al. (2016)	Foods of plant origin, cereals and honey	Glyphosate, Glufosinate, AMPA and others	4–26 ng g^{-1}	Liquid chromatography/ tandem mass spectrometry
Liao et al. (2018)	Milk-based baby foods and other milk products, infant formulae, yogurt	Glyphosate, Glufosinate	/	Liquid chromatography/ tandem mass spectrometry
Chamkasem et al. (2018)	Honey	Glyphosate, Glufosinate, and AMPA	0.25 μg mL^{-1}	Liquid chromatography/ tandem mass spectrometer

Application to real commercial samples

Finally, we applied the proposed method for the analysis of 30 real samples: 10 organic honeys, 10 beef muscle pools and 10 sea bass muscle pools, each thoroughly homogenised. All the samples were of Italian origin, taken from different supermarkets of Milan. None of the selected samples showed any traces of glyphosate or metabolites, ensuring the good quality of the samples, especially when it comes to organic products such as honey, demonstrating the absence of pesticide contamination both of the sample and of the production area.

Conclusions

In this study, we developed and validated a new and versatile IC-HRMS method for the detection of glyphosate, AMPA and glufosinate in three complex different matrices, honey, bass fish and bovine muscle. These results are of great importance and topical in the field of food safety because of the scarce data regarding this topic, the extractive and analytical difficulties related to these analytes in relation to complex matrices, and the legislative situation not yet outlined on the use of glyphosate and residues in consumer products. The application of the method to real commercial samples did not show any traces of the pesticides. Further studies of the method's application and statistical evaluation are necessary to form a more complete view on this matter.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The purpose of the publication is to describe and discuss the performance of a residue analytical method for glyphosate, AMPA and glufosinate in, food of animal origin. As such, the publication is not relevant to risk assessment. However, since it also reports residue levels for the investigated compounds in 10 honey samples and since according to SANTE/11956/2016 rev. 9 it is possible to derive EU MRLs in honey based on monitoring data, the publication may be considered relevant to risk assessment and MRL setting. Based on the provided validation results, the method is considered reliable. The LOQ (defined as the lowest fortification level yielding acceptable recoveries) was 0.010 mg/kg for both glyphosate and AMPA (although different values, presumably estimated from the signal to noise ratio, are stated in Table 2). None of the 10 analysed honey samples showed residues of glyphosate or AMPA above the LOQ. However, it is important to note that all the samples were from organic production and this may need to be taken into account in the evaluation.

1. Information on the study

Data point	CA 6.10.1
Report author	El Agrebi N. et al.
Report year	2020
Report title	Honeybee and consumer's exposure and risk characterisation to glyphosate-based herbicide (GBH) and its degradation product (AMPA): Residues in beebread, wax, and honey
Document No.	Science of the Total Environment 704 (2020) 135312
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

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

Materials and Methods

Study areas

Three different bee matrices were sampled for the analysis of GBH residues and AMPA: (i) beebread (N = 179), (ii) wax from the brood chamber (N = 100) and additionally (iii) a combination of wax from the honey super and corresponding extracted honey (N = 10). We used 379 non-professional apiary sites located in Belgium, including 2,997 colonies of *Apis mellifera*. For beebread and wax sampling, apiaries were selected (193 for beebread, 186 for wax and honey) from the Federal Agency for the Safety of the Food Chain (FASFC) apiaries database that included 4,949 registered beekeepers in 2015. The apiaries were stratified by province (N = 20/province and 10 provinces in Belgium) and randomly distributed in Flanders (northern Belgium) and Wallonia (southern Belgium). All sampled bee colonies seemed healthy, with no clinical signs of infectious diseases or acute intoxication (Ravoet

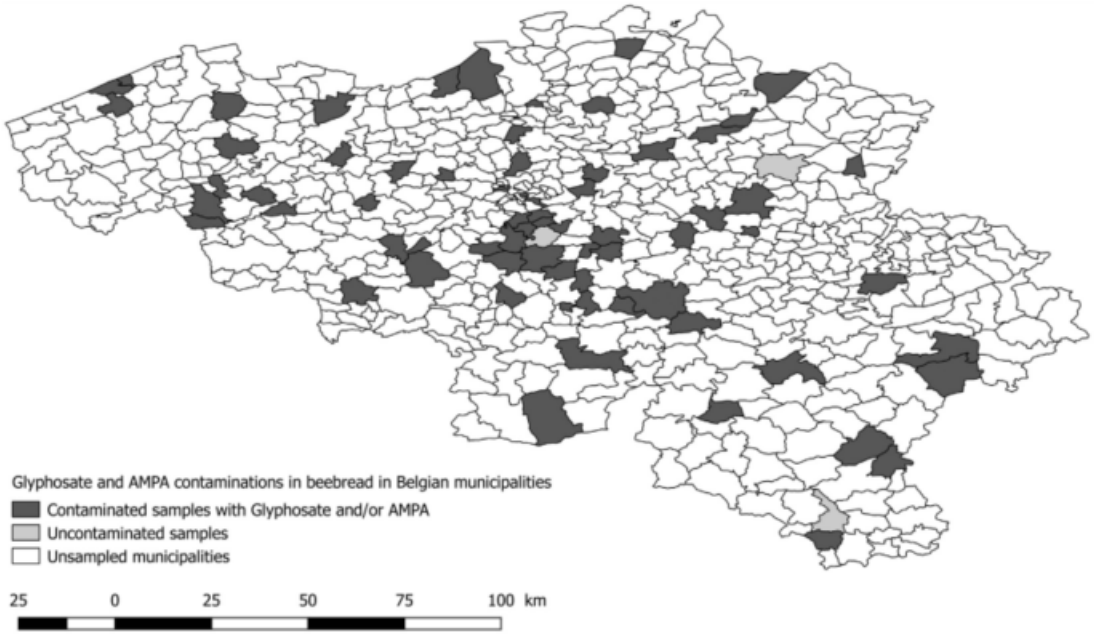
et al., 2015). Quantum GIS (QGIS Development Team, 2009; http://qgis.osgeo.org) was used to create the maps in **Figure 1** and **Figure 2**.

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

Beebread collection

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

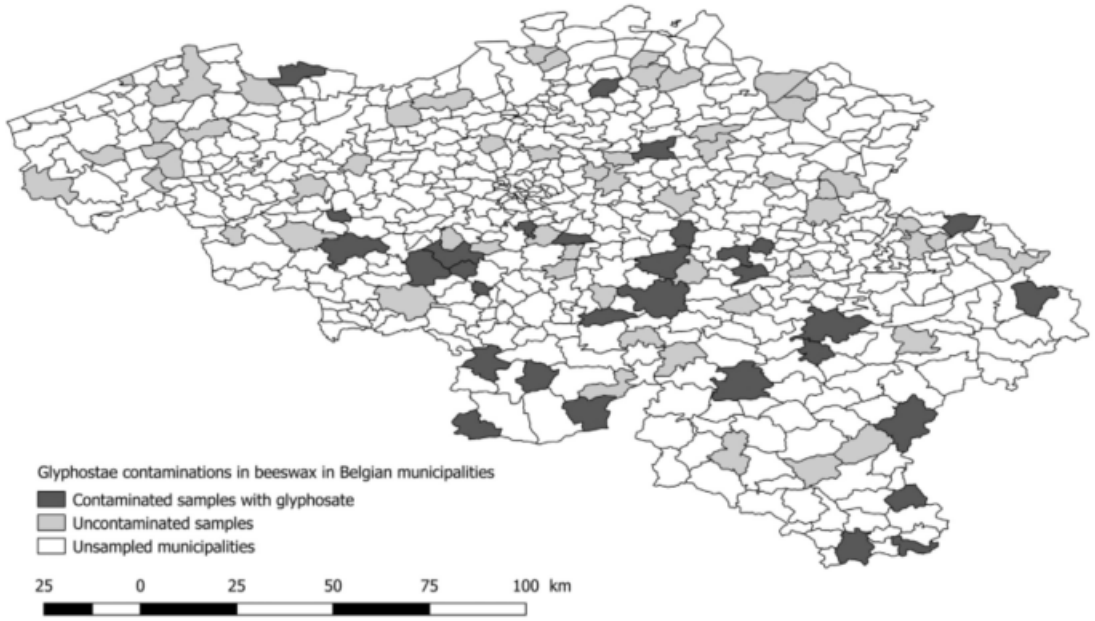
Figure 1: Glyphosate residues and AMPA contaminations in beebread across Belgium, in 2016.



Beebread extraction

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

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



Wax collection

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

Honey/wax sampling

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

Glyphosate-based herbicide residues and AMPA detection

The GBH residues and AMPA analyses were carried out between May and June 2017 (September 2017 for the 10-paired samples of wax/honey) by the Phytocontrol laboratory (France) ISO 17,025 accredited under the number No 1–1904 for the analysis of bee products by the French competent authority. The analysis method used for the targeted matrices (beebread, beeswax, and honey) was a high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS-MS). The analytes were extracted using an aqueous solution followed by a simple clean up with a C18 solid-phase extraction (SPE) cartridge, and then glyphosate and AMPA were derivatised using 9-fluorenyl-methoxycarbonyl (FMOC-Cl) in borate buffer. For beeswax, an additional hexane treatment was used in order to defat the extract. The derivatives of glyphosate and AMPA were separated on a C18 column (105 x 4.6 mm; 5 µm) with gradient elution with the mobile phase of acetonitrile and 5 mmol/L ammonium acetate (pH 9), and finally detected with negative ion

electrospray ionization-mass spectrometry (ESI-MS) in multiple reaction monitoring (MRM) mode (drying gas flow at 15 mL/min, nebulizing gas flow at 3 L/min). Limits of quantification (LOQ) for both glyphosate and AMPA in the 3 matrices were 10 ng/g, while limits of detection (LOD) were 1 ng/g. Matrix effects were compensated by the addition of ^{13}C labeled glyphosate (used as internal standard) to the sample prior extraction, as well as in spiked samples used to set up the calibration curve. Three levels of spiking, including the LOQ, were performed on several matrices of different categories, which were analysed in condition of repeatability and intermediate fidelity. The mean spiked recoveries of glyphosate and AMPA at 3 spiked levels ranged from 72.2% to 112.9% with the relative standard deviations (RSD, n = 5) of 0.1% – 4.5%. The tolerance interval was plotted with a beta probability of 80%, which represents the proportion of future values that the routine method will produce over the entire field of application. This allows to ensure that the molecule of glyphosate is extracted correctly and to correct any matrix effects.

Exposure assessment and risk characterisation to honeybee health

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

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

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

Risk to consumer's health

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

Then, we assessed the risk posed by GBH residues and AMPA in beebread and beeswax to consumer's health through the assessment of the consumer exposure to these compounds through pollen and beeswax consumption. Thus, we assumed that beebread contamination levels correspond to pollen contamination levels. To estimate the pollen and beeswax consumption, we used published consumption data. According to EFSA (EFSA, 2007), the 95th percentile of the daily consumption of beeswax corresponds to 1.29 g/person, which is 0.022 g/kg b.w. for a 60 kg individual. Concerning the daily consumption of pollen, the highest 95th percentile value recorded in the EFSA Comprehensive

European Food Consumption Database (EFSA, 2018) corresponds to 69.55 g/person, that is 1.35 g/kg b.w. for a 52 kg individual, in France (according to the second version of the FoodEx food classification system). Then, as a worst-case scenario, we multiplied these high levels of consumption with the highest GBH residues and AMPA concentrations. Finally, we compared the exposure levels with the reference toxicological values of these compounds (above mentioned) to characterise the risk.

Statistical analysis

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

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

A logistic regression (odds ratio's (OR) with 95% confidence intervals (95% CI)) was used to test a possible risk factor of GBH residues detection in beeswax and regions (Stata SE 14.1®, Stata-Corp LP, College Station, TX, USA). For all tests, a level of significance of 5% was used and divided, if needed, by the number of comparisons performed for the Bonferroni correction.

Results

Glyphosate-based herbicide residues and AMPA in beebread

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

Table 1: Glyphosate and AMPA detection, residue levels and hazard quotient to bees in beebread, beeswax and honey samples in Flanders (North Belgium), Wallonia (South Belgium) and Belgium.

Matrix	Region	Sampling period	Nb. analysed samples	Nb. samples > LOQ	Nb. samples < LOQ	Nb. samples detected	% samples > LOQ	% samples < LOQ	% samples detected	Multi-test for detection	Average [] ng g^{-1}	S.D. [] ng g^{-1}	Multi-test for []	Max [] ng g^{-1}	Median [] ng g^{-1}	Max HQ
Beebread	**GBH**															
	Flanders	Fall 2016	39	34	3	37	87.2%	7.7%	94.9%	aa	58.44	133.28	aa	700	23	7
	Wallonia		42	32	5	37	76.2%	11.9%	88.1%	aa	52.41	39.70	aa	160	49.5	1.6
	Belgium		81	66	8	74	81.5%	9.9%	91.4%	aa	55.52	98.89	aa	700	26	7
	AMPA															
	Flanders	Fall 2016	39	5	3	8	12.8%	7.69%	20.5%	a-	39.8	25.16	a-	77	38	0.8
	Wallonia		42	10	3	13	23.8%	7.14%	30.9%	a-	80.8	78.09	a-	250	58.5	2.5
	Belgium		81	15	6	21	18.5%	7.4%	25.9%	a-	67.13	67.09	a-	250	44	2.5
	GBH															
Beeswax	Flanders	Spring	48	3	1	4	6.3%	2.08%	8.3%	ab	28.33	22.90	aa	54	21	0.5
	Wallonia	2016	52	23	5	28	44.2%	9.62%	53.8%	bb	66.43	84.01	aa	320	40	3.2
	Belgium		100	26	6	32	26%	6%	32%	cb	62.04	80.05	aa	320	36	3.2
Honey	Flanders	Summer	2	0	1	1	0%	50%	50%	/	/	/	/	/	/	/
	Wallonia	2017	8	1	0	1	12.5%	0%	13%	/	11	/	/	11	11	/
	Belgium		10	1	1	2	10%	10%	20%	/	11	/	/	11	11	/

GBH: Glyphosate based herbicide, Nb.: number; > LOQ: detection with quantification, <LOQ: detection without quantification, []: concentration; AMPA: aminomethylphosphonic acid; HQ beebread (oral) threshold value = 1000; HQ wax (contact) threshold value = 5000, + detection is the sum of samples > LOQ and < LOD; S.D.: standard deviation; /: not determined. Multi-testing: a Fisher's exact test and a two-sample Wilcoxon rank-sum (Mann-Whitney) test were respectively used for each pairwise comparison of frequency of detection and mean concentration of the compounds. Different letters were used for significant differences. The first position letter corresponds to the comparison of regions for a same matrix; the second position letter corresponds to the comparison of breebread and beeswax for the mean concentration of GBH. A level of significance of 5% was used, divided by the number of tests performed for the Bonferroni correction.

