

Validation of an Analytical Method for the Detection and Quantification of Glyphosate and Related Residues in Food

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ABSTRACT

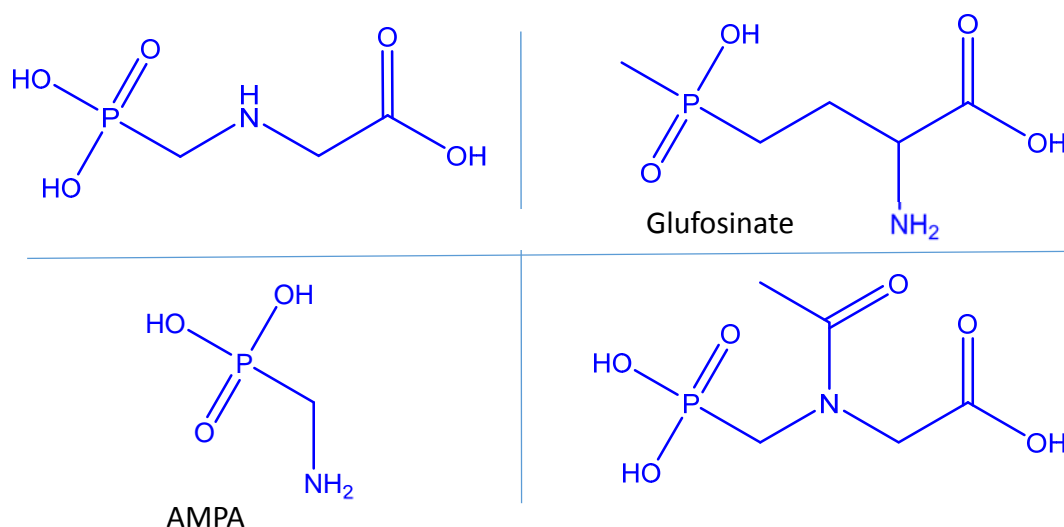
This work describes a method to detect and quantify glyphosate, N-acetyl glyphosate, and glufosinate in foods. The analysis of aminomethylphosphonic acid (AMPA) was only investigated for qualitative purposes since AMPA is not part of the tolerance expression for glyphosate. The method uses an aqueous extraction, consisting of 50 mM Acetic acid and 10 mM EDTA in water, followed by an Oasis HLB column cleanup. After filtration, the final extract was analyzed by LC-MS/MS using electrospray ionization (ESI) in the negative ionization mode. Stable isotopically labeled standards were used for the quantitation of glyphosate and glufosinate and matrix matched standard calibrations were used for all four analytes. A single lab validation (Level 2) was performed by the pesticides section of FDA Pacific Southwest Food and Feed Laboratory (PSFFL) which showed adequate results across twelve different commodity types encountered in the pesticide program. Recoveries, precision, linearity, and limits of quantitation were determined and found to be acceptable for almost all analyte/commodity combinations.

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INTRODUCTION

Glyphosate has been reported to be the most widely used agricultural chemical in the world¹. It is found on an increasing variety of foods, and there are EPA tolerances on various agricultural goods. In response, FDA has developed analytical methods for glyphosate, glufosinate, N-acetyl glyphosate, and aminomethylphosphonic acid (AMPA)²⁻⁵. Structures are shown in Scheme 1.

Scheme 1. Chemical structures of the analytes in this method.



The inclusion of three analytes, in addition to glyphosate, provides greater herbicide coverage and supports the detection of glyphosate. Glufosinate is an herbicide with a similar mode of action to glyphosate and similar structure. Both AMPA and N-acetyl glyphosate are metabolites of glyphosate. To determine compliance with the law when analyzing commodities such as meat and egg, matrices in which the metabolite might be expected, the quantified amount of glyphosate and N-acetyl glyphosate are summed. While the presence of AMPA can be used to confirm the presence of glyphosate, it is not part of the tolerance expression for glyphosate, and quantitation of AMPA is not necessary. As of September 2017, EPA tolerances for glyphosate ranged from 0.05 $\mu\text{g/g}$ to 200 $\mu\text{g/g}$, and tolerances for glufosinate ranged from 0.05 $\mu\text{g/g}$ to 25 $\mu\text{g/g}$ depending on the commodity⁶.

Glyphosate is a difficult compound to include in the current FDA multi-residue pesticide methods. Common multi-class extraction (e.g. QuEChERS) and detection (e.g. C18-based chromatography/positive mode mass spectrometry) methods are not effective due to the high polarity of the compound. To compensate, several methods have been developed for the analysis of glyphosate. Methods involving derivatizing the compound are especially sensitive but cumbersome and time consuming to carry out⁷⁻⁹. Reports have described aqueous extraction methods for detecting glyphosate in egg, honey, corn, milk, and soybean²⁻⁵. In conjunction with ORS and CFSAN, PSFFL worked to unify the methods to make them more practical for routine use. The unified method utilizes an aqueous extraction coupled to reverse phase chromatography that makes use of an ion pairing agent²⁻⁵. To test the method, PSFFL developed a validation scheme that included a wide range of matrices. Twelve matrices were chosen as

characteristic for different categories of foods encountered in the pesticides program¹⁰: high water content (carrot), high acid content (orange), dry/high sugar (honey), dry/high oil (soybean), some water/high oil (avocado), dry/high starch (corn), meat