Exposure assessment and risk characterisation of GBH residues in beebread for honey bees

Based on the honeybee oral acute LD_{50} (48 h) of glyphosate (100 mg/bee = moderate toxicity for adult bees) (Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate 2015; Lewis *et al.*, 2016) and on the maximum concentration of GBH residues detected in beebread (700 ng/g), the estimated maximum HQ (oral) of GBH residues for beebread found in Belgium is equal to 7 (=700/100). Because the honeybee oral acute LD_{50} (48 h) of AMPA is currently unknown in published data, it was impossible to estimate its corresponding HQ.

Considering the maximum consumption level of 12 mg of pollen per day (Rortais *et al*., 2005) (worst-case) and the maximum concentration of GBH residues detected in beebread (700 ng/g), this would correspond to a dose of 84 ng of GBH residues ingested per nurse bee over 10 days (0.012 g x 700 ng/g x 10 days). This exposure level corresponds to about 0.08% of the oral glyphosate LD_{50}. As mentioned, in the open literature, no oral acute LD_{50} (48 h) for AMPA is available. To assess the risk of AMPA to bees, we used, therefore, the parent compound glyphosate LD_{50} (Traynor *et al*., 2016). AMPA detection in beebread (250 ng/g) would correspond to about 0.03% of the oral glyphosate LD_{50}. Cumulatively, GBH and AMPA maximal concentration would correspond to about 0.12% of oral glyphosate LD_{50}.

Glyphosate-based herbicide residues and AMPA in beeswax

GBH residues were found in 32% of Belgian beeswax samples (N = 100, T1). A significantly higher GBH residues prevalence was found in Wallonia (53.8% positive sample, **Figure 2**), as compared to Flanders (8.3% positive samples, one-tailed Fisher's exact test (1 degree of freedom; $\alpha = 0.05$), $p < 0.001$); confirmed by a logistic regression comparing contaminations in both regions (with Flanders as a reference): OR = 18.4, 95% CI = 4.66–72.60, $p < 0.001$). A two-sample Wilcoxon rank-sum (Mann-Whitney) test showed that the average GBH residue concentration observed in Wallonia is not significantly higher than in Flanders ($p = 0.33$) (**Table 1**).

Exposure assessment of GBH residues in beeswax

No trace of AMPA has been detected in beeswax. HQ (contact) of beeswax for the maximum GBH residues concentration in Belgium is equal to 3.2 (= 320/100).

Wax renewal rate in Flanders and Wallonia

Beekeepers should renew the wax foundation of their bee colonies periodically. This improves bee health reducing the disease and chemical load of beeswax and allowing bees to rear their brood in a freshly built environment.

Flemish beekeepers had a significant higher wax renewal rate ($\geq 50\%$ per year) as compared to Walloon ones (N = 98, one-tailed Fisher's exact test (1 degree of freedom; $\alpha = 0.05$) , $p = 0.017$) (data not shown).

Risk assessment for the consumer of contaminated beebread and beeswax

As shown in **Table 1**, GBH residues contaminated significantly more frequently beebread (87.2% >LOQ) than beeswax (26% >LOQ) (one-tailed Fisher's exact test (1 degree of freedom; a =0.05), N = 181; $p < 0.001$) but the average concentration found in beebread (55.52 ng/g) and wax (51.3 ng/g) were statistically comparable (two-sample Wilcoxon rank-sum (Mann-Whitney) test; $p > 0.05$).

A high consumption level (95th percentile) of the most contaminated pollen and beeswax by GBH residues, according to our results, leads to an exposure of respectively 0.936 and 0.007 mg GBH residues/kg b.w./day through beeswax and pollen consumption. Concerning AMPA, the highest exposure corresponds to 0.334 mg AMPA/kg b.w./day through pollen consumption).

Transfer of GBH residues and AMPA from beeswax to honey

We wondered if a transfer of GBH residues and AMPA from beeswax to honey was possible. Thus, to further test this hypothesis, we concomitantly collected both wax and honey from the bee colony honey supers of 10 apiaries out of the 32 beekeepers with the highest GBH residues contaminations in wax from the brood chamber. We found 1 out of 10 wax samples (10%) contaminated with GBH residues (concentration: 48 ng/g). In honey, 2 out of 10 samples were contaminated by GBH residues (20%; 11 ng/g for the first sample and a detection lower than the quantification limit [LOQ] < 10 ng/g for the second sample). These 3 positive GBH residues samples came from different bee colonies. No trace of AMPA was detected in any of the matrices. The highest GBH residues concentration detected

in honey was about 5 times lower than the MRL (50 ng/g).

Discussion

Beebread

Our study showed an extended presence of GBH residues in beebread (81.5% positive samples at the national level) in both Belgian regions. AMPA was found in 18.5% of beebread samples at the national level. Only 2 samples contained AMPA without GBH residue. The LOQ values for glyphosate and AMPA are of 10 ng/g, which makes the analysis method very sensitive. Simultaneous AMPA/GBH residues detection in beebread could be explained by the GBH residues degradation in the matrix or by their simultaneous occurrence in the environment. In soil, the primary pathway degradation of glyphosate residues is microbial action, which yields AMPA and glyoxylic acid (Roberts *et al*., 1999). The maximum GBH residues concentration found (700 ng/g) led to sublethal exposure (not acutely toxic to bees), corresponding to a dose of 84 ng/bee (0.08% of its LD_{50}), ingested over the first 10 days of life of a nurse bee. AMPA dose in beebread also corresponded to a sub-lethal exposure (to about 0.03% of oral glyphosate LD_{50}) alone or cumulated with GBH residues (about 0.12% of oral glyphosate LD_{50}). However, while the LD_{50} is measured as a one-time dose, bees could be exposed to GBH residues contaminated beebread for a longer period, when re-contamination occurs, since glyphosate degradation time DT_{50} ranges between 1.0 and 67.7 days. Therefore, the use of the LD_{50} as a single benchmark could underestimate the exposure risk to bees.

Bee and bee colony health is significantly impaired by doses that are lower than those we found through sub-lethal effects. Helmer *et al.* (Helmer *et al*., 2015) orally exposed bees to sub-lethal field-realistic doses of GBH residues (1.25, 2.50, and 5.00 ng/bee) and showed a significant decrease ($p < 0.05$; $n = 40$) of beta-carotene and protein levels in their bodies after 10 days. Our results confirm Helmer's field-realistic doses (lower than 700 ppb, corresponding to 84 ng/bee). Other studies (Herbert *et al*., 2014), showed that adult *A. mellifera* workers exposed orally to 2.5 and 5 mg/L of GBH residues (field-realistic doses equivalent) presented reduced sucrose sensitivity leading to loss and difficulty in establishing associative memories, which, in turn, could cause inefficient collection of nectar and pollen for the colony and, finally, compromise its survival. Oral exposure to GBH residues concentrations (2.5, 5.0, and 10.0 mg/L, corresponding to a dose of 0.125, 0.25, and 0.5 µg/bee) affects honeybee cognitive abilities, with potential long-term negative consequences for colony foraging success (Balbuena *et al*., 2015). Exposures to 5 and 10 mg/L of GBH residues (dose of 0.25 and 0.5 µg/bee) perturb the gut microbiota of honeybees. Bee gut symbionts influence bee development, nutrition, and defence against natural enemies (Motta *et al*., 2018). Perturbations of these gut communities may affect bee susceptibility to environmental stressors, including poor nutrition (Tosi *et al*., 2017) and pathogens (Motta *et al*., 2018). Moreover, in evaluating the effect of Roundup® on the royal jelly-producing glands, Faita *et al.* (2018) showed that exposure to GBH residues resulted in the alteration of these glands that can trigger damage to the development and survival of bee colonies.

Regarding AMPA, no trace was found in honey and beeswax. In beebread, the maximum AMPA concentration was 250 ng/g. Because no information on AMPA toxicity to bees is available yet in the open literature, we were not able to assess its risks to bees. Nevertheless, Blot *et al.* (2019) confirmed that glyphosate have sub-lethal effects on the honeybee microbiota, while AMPA did not induce any significant change.

Beeswax

Measured GBH residues concentrations should not cause acute lethal effects since the estimated HQ for beebread and beeswax (7 and 3.2, respectively) were far below the "safety" oral and contact thresholds (1000 and 5000, respectively). Since beebread can be stored in the hive for months after collection in the field, glyphosate degradation have likely reduced its concentration over time. Furthermore, bees typically collect multiple chemicals simultaneously (Tosi *et al*., 2018). Because bees are bio-indicators of environmental health and pollution, residues found in bee products provide valuable information on environmental punctual contamination or accumulation which, nevertheless, might be underestimated (i.e. residue degradation, dilution of highly-concentrated samples, technical

limitations such as LOD) or overestimated (i.e. accumulation of contaminated pollen) (Tosi *et al.*, 2018).

Due to glyphosate high water solubility and a very low octanol/water partition coefficient (Log P (= Log Kow) at pH 7 and at 20°C = –3.2), GBH residues were expected to be found only in beebread but not in wax (a very hydrophobic matrix). Beeswax samples contamination rate was of 26% at the national level. The addition of surfactant in the formulation of end-use pesticide products is at the origin of the phenomenon allowing glyphosate, which is water-soluble, to penetrate lipid-based structures (Shokri *et al.*, 2001). Nevertheless, the risk assessment for honey bees and the consumer has been evaluated for glyphosate molecule solely without the concomitant formulation ingredients and adjuvants, nor other possibly concurring pesticides (Tosi *et al.*, 2018). The use of the glyphosate/AMPA molecule solely does not render the combined toxic effects of the formulation constituents nor the synergetic potential effects of pesticide combinations.

Wallonia had both a higher GBH residue detection rate (53.8%) and a significantly lower rate of wax foundation renewal rate, as compared to Flanders ($p = 0.017$). This supports our hypothesis that the beekeeping management practice of renewing wax foundation can protect bees from the accumulation of pesticide residues inside the hive. No trace of AMPA could be detected in beeswax, probably because the matrix is not suitable for microorganism growth due to its rich hydrophobic protective properties (Fratini *et al.*, 2016), resulting in no degradation of glyphosate in AMPA. Beeswax's conservative properties for pesticide residues combined with the beekeeping practice of wax recycling (Perugini *et al.*, 2018), may be at the origin of the unequal detection of GBH residues in Flanders and Wallonia. This result highlights the importance of replacing at least 50% of wax frames per year, the current recommendation being the yearly replacement of 25 to 33% of the wax from the brood chamber (ITSAP, 2017; Vergaert, 2017).

For human health, the highest exposure to GBH residues in pollen corresponds to 0.312% and 0.187% respectively of the ADI and of the ARfD, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 700 ng of GBH residues/g). The exposure to GBH residues through the beeswax consumption (1.29 g/day/person of contaminated beeswax with 320 ng of GBH residues/g) corresponds to only 0.002% and 0.001% respectively of the ADI and of the ARfD. Concerning AMPA, the highest exposure to this compound corresponds to 0.111% of the ADI, and this through the pollen consumption (69.55 g/day/person of contaminated pollen with 250 ng of AMPA/g).

Honey

The honey analysis resulted in a maximum GBH residues concentration of 11 ng/g, not exceeding the EU MRL (50 ng/g) for honey and theoretically meaning no risk for the consumer. In a survey on GBH residues in honey samples originating from different countries (Brazil, Canada, China, Germany, Greece, Hungary, India, Korea, Mexico, Uruguay, New Zealand, Spain, Taiwan, Ukraine, Vietnam and USA), GBH residues were found in fifty nine percent (59%) of analysed samples, with concentrations ranging between 17 and 163 ng/g (mean = 64 ng/g) (Rubio *et al.*, 2014).

Our concomitant analyses of wax and honey in samples (N = 10) from honey supers resulted in one wax sample being contaminated (48 ng/g). The low contamination in honey supers suggests that GBH residues are mostly stored in the brood chamber, where pollen and nectar are stored and where most bee activity occurs. This preliminary study showed no transfer from wax to honey. Because our results on the concomitant honey/wax contamination are based on limited data (N = 10), they should be confirmed with further studies.

For human health, considering our results and the assumptions we made with the available regulatory data, the consumption of these three contaminated food matrices (pollen, beeswax, and honey) would not be a food safety issue, nonetheless, caution should be taken in the interpretation the results as new studies confirmed glyphosate toxicity below regulatory limits (Mesnage *et al.*, 2015), and the genotoxicity of AMPA (Mañas *et al.*, 2009).

Bees are major pollinators in agricultural systems. Beebread, beeswax, and honey pesticide residue contamination can impact the viability of a colony when larvae develop on highly contaminated

beeswax and feed with contaminated food (Orantes-Bermejo *et al*., 2010). Even a low concentration of pesticide residues can have amplified toxic effects on animals, including bees, through interactions with other chemicals (Zhu *et al*., 2017) or environmental stressors. The pesticide risk to bees can synergistically amplify the adverse effect of non-chemical stressors too and conversely, nutritional stress can synergistically increase the toxicity of pesticides (Tosi *et al*., 2017).

Conclusions

Our study gives a glimpse of bees and human exposures to GBH residues. At this stage, glyphosate is analysed alone, even though it is never used in this form but only as part of a mixture with adjuvants in commercial formulations. Clarifications and further research are needed to estimate the risk of the herbicide alone and in formulations (i.e. with the adjuvants), especially at levels below the regulatory safe limits and over longer durations. More studies are needed to assess synergies with other pesticides, and longer term exposures at sub-lethal doses. More transparency is needed regarding the commercial formulation products.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a survey of pesticide residues (glyphosate/AMPA) in various bee-related matrices (beebread, wax, honey) from Belgium. While the representativeness of the sampling procedures may be questioned and although the results of the analytical method validations are not provided in a high level of details, the results are considered reliable. A considerable number of samples of beebread/pollen (n = 82) and beeswax (n = 100) were analysed for parent glyphosate and its metabolite AMPA. However, according to the guideline SANTE/11956/2016 rev. 9 the intake of pollen and wax by consumers is negligible and, therefore, it is not a regulatory requirement to investigate the residue levels in these commodities. The publication also provides analytical results for 10 honey samples. Only one of these samples was found to contain residues of parent glyphosate above the LOQ of 0.010 mg/kg (at 0.011 mg/kg). None of the honey samples showed detectable residues of AMPA (i.e. these residues were < 0.001 mg/kg). Since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data, these results are deemed relevant.

The publication concludes that, based on the observed residue levels, the intake of pollen, beeswax and honey by consumers does not cause any health issue. While this conclusion is certainly correct some of the details of the risk assessment are questionable. For instance, the considered ADI of 0.3 mg/kg bw/day for parent glyphosate is obsolete (and was already obsolete at the time when the publication was issued). Furthermore, the long-term residue intakes were calculated based on maximum residue levels and high percentile consumption figures, which does not correspond to the standard approach.

The publication also includes extensive considerations on bee safety, which, however, are not relevant to this section of the dossier and, therefore, are not discussed here.

1. Information on the study

Data point	CA 6.10.1
Report author	Karise R. *et al.*
Report year	2017
Report title	Are pesticide residues in honey related to oilseed rape treatments?
Document No.	Chemosphere 188 (2017) 389-396
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Executive summary

Pesticide treatments before and during the flowering of honey bee forage crops may lead to residues in honey. In northern regions oilseed rape belongs to the main forage crops that is mostly cultivated by means of intensive agriculture, including several pesticide treatments. However, in addition to the focal forage crops, pesticides from non-forage crops can spread to wild flowers around fields, and thus the residues in honey would reflect the whole range of pesticides used in the agricultural landscape. The aim of our study was to clarify which currently used pesticides are present in honey gathered from heterogeneous agricultural landscapes after the end of flowering of oilseed crops.

Honey samples (N = 33) were collected from beehives of Estonia during 2013 and 2014, and analysed for residues of 47 currently used agricultural pesticides using the multiresidue method with HPLC-MS/MS and GC-MS and a single residue method for glyphosate, aminopyralid and clopyralid. Residues of eight different active ingredients with representatives from all three basic pesticide classes were determined. Although no correlation was detected between the cumulative amount of pesticide residues and percent of oilseed crops in the foraging territory, most of the residues are those allowed for oilseed rape treatments. Among all pesticides, herbicide residues prevailed in 2013 but not in 2014. Despite the relatively small agricultural impact of Estonia, the detected levels of pesticide residues sometimes exceeded maximum residue level; however, these concentrations do not pose a health risk to consumers, also acute toxicity to honey bees would be very unlikely.

Materials and methods

Study location

Honey samples were gathered from Eastern and Southern Estonia (Ida-Viru, Tartu, Polva and Valga Counties) in 2013 (N = 14) and 2014 (N = 19). This area is representative of typical agricultural landscapes in Estonia with mostly intensively managed fields, forested areas and human settlements. Among other field crops, both winter and spring oilseed rape are often grown in Estonia, and both belong to the common forage crops of honey bees. Within a 2 km radius of each hive there is on average 34.6 ± 20.7% cultivated land (min. 0.81%, max. 70.2%), 48.1 ± 20.6% forest, 5.3 ± 7.6% waste and vacant land, 7.6 ± 5.0% grassland and 2.1 ± 3.6% garden. The average oilseed crop coverage within the foraging territory remained between 0 and 12.9%.

Pesticide selection

The 47 active ingredients analysed were selected for the survey as being the most commonly used in Estonian fields according to the pesticide ordering lists of the Tartu County Farmers Association for the year 2013-2014. These include the most commonly used contemporary herbicides (21), fungicides (15) and insecticides (10), and plant growth regulator and retardant (1). The active ingredients

searched for were: 2,4D, alpha-cypermethrin, amido-sulphuron, aminopyralid, azoxystrobin, clopyralid, cypermethrin, cyproconazole, deltamethrin, dicamba, dimethachlor, dimethoate, ethyl trinexapac, fenoxaprop-p-ethyl, fenpropidin, florasulam, fludioxonil, fluoxastrobin, flutriafol, fuberidazole, glyphosate, imazalil, imidacloprid, indoxacarb, iodosulfuron-methyl-sodium, lambda-cyhalotrin, MCPA, mefenpyr-diethyl, pencycuron, picloram, pinoxaden, prochloraz, propaquizafop, propiconazole, propoxycarbazone-sodium, prothioconazole, pymetrozine, pyroxsulam, quizalofop-p-ethyl, spiroxamine, sulfosulfuron, tau-fluvalinate, tebuconazole, thiacloprid, triadimenol, triasulfuron and tribenuron-methyl.

Sample collection and handling

A total of 33 honey samples were collected from beehives in the eastern and southern part of Estonia (Tartu County and its near vicinity) during 2013 and 2014 for analysis of pesticide residues. Each honey sample originated from a different apiary, each of which consisted of 10-20 honey bee hives. The sampled hive was selected randomly for testing. The distance between sampled apiaries was at least 4 km in 2013 and at least 8 km in 2014 to preclude overlapping of the main forage area. The samples were gathered from honeycombs within beehives during the honey harvest in mid-July after the end of oilseed rape flowering. Due to the funding allocated for this study, it was decided to concentrate only on honey samples, and in order to cover more apiaries from the largest possible territory, we sampled only one hive per apiary. The honey was extracted from the comb wax and thereafter kept at 5°C until analysis.

Chemicals and materials

The reference standards of pesticides were purchased from AccuStandard (New Haven, USA) and Dr. Ehrenstorfer (Germany). HPLC grade acetonitrile and methanol were purchased from Merck-Millipore (Darmstadt, Germany). ACS grade formic acid (≥ 96.0%), acetic acid (glacial, ≥ 99.85%), and ammonium formate (99%) were purchased from SigmaeAldrich (St. Louis, MO, USA). Ultrapure deionised water was generated by a Millipore Milli-Q™ system (Billerica, MA, USA). A buffer-salt mixture (1 g trisodium citrate dihydrate, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate and 4 g of anhydrous magnesium sulphate) and a mixture of dSPE (900 mg anhydrous magnesium sulphate, 150 mg PSA and 150 mg C18E) were obtained from Phenomenex (Torrance, CA, USA).

Stock solutions of approximately 1000 mg/L concentration were prepared by weighing 10 mg of standard in a 10 mL graduated flask and dissolving it in acetonitrile. The purity of the standard was taken into account in the preparation of standard solutions of final concentration. The mix of working standard solution with a concentration of 0.01 mg/L was prepared by diluting the appropriate volume of stock solution in acetonitrile. The stock and working standard solution were stored at – 20°C.

Sample preparation

Different sample extraction and detection procedures were used for analysis of the selected pesticides. Most compounds were analysed using QuEChERS extraction methodology followed by detection using GC-MS and UHPLC-MS/MS. Analysis of glyphosate, aminopyralid and clopyralid was performed as single analyses using extraction with methanol.

For analysis of glyphosate, aminopyralid and clopyralid, 5.0 ± 0.1 g of samples were weighed into a 50 mL polypropylene centrifuge tube, then 10 mL of water and 10 mL of methanol were added for extraction. The samples were shaken for 20 min and centrifuged for 10 min at 4500 rpm. An aliquot of extract was transferred to an autosampler vial for analysis by UHPLC-MS/MS.

UHPLC-MS/MS analysis

An Acquity UHPLC system (Waters, USA) coupled to QTrap 5500 (AB SCIEX, USA) equipped with an electrospray ionisation source was used for the analysis of pesticides in honey. The chromatographic conditions for analysis of glyphosate residues in honey are summarised in Table 1 below.

Table 1: Chromatographic conditions for analysis of glyphosate in honey

UHPLC system and conditions

Column:	Thermo Scientific, Hypercarb, 100 x 2.1 mm, 5 µm
Column temperature:	40°C
Injection volume:	10 µL
Mobile phase:	1% acetic acid in water
Column flow:	0.3 mL/min

MS system and conditions

	Quantification	*Confirmation*
Scan type:	MRM	MRM
Ionisation mode:	ESI negative	ESI negative
Ion source temperature:	500°C	500°C
Ion spray voltage [V]:	-4500	-4500
Curtain gas nebulizer [psi]:	45	45
Ion source gas 1 [psi]:	40	40
Ion source gas 2 [psi]:	60	60
Declustering potential [V]:	-50	-50
Collision energy [V]:	-20	-16
Mass transition for evaluation [*m/z*]:	168 → 63	168 → 150

Results

Performance of the method

The performance of the method was evaluated according to the EC guidance document SANCO/12571/2013. The method showed good linearity with the determination coefficients, higher than 0.990 for all compounds included in the study. The mean variation of coefficients for repeatability of the method ranged from 3.0% to 16%, and the recovery ranged from 78% to 115%.

The limit of quantification (LOQ) for which the S/N ratio exceeds 10 was assumed at a concentration level of 0.01 mg kg for all pesticides with the exception of aminopyralid, clopyralid, glyphosate, dicamba and picloram for which the LOQ was 0.05 mg/kg.

Analysis of the honey samples

The amounts and composition of pesticide residues found in the honey samples differed between years. The residues of glyphosate in honey samples are summarised in Table 2. The agricultural practices generally do not vary so much, but the need for different kinds of pesticides can vary widely from year to year.

Table 2: Concentrations of glyphosate residues found in honey samples in Estonia 2013-2014

Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [a] [µg/kg]**
1	2013	3.4	n.d.
2	2013	5.7	14
3	2013	6.2	**56**

Honey sample	**Year**	**% of oilseed rape in foraging range**	**Glyphosate residues [a] [µg/kg]**
4	2013	12.1	n.d.
5	2013	10	n.d.
6	2013	9.2	n.d.
7	2013	12.9	**62**
8	2013	9.2	n.d.
9	2013	9.1	n.d.
10	2013	14	n.d.
11	2013	8.6	n.d.
12	2013	5.1	n.d.
13	2013	9.2	n.d.
14	2013	9.3	n.d.
Average	*2013*	*8.86*	***44***
% of samples	*2013*		*21*
15	2014	0	n.d.
16	2014	8.6	n.d.
17	2014	1	n.d.
18	2014	3.8	n.d.
19	2014	0	n.d.
20	2014	2.8	n.d.
21	2014	8.8	n.d.
22	2014	2.7	n.d.
23	2014	11.6	(9)
24	2014	12.3	n.d.
25	2014	8.9	n.d.
26	2014	13.3	n.d.
27	2014	11.6	n.d.
28	2014	1.8	n.d.
29	2014	5.9	n.d.
30	2014	5.4	n.d.
31	2014	8.7	n.d.
32	2014	3.5	n.d.
33	2014	4.9	n.d.
Average	*2014*	*6.08*	*9*
% of samples	*2014*		*16*

(a): The numbers in parenthesis represent values under the limits of detection (LOD). The numbers in bold represent values above the maximum residue limits (MRL).

Honey as a product contains surprisingly few pesticide residues compared to bee bread or pollen (Thompson et al., 2014). Pesticide residues in different matrixes differ in their chemical composition and physical characteristics. Fat or lipid soluble compounds tend to contaminate wax, whereas water-

soluble compounds are more readily found in nectar or honey. Besides contaminated nectar, honey contamination may also occur via translocation of the compounds from comb wax to honey (Kochansky et al., 2001; Tremolada et al., 2004).

The relatively large areas with natural vegetation, and the low amounts of pesticides used in Estonian agriculture (Eurostat, 2015) has shaped the notion that the bee forage environment should be unpolluted in Estonia and probably also in other Nordic countries. Our results, however, suggest the situation may be of concern.

Despite the general low input of pesticides compared to the average usage over the European countries (Eurostat, 2015), some compounds found in honey samples exceeded the MRL. On the background of landscape characteristics, this might arise from relatively homogeneous land cover type – in Estonia, as in Ireland and the United Kingdom, the landscape in 2015 is dominated by larger areas composed of the same land cover type, also the number of structural green elements in the landscape is small (Eurostat, 2015). Larger forest areas may serve as barriers for bees, for instance. Forests have been shown to negatively affect bumble bees with larger foraging territories (Diaz-Forero et al., 2011). Such barriers may concentrate bees on other land, thus increasing the risk of forage on polluted plants. Honey bees prefer to forage in larger open areas rich in flowers, and flowering crops make up an important part of the forage. Since it is one of the most profitable crops, oilseed rape crops are common in crop rotations: covering 15% and 11% of total cultivated land in 2010 and 2015 accordingly (Statistics Estonia, 2012).

In northern regions, the most common group of pesticides sold are herbicides: these comprise more than 70% of pesticides sold in Estonia (Eurostat, 2015). The higher amounts of herbicide active ingredients needed for effective treatments compared to insecticides, for instance, may also be one reason why herbicide residues in particular were higher in our samples. The amounts of herbicides used on fields may differ from year to year depending on the weather conditions throughout the spring and summer. The amounts of herbicides sold in Estonia were higher in 2013 compared to 2014 (Eurostat, 2015) and this appears to have been reflected in our honey samples. Although pesticide residues may be retained in soils from the previous year or even from treatments made decades ago (Hilber et al., 2008; Lozowicka et al., 2016), the authors believe this probably did not affect our results because the samples with higher concentrations in 2013 did not show higher residue level in 2014. Most of the locations sampled in 2013 were also sampled in 2014. We suppose that in those cases where we found herbicide residues higher than the MRL, the bees must have foraged on recently treated fields. For instance, glyphosate residues may remain very high in nectar for up to seven days after treatment, as demonstrated by Thompson et al. (2014). Glyphosate-based herbicides are the most common herbicides worldwide. Moreover, its usage nowadays has gone beyond pest control purposes – being more of an agricultural instead of a pest management tool (Steinmann et al., 2012). We believe that this is something to consider for reducing the levels of pesticide residue found in food: by excluding the routine spray applications and retaining the weed management purpose of glyphosate, one could facilitate a less polluted environment.

The concentrations of all residues found from honey samples in this study remained below the lethal dose to honey bees. LD50 is measured for 2,4D was 0.0115 mg/bee (Extension Toxicology Network, 1996), clopyralid > 100 mg/bee (Dow AgroSciences, 2007) and glyphosate 100 mg/bee (Thompson et al., 2014), tebuconazole 83 mg/bee, azoxystrobin 200 mg/bee, dimethoate 0.11 mg/bee, thiacloprid 27.89 mg/bee, and tau-fluvalinlate 45 mg/bee (Sanchez-Bayo and Goka, 2014). This means that the concentrations found are definitely below acute lethal dosages, although sub-lethal effects cannot be excluded when considering that at least nurse bees consume the contaminated food until they produce the royal jelly, and also larger larvae are fed with nectar and pollen collected by foragers.

Conclusion

Our results demonstrate that intensively treated oilseed rape fields can be a source for pesticide residue contamination in honey, however no direct correlation was found. We believe that pesticides escape from fields over larger neighbouring areas with wild vegetation and contaminate the nectar of wild plants. Our study indicates that most of the agrochemical residues in Estonian honey can originate from oilseed treatments, however the same active ingredients are used for different crops, which is

why no direct references can be made. The compounds that were represented in the highest amounts belonged to herbicides, the most frequently used pesticide group in Northern European climatic conditions. In the context of honey as human food, the concentrations of pesticide residues do not pose any health risk to consumers, although in some cases the levels detected exceeded the MRLs. Concerning the health of bees, the residues remained below acute lethality, however some sub-lethal effects cannot be excluded.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The publication is considered relevant to the setting of a suitable MRL for glyphosate in honey since according to SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Although only limited information is given about the validation of the method for the determination of glyphosate residues, the analytical results are most likely reliable. The residue levels found for glyphosate are consistent with the EU-monitoring data published by EFSA for 2016-2017 in that: 1. Most of the samples do not show quantifiable residues of glyphosate. 2. Some samples show residues > 0.05 mg/kg, which indicates that it is appropriate to increase the existing MRL. 3. The measured residue levels are far below the levels found in the tunnel residue study.

1. Information on the study

Data point	CA 6.10.1
Report author	Rubio, R. et al.
Report year	2014
Report title	Survey of glyphosate residues in honey, corn and soy products
Document No.	J Environ Anal Toxicol 2014, Vol 5(1): 249
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

Samples of honey (sixty nine), pancake and corn syrup (twenty six), soy sauce (twenty eight), soy milk (eleven), and tofu (twenty) purchased in the Philadelphia, US metropolitan area were analyzed for glyphosate residue using ELISA. The limit of quantification (LOQ) and range of the method were determined for honey, pancake syrup, and corn syrup to be 15 to 800 ppb; soy sauce, soy milk, and tofu 75 to 4000 ppb. Glyphosate residues above the limit of quantification were not found in pancake and corn syrup, soy milk, and tofu. Of the sixty-nine honey samples analyzed, forty-one samples, or fifty-nine percent (59%), had glyphosate concentrations above the method LOQ (15 ppb), with a concentration range between 17 and 163 ppb and a mean of 64 ppb. Eleven of the tested honey samples were organic; five of the organic honey samples, or forty-five percent (45%), contained glyphosate concentrations above the method LOQ, with a range of 26 to 93 ppb and a mean of 50 ppb. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62%), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb. In addition to comparison of production method (organic *vs.* conventional), the honey results were evaluated according to pollen source and by country of origin, grouped by GMO usage (prohibited, limited, or permitted). Glyphosate concentrations above the method LOQ (75 ppb) were also found in ten of the twenty-eight soy sauce samples evaluated (36%), with a concentration range between 88 and 564 ppb and a mean of 242 ppb; all organic soy sauce samples tested were below the method LOQ.

Materials and Methods

Chemicals and reagents

Chemicals were of reagent grade and were purchased from Sigma Chemical Company, St. Louis MO, USA, except as indicated. Glyphosate (> 98% purity), Chem Service, West Chester, PA, USA. Glyphosate micro titer plate ELISA, Abraxis PN 500086; Glyphosate sample diluent, PN 500082, Abraxis LLC, Warminster, PA, USA. Glyphosate stock solution was prepared in deionized water to 1.0 mg/mL; spiking solutions were prepared from the working solution using deionized water.

Samples and sample preparation/extraction

In total, 153 representative samples were purchased from markets in the Philadelphia metropolitan area (69 honey, 26 corn and pancake syrup, 28 soy sauce, 11 soy milk, and 20 tofu products).

Honey, corn and pancake syrup samples: A 0.50 g aliquot of sample was weighed into a micro centrifuge tube and 0.50 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/

extraction described above produced a 1:200 sample dilution.

Soy sauce: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, then diluted by adding 40 µL of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Soy milk: A 0.10 mL aliquot of sample was transferred into a micro centrifuge tube and 0.90 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then centrifuged at 6000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Tofu: A 1.0 g aliquot of sample was weighed into a 20 mL vial and 10.0 mL of 1N HCl was added. The sample was mixed for 2 minutes using a vortex mixer, and then allowed to separate for 2 minutes. Approximately 1 mL of the mixture was transferred into a micro centrifuge tube and centrifuged at 6,000 x g for 5 minutes. The sample was then diluted by adding 40 µL of the middle layer of the acid treated sample into 3.96 mL of glyphosate sample diluent and mixed using a vortex mixer. The sample was then analyzed in the ELISA. The sample preparation/extraction described above produced a 1:1000 sample dilution.

Determination of glyphosate in samples

The instructions provided in the ELISA kit user's guide were followed, in brief, glyphosate calibrators provided in the kit and the samples to be tested are derivatized for ten minutes and then added, along with an antibody specific for glyphosate to micro titer wells coated with goat anti-rabbit antibody and incubated for thirty minutes with shaking. A glyphosate horseradish peroxidase (HRP) enzyme conjugate is then added. At this point a competitive reaction occurs between the glyphosate, in the calibrators or samples, and the enzyme labeled glyphosate for the antibody binding sites on the micro titer well. The reaction is allowed to continue for sixty minutes. After a washing step an enzyme substrate (hydrogen peroxide) and the chromogen (3,3',5,5'-tetramethylbenzidine) are added. The enzyme-labeled glyphosate bound to the glyphosate antibody catalyzes the conversion of the substrate /chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilized by the addition of diluted acid and read in a Molecular Devices micro titer plate reader (450 nm). Since the labeled glyphosate (conjugate) was in competition with the unlabeled glyphosate (sample) for the antibody sites, the color developed is inversely proportional to the concentration of glyphosate in the sample.

Data analysis

The evaluation of the assay was performed using Molecular Devices Soft max pro evaluation program (4-Parameter). The program calculates the mean absorbance value for each of the standards (Bi) and calculates the %Bi /B0 for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance (B0). The program then constructs a non-linear regression model of a standard curve by plotting the % Bi/B0 for each standard on the vertical linear (y) axis versus the corresponding glyphosate concentration on the horizontal logarithmic (x) axis. The % Bi/B0 for samples is interpolated using the standard curve yielding sample concentration levels of glyphosate from the standard curve. Correlation coefficients of the assays were >0.995 and standard deviation between standard replicate analysis were < 10%.

Validation, performance and quality control

Specificity had been previously determined (ELISA user's guide), (**Table 1**). Recovery, limit of quantitation, range and limit of quantification were determined to test the validity of the dilution/ extraction procedures of each of the matrices used in combination with the glyphosate ELISA.

Table 1: Cross-reactivity table. The reactivity of glyphosate to various related compounds expressed as LOD and as the dose required for 50% absorbance inhibition (50% B/Bo).

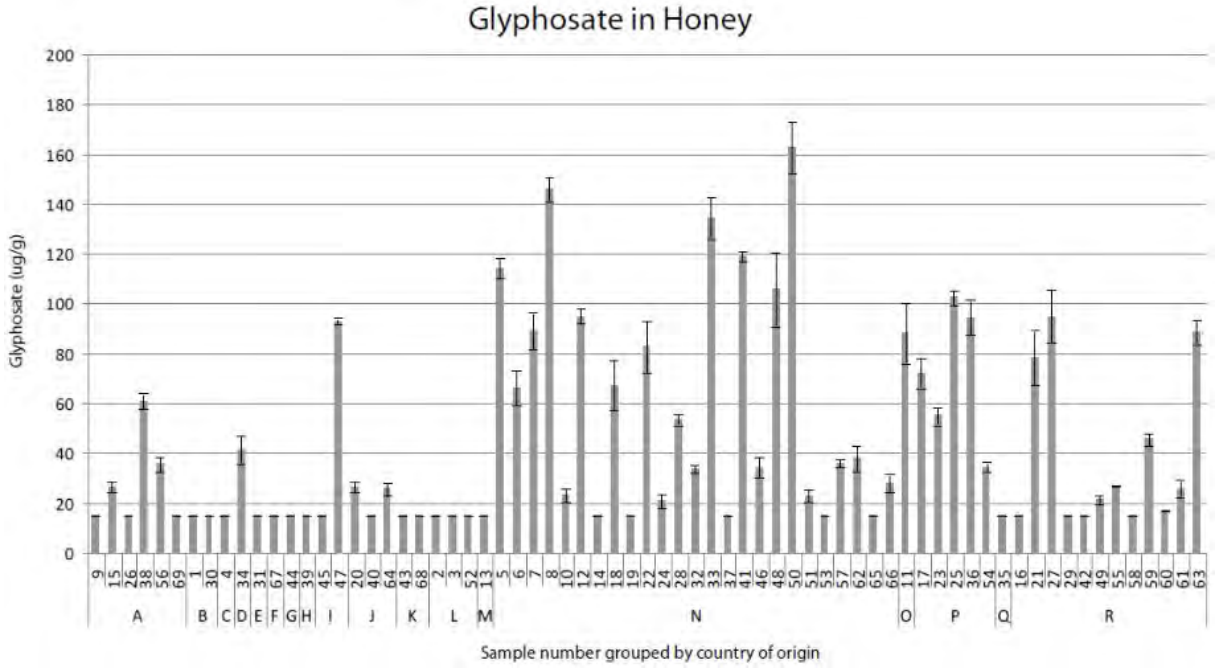
COMPOUND (B/Bo)	LOD(ng/mL)	50% B/Bo(ng/mL)
Glyphosate	0.05	0.5
Glyphosine	50	3,000
Glufosinate	2,000	70,000
AMPA	35,000	>1,000,000
Glycine	>10,000	>1,000,000

Results and Discussion

The method performance for glyphosate analysis was determined by conducting recovery tests on each of the matrices. To determine the accuracy of the glyphosate analysis for the sample matrices analyzed in this study, matrix samples that were glyphosate negative and positive (positive samples were not encountered with tofu, soy milk, pancake and corn syrup) were spiked as follows: 15, 40, 100, 200 and 400 ng/ mL (honey, pancake and corn syrup); 75, 200, 500, 1000 and 4000 ng/mL [soy sauce, soy milk and tofu (ng/g)]. Analysis was performed in duplicate for all unspiked and spiked samples at all levels. Average recovery obtained for glyphosate negative honey samples fortified with glyphosate was 119%, (SD = 10). Average recovery for glyphosate positive honey (unspiked contained 44 ng/g glyphosate) after fortification was 116% (SD = 10). Average recovery for negative soy sauce was 94% (SD = 5), and for positive fortified soy sauce (unspiked contained 417 ng/mL) was 86% (SD = 5). The limit of quantification and range of the method were determined for honey, pancake and corn syrup to be 15 to 800 ng/g; soy sauce, soy milk, and tofu 75 to 4000 ng/ mL or ng/g, respectively.

In this study, the first sample matrix analyzed for the presence of glyphosate was honey; 69 samples were analyzed and classified into 18 groups depending on the country of origin listed on the bottles: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. The glyphosate concentrations obtained are shown in (**Figure 2**). Forty-one out of the sixty-nine honey samples analyzed, or fifty nine percent (59%), had glyphosate concentrations above the method LOQ (15 ng/g) with a concentration range between 17 and 163 ng/g and a mean of 64 ng/g.

Figure 2: Concentration of glyphosate (ng/g) in honey samples listed by honey origin: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis.



The glyphosate concentration in honey grouped by flower (pollen) source is shown in (**Figure 3**). The pollen types listed on the bottles were: clover (12 samples), exotic (11 samples), wildflower (11 samples), unknown (35 samples). (**Figure 4**) depicts the concentration of glyphosate in honey samples grouped by growing method of source pollen: organic (11 samples) and traditional (58 samples); 5 of the 11 organic samples had glyphosate concentrations above the method LOQ with a range of 26 to 93 ng/g and a mean of 50 ng /g. Of the fifty-eight non-organic honey samples, thirty-six samples, or sixty-two percent (62%), contained glyphosate concentrations above the method LOQ, with a range of 17 to 163 ppb and a mean of 66 ppb.

Figure 3: Concentration of glyphosate (ng/g) in honey samples by flower (pollen) source. Dashed line represents LOQ of method (15 ng/g). Exotic flowers were sophora, manuka, orange, cactus, summer flower, lychee, alfalfa, acacia). Error bars represent concentrations obtained during duplicate analysis.

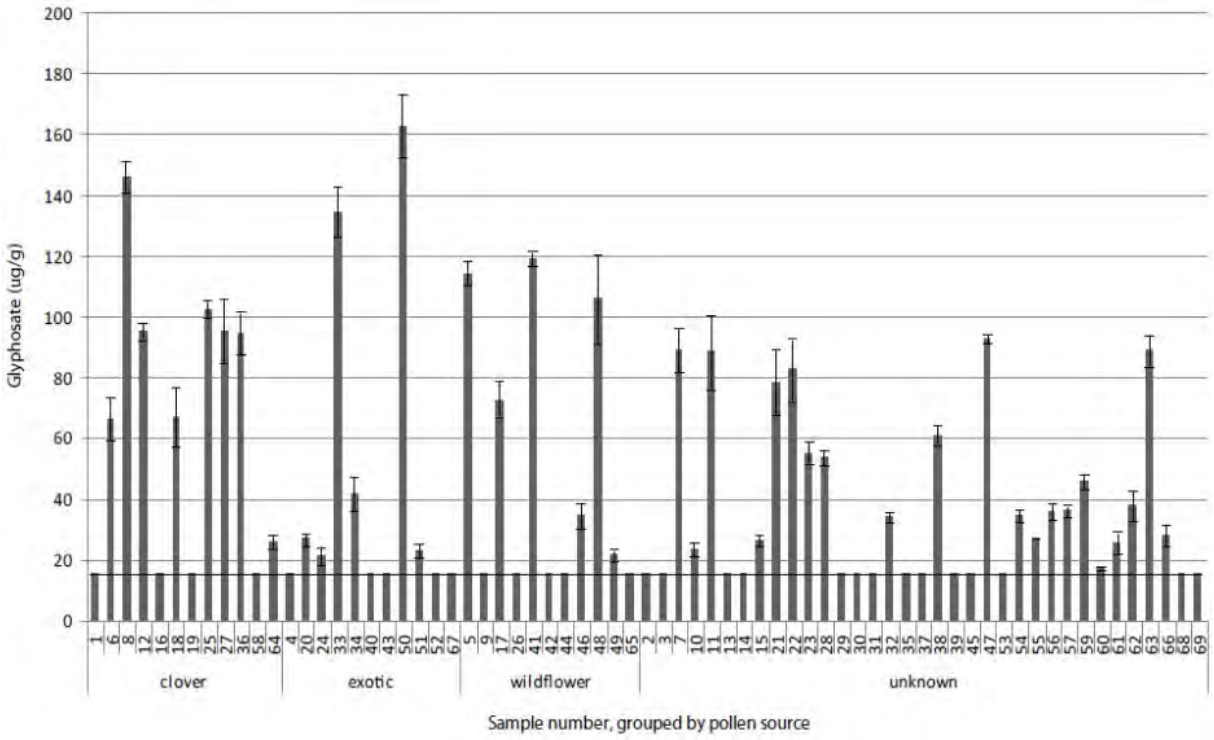


Figure 4: Concentration of glyphosate (ng/g) in honey samples by growing method of source pollen (Organic *vs.* Traditional). Dashed line represents LOQ of method (15 ng/g).) Error bars represent concentrations obtained during duplicate analysis.

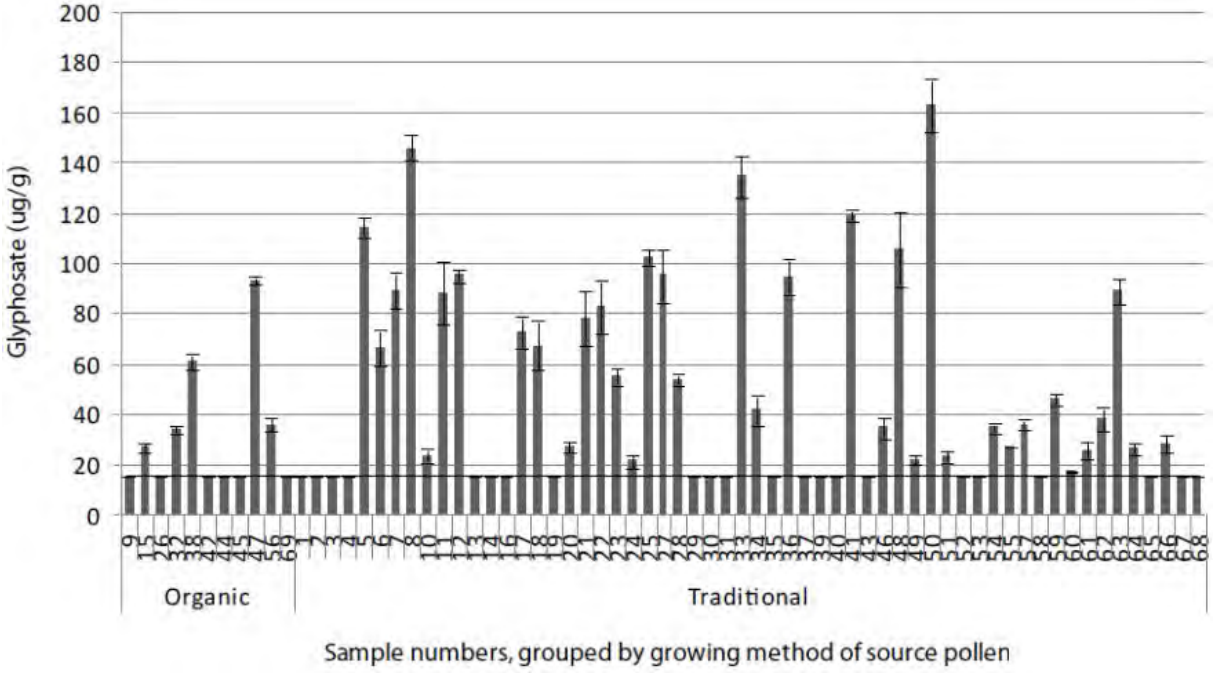
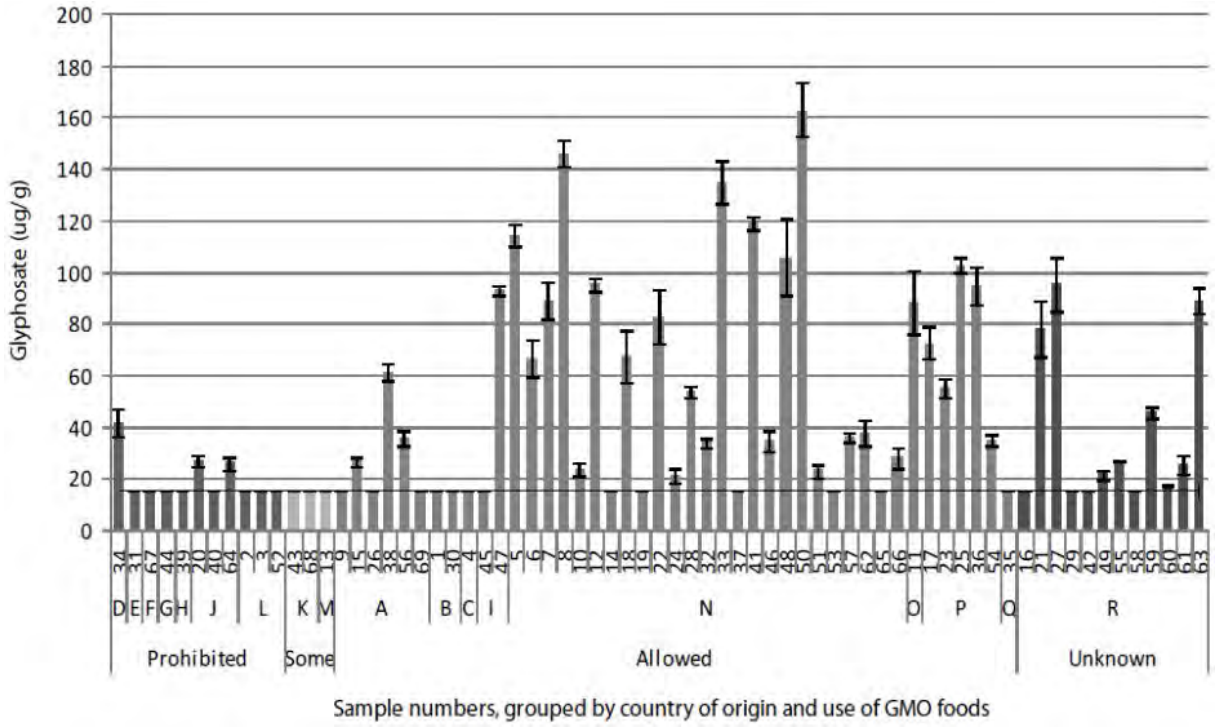


Figure 5 depicts the concentration of glyphosate in honey by country and whether the use of genetically modified organisms (GMO) seeds is prohibited or permitted. The graph also shows where some minimum uses of GMO traits are allowed (Spain, and blend of Vietnam/Ukraine). The glyphosate

concentration in honey originating in countries that do not allow or allow limited GMO traits (3 out of 14 samples above the LOQ) ranged from 26 to 41 ng/g with a mean of 31 ng/g. The glyphosate range for those countries that allow GMO (30 out of 43 samples above LOQ) was 21 to 163 ng/g with a mean of 71 ng/g. Samples of unknown origin (8 out of 12 samples above LOQ) ranged from 17 to 95 ng/g with a mean of 50 ng/g.

Figure 5: Concentration of glyphosate (ng/g) in honey samples listed by honey origin and the allowance of GMO use: (A) Brazil, (B) Canada, (C) China, (D) Germany, (E) Greece, (F) Hungary, (G) India, (H) Korea, (I) blend of Mexico, Brazil, and Uruguay, (J) New Zealand, (K) Spain, (L) Taiwan, (M) blend of Ukraine and Vietnam, (N) USA, (O) blend of USA and Argentina, (P) blend of USA, Argentina and Canada, (Q) blend of USA, South America, (R) unknown origin. Dashed line represents LOQ of method (15 ng/g). Error bars represent concentrations obtained during duplicate analysis.



The second matrix group analyzed for glyphosate was soy sauce. The analysis consisted of 28 samples, (**Figure 6**). Ten out of 28 samples (36%) had glyphosate concentrations above the method LOQ (75 ng/mL) with a concentration range between 88 and 564 ng/mL and a mean of 242 ng/mL. (**Figure 7**) shows the concentration of glyphosate in soy sauce by method of soy bean growing (organic vs. traditional). The recent report from the Chinese Academy of Medical Science and the Beijing Union Hospital [20] reported an average glyphosate concentration in soy sauce of 133 ng/mL in samples that did not specify on the bottle whether or not the raw material was GM soybean. In our study, the small subset of organic labeled samples (three) was all below the limit of quantitation of the test.

Figure 6: Concentration of glyphosate (ng/mL) in soy sauce samples. Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.

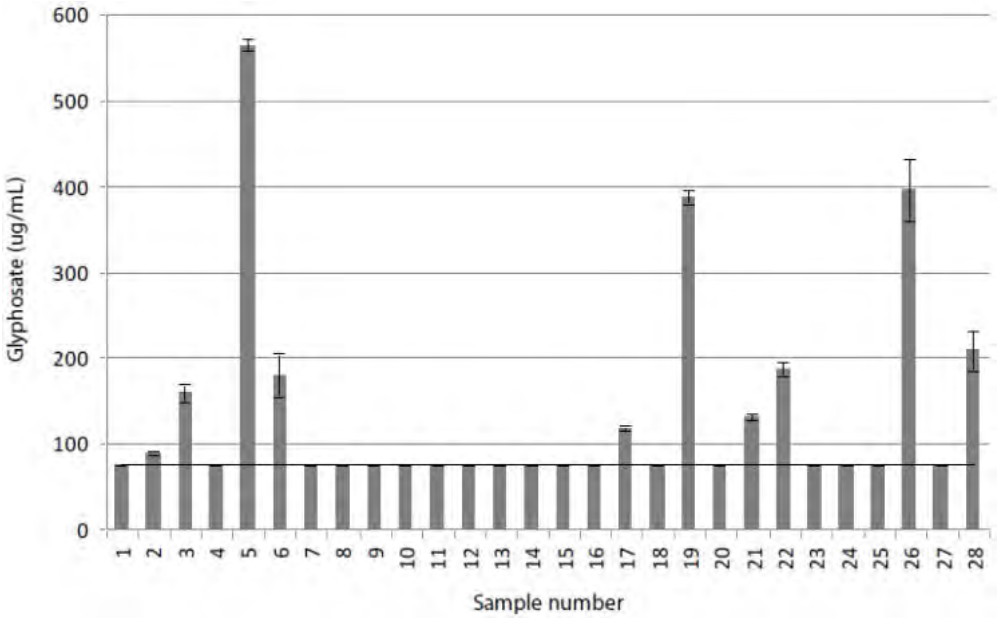
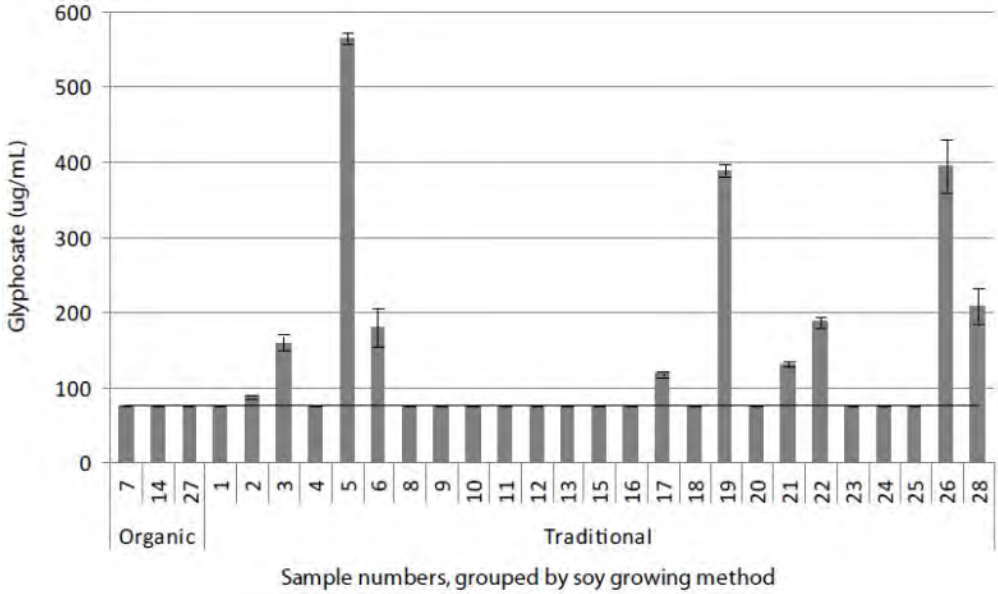


Figure 7: Concentration of glyphosate (ng/mL) in soy sauce samples by growing method of soy beans (Organic *vs.* Traditional). Dashed line represents LOQ of method (75 ng/mL). Error bars represent concentrations obtained during duplicate analysis.



Corn and pancake syrup (26 samples), soy milk (11 samples), and tofu (20 samples) tested were negative for glyphosate at the LOQ of the method (15 ng/g for pancake and corn syrup, and 75 ng/mL or ng/g for soy milk and tofu, respectively).

Studies on glyphosate residues in food are scarce. Among the few studies found was a recent report published on the incidence of glyphosate in soy sauce, conducted by the Chinese government [20]. Searches were conducted by the authors using various scientific databases on the concentration and incidence of glyphosate in honey, but these failed to provide any information. The honey samples analyzed in the present study show that 59% of all samples contained glyphosate residues (ranging from 17 to 163 ng/g, mean 64 ng/g); the residue concentration does not seem to depend on pollen source or growing method, even organic honey contained glyphosate residues (5 out 11 samples, or 45%, mean

glyphosate concentration 50 ng/g). Comparing the concentration of glyphosate in honey by countries that use GMO extensively with countries that allow the use of some GMO traits and those that do not allow GMO, shows that, in general, glyphosate levels are lower in samples from countries that do not allow or allow limited use of some GMO traits, such as Spain and Vietnam/ Ukraine blend (mean 31 ng/g), compared to those countries that allow planting of GMO traits (71 ng/g). It should be noted, however, that some residues of glyphosate (although < 50 ng/g) were found in honeys originating from Germany and New Zealand, countries where no GMO planting is allowed.

The European Union has specific guidelines for the labeling of organic honey [25,26]. According to those guidelines, the location of apiaries is strictly controlled and states that "Nectar and pollen sources available over a three-kilometer radius around the apiary sites must consist essentially of organically produced crops or crops treated with low-environmental-impact methods. Apiaries must also be far enough away from any non-agricultural production source that could lead to contamination (*e.g.* urban centers, waste dumps, waste incinerators, etc.). Member States have the option of prohibiting the production of organic honey in certain regions or areas that do not meet these conditions. Organic honey must not contain chemicals residues (synthetic pesticides, etc.)." The United States has no such guidelines for the organic production of honey, but uses organic farming certification for honey labeling purposes; one reason is that it is practically impossible to regulate without testing all honey for residues since bees can fly up to 3 miles in search of nectar and it is difficult to be certain that they do not feed on nectar contaminated by crop spraying or industrial sources. In the EU, glyphosate residues in non-organic honey regulatory limits are 50 ng/g [27], the United States does not have a limit in honey. The limit in drinking water in the United States is 700 ng/mL; the reference dose is 1.75 mg/kg/day; the One-Day Health Advisory level is 20 mg/L [28]. Also, it is widely known that like milk and olive oil, honey is one of the foods that is most commonly mislabeled and adulterated [29] providing yet another source of glyphosate contamination in honeys that, according to the bottle label, originated in non-GMO countries.

Bee colony collapse disorder (CCD) is a growing threat to the efficient production of food around the world. Honey bees pollinate nearly 130 species of plant life [30], such as fruits, vegetables, nuts, and seed crops. Honeybees are therefore indirectly responsible for an estimated one-third of the world food supply [31]. Although several factors are involved in CCD, including numerous pathogens and parasites, the extensive use of pesticides [32,33] such as neonicotinoids have provided evidence that these products are harmful to honey bees and have lead to a recent ban or restriction in the use of three neonicotoids by the European Union [34]. Although glyphosate is not acutely toxic to bees, it is chronically toxic to animals and is reported to disrupt the endocrine system [35,36] and a recent study indicates that honey bees exposed to increasing sub-lethal concentrations of glyphosate exhibit a decrease in acetyl cholinesterase (AChE) activity [37]. The high rate of glyphosate use creates the potential for wide-spread contamination of our food chain. Glyphosate is used throughout the bee foraging period in high amounts and is found in the air, water, and in plant parts frequented by bees, such as flowers and buds, potentially contaminating the nectar collected by bees from contaminated plants [38]. Based on its prevalence in the environment, as well as our findings in honey samples, we propose that future studies should be conducted to determine if glyphosate is in fact a contributing factor in CCD.

Conclusions

This study indicates the presence of glyphosate residues in honey and soy sauce, but not in pancake and corn syrups or soy based products such as soy milk and tofu. Forty one out of sixty nine (59%) honey samples analyzed contained glyphosate at a concentration above the method LOQ (15 ng/g) with a range between 17-163 ng/g and a mean of 64 ng/g. Ten out of twenty eight (36%) soy sauce samples contained glyphosate at a concentration above the method LOQ (75 ng/mL) with a range between 88-564 ng /mL and a mean of 242 ng /mL. Future studies should be conducted on many other food products to determine the extent of glyphosate residue contamination.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a survey of glyphosate residues in honey (n = 69), pancake and corn syrup (n = 26), soy sauce (n = 28), soy milk (n = 11) and tofu (n = 20) purchased in USA, but originating from various countries around the globe. In the context of the dossier for the renewal of the EU approval of glyphosate and with regard to the supported representative uses, the residue data for pancake and corn syrup, soy sauce, soy milk and tofu are not considered relevant. However, the residue data for glyphosate in honey are potentially relevant since according to the guideline SANTE/11956/2016 rev. 9 it is possible to derive MRLs in honey based on monitoring data. Only few of the analysed honey samples originated from Europe but, as honey available to European consumers may originate from outside the EU, it is appropriate to consider honey residue data from outside the EU to derive the EU MRL.

The samples were analysed by means of an ELISA method which was validated by determining the recovery rates from fortified samples. The validation results are not provided in detail, but the average recoveries and relative standard deviations were satisfactory, although the validation was not conducted exactly in accordance with EU or OECD guidelines (i.e. with at least 5 replicates at the LOQ and 5 replicates at a higher level). The limit of quantification was estimated at 0.015 mg/kg. The specificity of the method was investigated by assessing the response of the ELISA test to a series of substances chemically related to glyphosate and it was shown that the response of these substances was at least 1000 times less than that of glyphosate. While this experiment allows to exclude some possible sources of false-positive results, it does not allow to completely rule out that other (not tested compounds) may yield false positive results. Despite these limitations, the obtained analytical results are considered fairly reliable.

59% percent of the 69 honey samples contained glyphosate residues above the method LOQ (0.015 mg/kg) with a concentration range between 0.017 and 0.163 mg/kg and a mean of 0.064 mg/kg. While the individual results are not provided, it seems that about 31% of the samples (22 from 69) showed residues of glyphosate above the EU MRL of 0.05 mg/kg. The samples originating from the EU all showed residues < 0.05 mg/kg. Overall, the findings reported in the publication are in line with the results of the EU-monitoring since the publication shows that glyphosate can occur in honey at levels > 0.05 mg/kg and that it is, therefore, appropriate to increase the existing EU-MRL. The highest measured residue level was 0.163 mg/kg, which is less than the maximum value found during the EU-monitoring for 2016-2017.

1. Information on the study

Data point	CA 6.4.2
Report author	Schnabel K. *et al.*
Report year	2017
Report title	Effects of glyphosate residues and different concentrate feed proportions on performance, energy metabolism and health characteristics in lactating dairy cows
Document No.	Archives of Animal Nutrition, 2017, Vol. 71, No. 6, 413-427
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 aim of this study was to examine the influence of glyphosate (GL) residues in feedstuffs on performance, energy balance and health-related characteristics of lactating dairy cows fed diets with different concentrate feed proportions. After an adaption period, 64 German Holstein cows (207 ± 49 d in milk; mean ± SD) were assigned to either groups receiving a GL contaminated total mixed ration (TMR) (GL groups) or an uncontaminated TMR (CON groups) during a 16 weeks trial. Contaminated feedstuffs used were legally GL-treated peas and wheat (straw and grain). GL and CON groups were subdivided into a "low concentrate" group (LC) fed on dry matter (DM) basis of 21% maize silage, 42% grass silage, 7% straw and 30% concentrate and a "high concentrate" group (HC) composed of 11% maize silage, 22% grass silage, 7% straw and 60% concentrate for ad libitum consumption. Body condition score, body weight, DM intake and milk performance parameters were recorded. In blood serum, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and glucose were measured and energy balance was calculated. Milk was analysed for GL residues.

At week 0, 7 and 15, general health status was evaluated by a modified clinical score. The average individual GL intake amounted for Groups CON_{LC}, CON_{HC}, GL_{LC} and GL_{HC} to 0.8, 0.8, 73.8 and 84.5 mg/d, respectively. No GL residues were detected in milk. GL contamination did not affect body condition score, body weight, DM intake, nutrient digestibility, net energy intake, net energy balance or BHB, glucose, NEFA and milk performance parameters; whereas concentrate feed proportion and time did affect most parameters. The clinical examination showed no adverse effect of GL-contaminated feedstuffs on cows' health condition. In the present study, GL-contaminated feedstuffs showed no influence on performance and energy balance of lactating dairy cows, irrespective of feed concentrate proportion.

Materials and Methods

Experimental design

Sixty-four German Holstein cows (207 ± 49 d in milk; mean ± SD) were used in a 17 weeks trial. The experiment was designed as a 2×2 factorial design with GL contamination and concentrate proportion in feed as the main factors. At the start of the experiment (week 0), all animals were fed with an energetically adequate total mixed ration (TMR), based on the recommendations of the Society of Nutrition Physiology (GfE 2001) consisting of 30% maize silage, 30% grass silage and 40% concentrate on a dry matter (DM) basis. To provide equal conditions in the following 16 weeks of trial, 48 cows and 16 heifers were assigned to four different feeding groups by considering number of lactation (2.8 ± 0.7) and data that had been collected prior to the trial, presenting a 3-d mean of body weight (BW, 645 ± 21 kg), daily feed intake (40.9 ± 0.2 kg fresh matter of the ration) and fat corrected

milk (FCM, 4% fat; 29.1 ± 0.5 kg). Half of the animals received in their ration GL contaminated peas and wheat kernels, processed in concentrate and GL contaminated straw (Groups GL). As control group, the other half of cows received a non-contaminated ration (Groups CON). Both groups were subdivided into one group receiving a diet with a low concentrate proportion (LC) composed on DM basis of 21% maize silage, 42% grass silage, 7% straw and 30% concentrate, and another group receiving a diet with high concentrate proportion (HC) composed of 11% maize silage, 22% grass silage, 7% straw and 60% concentrate . TMR and water were provided *ad libitum*. Cows were kept in a free stall-barn, Groups GL and CON were separated by the feed alley, and within each group subgroups LC and HC were separated by fences inside the barn.

Feedstuff production, animal measurements and sample collection

Maize, grass, peas and wheat were grown on the acreage of the experimental station of the Friedrich-Loeffler-Institut (FLI), in Braunschweig, Germany, to generate equal growth and soil conditions. The acreage had not been treated before with GL for at least 3 years. Maize and grass were grown without GL-application. For GL contamination Roundup Record® (007525–60/MOT), Monsanto, Agrar Deutschland GmbH (Düsseldorf, Germany) was used as water-soluble granulate, containing as active ingredient 720 g GL per kg GL solution. A part of wheat and peas was treated with Roundup Record®, in pre-harvest application with 2.5 l/ha for wheat and 2 l/ha for peas, according to the legal regulations [Regulation (EC) No. 396/2005 of the European Parliament and of the council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC] while another part remained untreated and served for uncontaminated control feedstuffs. GL contaminated and non-contaminated feedstuffs were harvested and stored separately to avoid cross contamination.

During the trial, samples of maize and grass silage were taken twice a week, while samples of straw and concentrate were taken once a week and pooled over 4 weeks. Water and DMI were recorded daily by computerised feeding bins (type RIC; Insentec B.V., Marknesse, The Netherlands). Every second week the body condition score (BCS) was evaluated using a 5-point scale (Edmonson et al. 1989). Cows were milked twice daily beginning at 05:30 h and at 15:30 h and milk yield was recorded using automatic milk counters (Lemmer Fullwood GmbH, Lohmar, Germany). Morning and evening milk samples were collected twice a week. In week 0 and 16, an additional morning and evening milk sample was taken and pooled according to their proportion of total daily milk yield and frozen at −20°C. BW was recorded automatically by a scale after leaving the milking parlour. Blood samples were taken after morning milking from a jugular vein in serum tubes at week 0, 4, 8, 12 and 16.

Analyses

Feed samples were dried at 60°C before analysis for chemical composition according to the methods of the VDLUFA (1993) applying method number 3.1 (DM), 8.1 (crude ash), 4.1.2 (crude protein), 5.1.1 (ether extract), 6.1.1 (crude fibre), 6.5.1 (neutral detergent fibre without ash, amylase treated) and 6.5.2 (acid detergent fibre without ash). The TMR of each treatment group was tested for the apparent digestibility of crude nutrients and net energy for lactation (NE_L) content by using German Blackhead/SKF wethers according to the regulations published by the Society of Nutrition Physiology (GfE 1991).

GL and aminomethylphosphonic acid (AMPA) concentration in feed samples were measured by an accredited laboratory (Wessling GmbH, Altenberge, Germany). Samples were extracted with formic acid (0.1%) and methylene chloride. Derivatisation was conducted with fluorenylmethoxycarbonyl chloride. After solid-phase extraction, GL and AMPA were determined by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). GL and AMPA were quantified using internal standards containing 1.2-$^{13}C_2{}^{15}N$ GL (1 µl/ml) and $^{13}C^{15}N$ AMPA (1 µl/ml). The limit of detection (LOD) and the limit of quantification (LOQ) for each substance were calculated from the signal-to-noise ratio amounting to 3 for the LOD and 10 for the LOQ, whereby the LOQ and LOD of the feed samples were 0.02 and 0.007 mg/kg for GL and AMPA, respectively. The recoveries for GL and AMPA analyses in feed samples were 70–120% using an internal standard concentration of 0.625 mg/kg for feed analyses.

Milk samples were analysed for fat, protein, lactose and urea using an infrared milk analyser (Milkoscan FT 6000®; Foss Electric, Hillerød, Denmark). Somatic cell count (SCC) was detected by flow cytometric measurement (Fossomatic 500®, Hillerød, Denmark). GL was determined in milk samples by Federal Office of Consumer Protection and Food Safety (BVL, Marienfelde, Berlin). Based on QuPPe-Method (Anastassiades et al. 2015), the samples were homogenised, water content adjusted to 100% and glyphosate $^{13}C_2{}^{15}N$ was added as internal standard. Afterwards, samples were extracted with MeOH/Cyclohexan. and purified with acetonitrile. After degreasing by freezing, derivatisation was conducted with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). GL was analysed by LC-MS/MS equipped with electrospray ionisation source (negative mode). Confirmation was performed by diagnostic ions (precursor ion (*m/z*): 390, 167.9; production (*m/z*): 390, 149.9. The LOQ was 0.01 mg/kg (recovery rate 104%) which is according to (SANTE/11945/2015) the lowest spike level of the validation with recoveries between 70 and 120% and a within laboratory reproducibility $RSD_{wR} \leq 20\%$.

Blood samples were analysed for serum concentrations of β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and glucose, after centrifugation, using an automatic analysing system, based on photometric measurement (Eurolyser®, Type VET CCA, Salzburg, Austria).

Clinical examination

At weeks 0, 7 and 15, the general health status of all cows was evaluated by a modified clinical score according to Seyboldt et al. (2015); Dirksen et al. (2012). Scoring was performed by two veterinarians who were unaware of the treatment group at the examination. The score system for most parameters was 0–2 (0, no symptom; 1, moderate symptom; 2, severe symptom); however, the locomotion system got a score of 0–3 (0, no symptom; 1, mild symptom; 2, moderate symptom; 3, severe symptom). Those parameters leading to yes or no answers were scored only 0–1 (0, no symptom, 1, symptom). For evaluation, the data were summarised to four different symptom complexes, namely respiration and cardiovascular system including 22 tested parameters (max score of 35), gastrointestinal tract including 13 tested parameters (max score of 30). Furthermore, udder and locomotion system score were counted separately for each quarter of the udder and each leg. Consequently, udder health included five tested parameters (max score of 29), and locomotion system included 20 tested parameters (max score of 238). If a cow reached the maximal symptom score in all parameters of one symptom complex, we postulated 100% illness in that complex. For the total cumulative health score, each of the individual complexes then counted as 25%. For example, if one cow would reach 100% in one complex, the total illness would result in 25% whereas a cow with 0% in each complex would be considered as completely healthy.

Calculations

The energy content of the experimental TMR was calculated based on nutrient digestibility measured with wethers (GfE 1991) and on equations for calculation of energy content in feedstuffs published by the Society of Nutrition Physiology (GfE 2001), as well as net energy requirement for maintenance (NE M), feed content and requirement for NE_L and milk energy:

$$NE_M\,[\text{MJ } NE_L/\text{d}] = 0.293 \times BW^{0.75}\,[\text{kg}]$$

$$NE_L\,\text{content}\,[\text{MJ/kg feed}] = 0.6 \times [1 + 0.004 \times (\text{q - } 57)] \times \text{ME}\,[\,\text{MJ/kg}\,]$$

$$NE_L\,\text{requirement}\,[\text{MJ } NE_L/\text{d}] = [\text{Milk energy output}\,[\text{MJ } NE_L/\text{d}\,] + 0.086] \times \text{Milk yield}\,[\,\text{kg/d}\,]$$

$$\text{Milk energy}\,[\text{MJ } NE_L/\text{kg}] = 0.38 \times \text{Milk fat}\,[\%] + 0.21 \times \text{Milk protein}\,[\,\%] + 0.95$$

FCM (4% fat) was calculated based on the equation of Gaines (1928):

$$\text{FCM}\,[\text{kg/d}] = [(\text{Milk fat}\,[\%] \times 0.15) + 0.4] \times \text{Milk yield}\,[\,\text{kg/d}\,]$$

Energy-corrected milk (ECM) was calculated based on the equation of Sjaunja et al. (1990):

$$\text{ECM [kg/d]} = \text{Milk yield [kg/d]} \times$$
$$[(38.3 \times \text{Milk fat [g/kg]} + 24.2 \times \text{Milk protein [g/kg]} + 16.54 \times \text{Milk protein [g/kg]} + 20.7) / 3140]$$

Net energy (NE) balance was calculated as follows:

$$\text{NE balance [MJ NE}_L\text{/d]} = \text{Energy intake [MJ NE}_L\text{/d]} - \{\text{NE}_M\ [\text{MJ NE}_L\text{/d}] + \text{NE}_L\ [\text{MJ NE}_L\text{/d}]\}$$

Statistical analyses

Before data analyses, data of DMI, BW, NE balance and milk performance were condensed to a 14 d mean. Variables were all tested for normal distribution via visual histogram plot, only the number of cell counts had to be given as decimal logarithmic value. All statistical analyses were performed using the Software SAS (Version 9.2; SAS Institute Inc., Cary, North Carolina, USA). Parameters were analysed using the MIXED procedure for repeated measures (Littell et al. 1998). In case the variable showed significant effect between the groups in week 0, week 0 of that variable was set as covariable. For each variable covariance, structure was tested for compound symmetry (CS), autoregressive (1) AR (1) and unstructured (UN), and the model which proved the best Akaike information criterion for a finite sample size (AICC) was chosen. The model contained GL contamination (GL), concentrate feed proportion (CFP) and time (t) measured in trial weeks as fixed effects and the interaction between GL and CFP, GL and t, CFP and t and GL, CFP and t. Effects were declared as a trend if *p*-values were ≤ 0.10 and as significant if *p*-values were ≤ 0.05 after Tukey's test. Results are presented as Least Square (LS) Means ± standard error (SE) of LS means unless otherwise stated.

Results

In total, 61 out of the initial 64 cows completed the entire trial. Two cows were excluded because of diseases not related to the experimental treatments. In the first week of the trial, one cow of group GL HC had an abomasal displacement. A cow of Group GL LC developed a general peritonitis in week 8. Another cow of Group GL LC became dry in trial week 11 and was excluded from the trial.

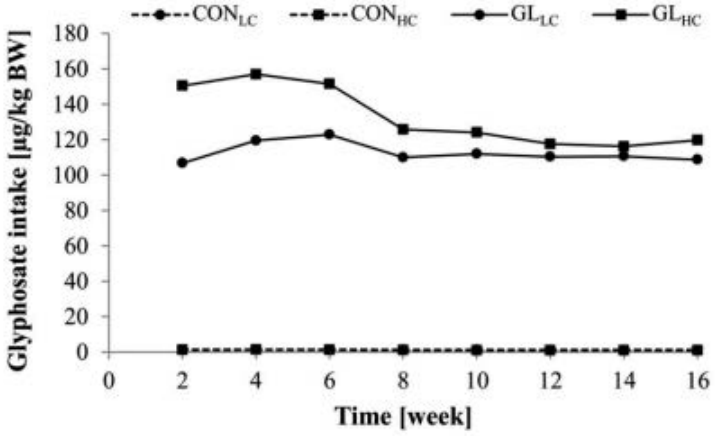
The chemical composition of the cows' ration components (Table 1) was within the normal range for the respective feedstuffs (DLG 1997). The average daily GL intake in Groups CON LC and CON HC was 0.8 and 0.8 mg/d, respectively, and in Groups GL LC and GL HC 73.8 and 84.5 mg/d, respectively. Figure 1 presents an overview of the cows' daily intake in the particular trial weeks in µg per kg BW).

Table 1: Ingredients of concentrate and chemical composition of the feedstuffs used in the total mixed ration (TMR).

	Concentrate composition[#]				Roughage[†]			
	Group CON_{LC}	Group CON_{HC}	Group GL_{LC}	Group GL_{HC}	Straw CON	Straw GL	Grass silage	Maize silage
Ingredients [% DM]								
Peas	29	29	-	-				
Peas GL treated	-	-	29	29				
Wheat	36	36	-	-				
Wheat GL treated	-	-	36	36				
Corn	26.8	30.8	26.8	30.8				
Urea	1.3	0.6	1.3	0.6				
Calcium carbonate	1.5	0.7	1.5	0.7				
Soybean oil	1	1	1	1				
Vitamin/mineral premix[§]	4.4	1.9	4.4	1.9				
Chemical composition								
Dry matter (DM) [g/kg]	881	884	885	881	899	895	324	345
Nutrients [g/kg DM]								
Crude ash	70	44	70	44	58	57	118	41
Crude protein	160	143	163	144	25	32	140	71
Ether extract	34	40	34	40	11	11	35	32
Crude fibre	34	34	34	35	436	440	298	230
$aNDF_{om}$[‡]	106	116	111	115	806	808	550	402
ADF_{om}[•]	43	45	42	46	504	507	326	237
Starch	589	610	591	612				322
Sugar	38	38	38	38				
Herbicide agent residue [mg/kg DM]								
Glyphosate	0.03	0.00	0.37	0.43	0.57	61.81	0.00	0.00
AMPA*	0.00	0.00	0.00	0.00	0.00	0.59	0.00	0.00

[#]CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR); [†]Composition (on DM basis of the TMR) in LC groups: 21% maize silage, 42% grass silage and 7% straw (GL or CON); in HC groups: 11% maize silage, 22% grass silage and 7% straw (GL or CON); [§]Provided per kg concentrate feed (according to manufacturer specification) for LC groups: 6.16 g Ca, 5.28 g Na, 3.52 g P, 2.2 g Mg, 0.31 g Zn, 0.21 g Mn, 0.06 g Cu, 4.4 mg I, 1.76 mg Se, 1.32 mg Co, 35 200 IU vitamin A, 4 400 IU vitamin D_3, 66 mg vitamin E; HC groups: 2.66 g Ca; 2.28 g Na; 1.52 g P; 0.95 g Mg; 0.13 g Zn; 0.09 g Mn; 0.02 g Cu; 1.9 mg I; 0.76 mg Se; 0.57 mg Co; 15 200 IU vitamin A; 1 900 IU vitamin D_3; 28.5 mg vitamin E. [‡]$aNDF_{om}$, neutral detergent fibre without ash, amylase treated, [•]ADF_{om}, acid detergent fibre without ash; *AMPA, aminomethylphosphonic acid (degradation product of GL). Values are presented as means.

Figure 1: Average daily glyphosate intake of the experimental groups per kg body weight (BW) (Values are presented as means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



BCS, BW, water intake, DMI, NE intake and NE balance are shown in Table 2. These variables were not affected by GL treatment, no matter which CFP, while an interaction for CFP and t was observed ($p < 0.001$). The interactions were driven by the concentrate proportion in the ration presented with DMI in Figure 2. The mentioned performance parameters increased in HC groups and decreased in LC groups over the experimental time. This is also illustrated by the data of measured energy content, which was lower in LC groups (in Groups CON_{LC} and GL_{LC}, 6.6 and 6.6 NE_L MJ/kg DM, respectively) than in HC groups (CON_{HC}, and GL_{HC}, 7.1 and 7.2 NE_L MJ/kg DM, respectively). No interactions between GL and CFP were detected.

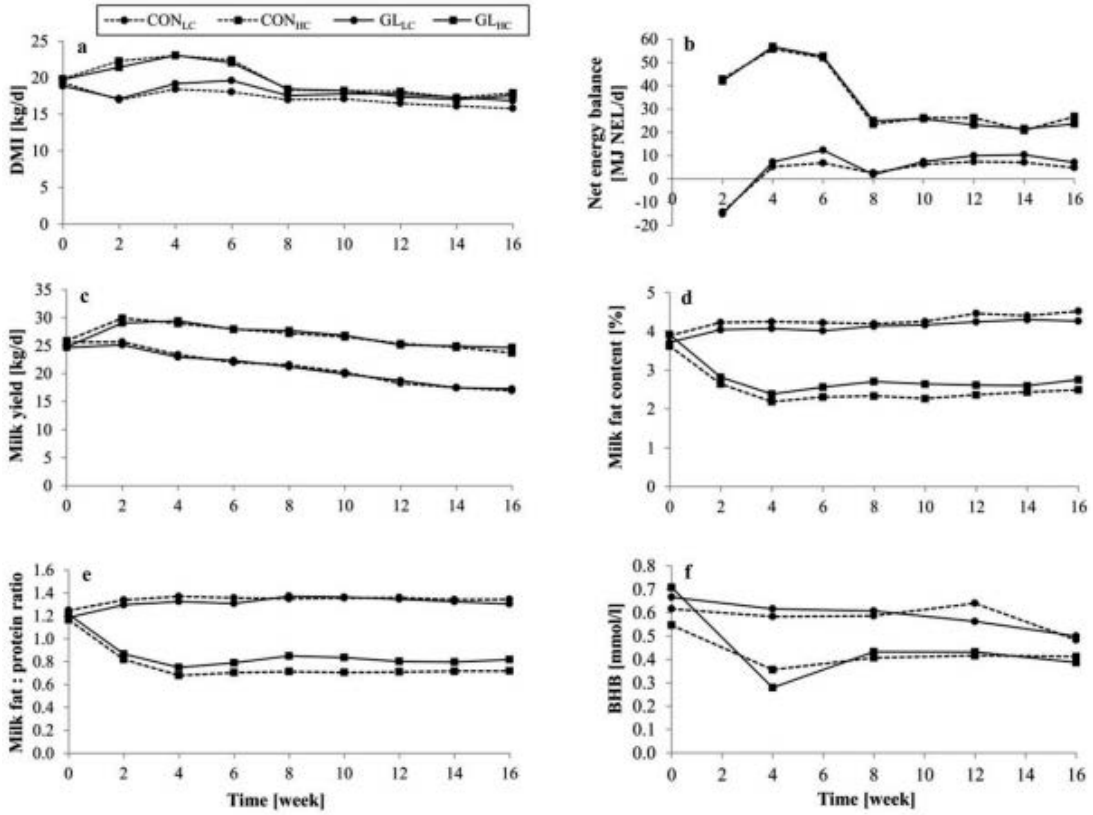
Table 2: Effects of glyphosate residues and concentrate feed proportion (CFP) in total mixed rations (TMR) on body condition score, body weight, dry matter intake (DMI), net energy intake and net energy balance.

	Control (CON)		Glyphosate (GL)		*p*-Value[*]					
	LC[#] (n = 16)	HC[†] (n = 16)	LC (n = 14)	HC (n = 15)	GL	CFP	t[§]	CFP × GL	CFP × t	GL × t
Body condition score	2.8 ± 0.1	3.2 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	0.469	0.018	0.021	0.673	<0.001	0.689
Body weight [kg]	646 ± 5	675 ± 5	641 ± 5	669 ± 5	0.232	0.870	<0.001	0.240	<0.001	0.070
DMI [kg/d]*	17.3 ± 0.3	19.7 ± 0.3	18.0 ± 0.3	19.4 ± 0.3	0.434	<0.001	<0.001	0.094	<0.001	0.707
Water intake [kg/d]	73.8 ± 2.7	82.3 ± 2.7	78.4 ± 2.9	84.1 ± 2.8	0.469	0.018	<0.001	0.619	0.007	0.281
Net energy intake [MJ NE_L/d]	112 ± 4	145 ± 4	112 ± 4	144 ± 4	0.990	<0.001	< 0.001	0.915	<0.001	0.845
Net energy balance [MJ NE_L/d]	3.3 ± 2.6	34.3 ± 2.6	5.1 ± 2.8	33.8 ± 2.7	0.769	<0.001	<0.001	0.631	<0.001	0.853
BHB[‡] [mmol/l]	0.58 ± 0.02	0.43 ± 0.02	0.59 ± 0.02	0.45 ± 0.02	0.545	<0.001	<0.001	0.805	<0.001	0.222
Glucose [mg/dl]	59.6 ± 1.13	61.0 ± 1.13	60.8 ± 1.21	59.9 ± 1.17	0.987	0.837	<0.001	0.333	0.030	0.418
NEFA[¶] [mmol/l]	0.22 ± 0.02	0.20 ± 0.02	0.22 ± 0.02	0.29 ± 0.02	0.012	0.175	<0.001	0.013	0.052	0.696

[#]LC, low concentrate proportion (30% concentrate in TMR); [†]HC, high concentrate proportion (60% concentrate in TMR); [§]t, time effect of trial week; [+]GL × CFP × t ($p > 0.05$) for all variables; *analysed with DMI week 0 as covariance factor; [‡]BHB, β-hydroxybutyrate; [¶]NEFA, non-esterified fatty acids. Values are presented as LS means ± SE (standard error).

The BHB concentrations in blood decreased in HC groups (CFP × t; $p < 0.001$), as presented in Figure 2. Glucose showed the same interaction (CFP × t; p = 0.030) but less pronounced, as presented in Table 2. NEFA concentrations in blood showed a trend for an interaction between CFP and t (p = 0.052). The higher NEFA concentrations in GL groups at the beginning of the trial and in Group GL_{HC} at the end of trial should not be interpreted due to the interaction between CFP and GL (p = 0.013) for this variable.

Figure 2: Effects of glyphosate residues and concentrate feed proportion in total mixed rations on dry matter intake (DMI) (A), net energy balance (B), milk yield (C), milk fat content (D), milk fat: protein ratio (E) and β-hydroxybutyrate (BHB) (F) during 16-weeks trial in established lactation period (Values are presented as LS means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



Measurements of pooled milk samples revealed virtually no incidences of GL in milk (LOQ < 0.01 mg/kg). The time-dependent increase of milk yield in the HC groups and decrease in the LC groups resulted in an interaction (CFP × t; $p < 0.001$) presented in Figure 2. In contrast, LC groups increased and HC decreased in milk fat content and milk fat yield, milk protein yield, milk lactose yield, milk fat:protein ratio, milk urea (CFP × t; $p < 0.001$) and slightly in FCM (CFP × t; $p = 0.007$) and ECM (CFP × t; $p = 0.076$); all milk variables are presented in Table 3. The data of milk protein content was affected by t ($p < 0.001$) and CFP ($p = 0.036$) for all groups, while milk lactose content displayed a significant time-effect and a trend for an interaction between CFP and GL (CFP × GL; $p = 0.090$). An interaction between GL, CFP and t was found for milk yield and FCM ($p = 0.011$ and $p = 0.023$). Milk urea showed an interaction (GL × t; $p = 0.004$) between t and GL; this was due to slight differences between Groups CON_{HC} and GL_{HC} at the beginning of the experiment and disappeared in the course of the experiment. These differences were not significant but an impact on the detected interactions cannot be excluded.

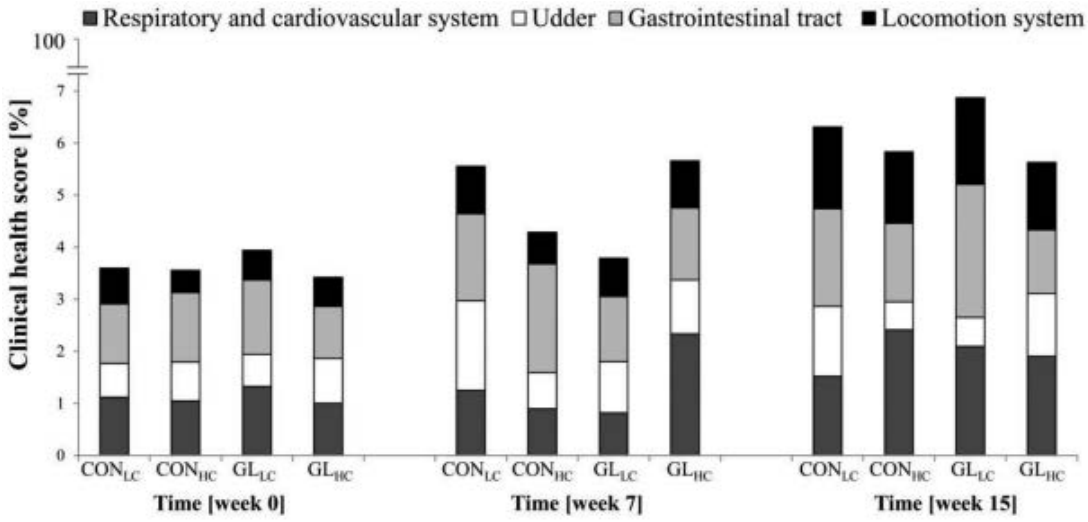
Table 3: Effects of glyphosate residues and concentrate feed proportion (CFP) in total mixed ration (TMR) on milk performance parameters.

	Control (CON)		Glyphosate (GL)		*p*-Value						
	LC[#] (n = 16)	HC[†] (n = 16)	LC (n = 14)	HC (n = 15)	GL	CFP	t[$]	CFP × GL	CFP × t	GL × t	GL × CFP × t
Milk yield [kg/d]	21.2 ± 1.0	26.7 ± 1.0	21.1 ± 1.1	26.7 ± 1.0	0.933	<0.001	<0.001	0.932	<0.001	0.855	0.011
Fat-corrected milk [kg/d]	22.6 ± 1.0	21.3 ± 1.0	21.9 ± 1.1	21.8 ± 1.0	0.905	0.523	<0.001	0.566	0.007	0.366	0.023
ECM[‡] [kg/d]	22.2 ± 0.9	22.1 ± 0.9	21.5 ± 1.0	22.7 ± 1.0	0.999	0.549	<0.001	0.484	0.076	0.696	0.179
ECM/dry matter intake [kg/kg][*]	1.26 ± 0.03	1.14 ± 0.03	1.22 ± 0.03	1.19 ± 0.03	0.945	0.036	<0.001	0.173	<0.001	0.931	0.980
Milk fat content [%]	4.27 ± 0.15	2.52 ± 0.15	4.11 ± 0.16	2.78 ± 0.15	0.749	<0.001	<0.001	0.170	<0.001	0.384	0.955
Milk fat yield [kg/d]	0.92 ± 0.04	0.69 ± 0.04	0.88 ± 0.05	0.74 ± 0.05	0.889	<0.001	<0.001	0.204	<0.001	0.612	0.910
Milk protein content [%]	3.19 ± 0.04	3.27 ± 0.04	3.13 ± 0.05	3.25 ± 0.05	0.343	0.036	<0.001	0.699	0.107	0.784	0.399
Milk protein yield [kg/d]	0.69 ± 0.03	0.88 ± 0.03	0.68 ± 0.03	0.87 ± 0.03	0.646	<0.001	<0.001	0.952	<0.001	0.633	0.240
Milk lactose content [%]	4.72 ± 0.05	4.69 ± 0.05	4.73 ± 0.05	4.86 ± 0.05	0.067	0.313	<0.001	0.090	0.249	0.194	0.221
Milk lactose yield [kg/d]	1.04 ± 0.05	1.27 ± 0.05	1.03 ± 0.05	1.31 ± 0.05	0.853	<0.001	<0.001	0.668	<0.001	0.709	0.509
Milk urea [mg/kg]	162 ± 7	77 ± 7	157 ± 7	88 ± 7	0.689	<0.001	<0.001	0.282	<0.001	0.004	0.453
Somatic cell count [log10/ml]	2.13 ± 0.10	2.31 ± 0.10	2.19 ± 0.10	2.28 ± 0.10	0.888	0.195	<0.001	0.649	0.289	0.770	0.911
Milk fat:protein ratio	1.34 ± 0.05	0.77 ± 0.05	1.31 ± 0.05	0.86 ± 0.05	0.520	<0.001	<0.001	0.230	<0.001	0.200	0.990

[#]LC, low concentrate proportion (30% concentrate in TMR); [†]HC, high concentrate proportion (60% concentrate in TMR); [$]t, time effect of trial week; [‡]ECM, energy corrected milk; [*]analysed with value of week 0 as covariance factor. Values are presented as LS means ± SE (standard error).

The average values of the total health score in Groups CON_{LC}, CON_{HC}, GL_{LC} and GL_{HC} were 5.2 ± 0.4, 4.5 ± 0.4, 4.9 ± 0.5 and 4.9 ± 0.5, respectively (LS means ± SE). The total health score (Figure 3) showed for all groups a time effect (t; $p < 0.001$) and an interaction between all three tested values (CFP × t × GL; $p = 0.010$).

Figure 3: Average daily glyphosate intake of the experimental groups per kg body weight (BW) (Values are presented as means). CON, non-contaminated ration; GL, glyphosate contaminated ration; LC, low concentrate proportion (30% concentrate in TMR); HC, high concentrate proportion (60% concentrate in TMR).



Discussion

GL is worldwide the most used active substance in non-selective herbicides in agriculture (Duke and Powles 2008). According to von Soosten et al. (2016), Krüger et al. (2013); Krüger et al. (2014a)) and Rulff et al. (2016) dairy cows are exposed to 0.08–0.9 mg GL per day due to GL contamination in common dairy cow rations. Up to now, the effects of GL on health of dairy cows was solely deduced from field observations and in vitro studies. Therefore, the present exact-feeding experiment on dairy cows in practical conditions was designed to investigate the effects of GL-contaminated feedstuffs which were generated by a legal application and represent a worst-case scenario. Here, the daily exposure was approximately four-fold higher than the maximum observed under average feeding conditions as outlined above.

The cows were fed two different crude fibre and concentrate proportions in their rations with the intention to investigate whether the overall effects of GL depend on different ruminal conditions as

triggered by different concentrate feed proportions. The average daily GL intake in GL groups amounted to 79.1 mg and in both groups straw formed the major GL source. GL was used by spray application, so that the plants' surface was the most contaminated part. This may explain the high straw contamination and the rather small contamination of peas and wheat kernels which are protected by their husks and pods. In CON groups, a small daily GL intake with an average value of 0.8 mg/d was observed. This corresponds to the average value of GL concentration in usual dairy rations (von Soosten et al. 2016). The half-life of GL soils residues varies between 2.8 and 500.3 d DT_{50} (50% dissipation time) (EFSA 2015). Therefore, GL contamination in plants might be originated from soil residues. Overall, GL exposure in GL groups was about 100 times higher than in CON groups. In this study, 121 milk samples were analysed for GL and AMPA. No positive findings above the validated LOQ could be reported. These findings correspond to the study of von Soosten et al. (2016), who reported that milk was virtually free from GL and AMPA, while 8% of daily consumed GL were excreted in urine and 61% passed the digestive tract of dairy cows unmetabolised and were excreted with faeces. The high proportion of GL excreted with faeces also indicates a high concentration of unmetabolised GL in the gastrointestinal tract and the possibility for GL to interact with microorganisms within the ingesta passage time. The chemical composition of the cows ration offered the aimed components within the normal range for the respective feedstuffs with a high fibre content and low energy in LC groups and a low fibre content and high energy in HC feed groups (DLG 1997). Therefore, our experimental feeding design offered adequate conditions to test the effect of GL in rations with different concentrate parts on performance, energy metabolism and health characteristics of dairy cows.

Performance and health

In the present trial, the drop of DMI and the negative energy balance in LC groups at the beginning might be caused by the required adaptation to the experimental ration. The ME and NE_L concentrations of feed confirm the intended differences in energy supply between LC and HC groups, GL showed no influence on both of them and no differences between GL and CON groups were detectable.

Consequently, BW and BCS, NE intake and NE balance were affected by the concentrate feed proportion of the ration, but GL contamination had no influence on the parameters in both rations. The results are in accordance with the results of Donkin et al. (2003), who found a similar DMI of GL-tolerant RoundupReady corn sprayed with Roundup Ultra® (Monsanto Company, St. Lois, MO) and non-transgenic control corn.

Milk yield differed in accordance to the concentrate proportion of the ration and dropped slightly over time due to the advanced lactation period. Based on field observations, Krüger et al. (2014b) postulated milk yield decrease in GL fed cows; this could not be proven by our feeding trial. There was no change in the amount and composition of milk provable in GL groups compared to CON groups. Donkin et al. (2003) could not detect any influence of GL on milk components and on dairy cow performance. The lack of influence of GL on milk components might be related to the absence of GL residues in milk, demonstrating that milk is no major excretion pathway of GL. Consequently, direct effects of GL residues on synthesis of milk components in the mammary gland can be most probably excluded.

On the contrary, different dietary energy levels exhibited significant effects on concentrations and amounts of milk protein, milk fat content, milk lactose, milk urea, SSC and milk fat/protein ratio.

General energy metabolism was not adversely influenced by dietary treatments as blood NEFA, BHB and glucose values were in the normal reference range (Kraft and Dürr 2005). However, BHB levels were significantly influenced by dietary energy level. The overall blood BHB levels might result either from ketogenesis or from ruminal nutrient metabolism. Thus, the higher BHB levels in cows fed the LC diets might reflect a diet induced higher ruminal release of butyrate and/or a slight energy deficit compared to their HC-fed counterparts. Both dietary energy levels were not influenced by GL.

Regarding the putative health effects of GL, Rulff et al. (2016) considered GL being a part of pathogenesis of downer cow syndrome. Furthermore, Krüger et al. (2013, 2014b) and Ackermann et al. (2015) related possible symptoms of *C. botulinum* disease (drop of milk production, mobility disorders, retracted abdomen and forced respiration) to a forced production of BoNT, probably as a result from a

decline of enterococci population in gastrointestinal tract. This effect should be more pronounced in high fibre rations. Krüger et al. (2013) termed GL reasonable for the imbalance of the microorganisms, whereas Riede et al. (2016) could not show any effect of GL on microorganisms in their RUSITEC study. It should be noted that both were in vitro studies which are not able to represent realistic exposure conditions. But similar results were found in a study about the effect of GL contaminated feed on wethers, where GL showed no indication of an impairment of rumen bacteria or a shift in rumen microbial population, neither the group of cellulolytic bacteria, nor the group of amylolytic bacterial species (Hüther et al. 2005).

In our study, the general health status including the previously mentioned symptoms were evaluated. The general health status of cows is, among other factors, related to the health of the rumen microbiome (Zebeli et al. 2015). In our study, the cows showed less than 10% symptoms in total clinical health score. Symptoms occurred without discernible pattern, for instance both GL groups were scored less than CON_{LC} but more than CON_{HC}. Despite the clear influence of the different concentrate proportions of the rations on several parameters, which probably caused very different gastro-intestinal microbial conditions, an effect of GL-residues in feed on performance, metabolism and health characteristics of dairy cows could not be observed in the present trial.

3. Assessment and conclusion

Assessment and conclusion by applicant:

About 30 cows (distributed in two subgroups) were fed with glyphosate-treated commodities for 17 weeks. During this period the exposure of these cows to parent glyphosate residues via feed was about 0.110-0.120 mg/kg bw/day (Figure 1). None of the analysed milk samples (presumably about 60 pooled samples from the two subgroups fed with glyphosate-treated commodities) showed residues of parent glyphosate or AMPA above the limit of quantification of 0.01 mg/kg. This is fully in line with the results of the GLP cow feeding studies submitted in the dossier, which also show that the transfer (if any) of glyphosate-derived residues in cow milk is extremely low. 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.

However, the main objective of the publication was to investigate the impact of glyphosate residues in feed on health and performance of dairy cows. No significant effects were identified but this part of the publication is not considered relevant to the residue section.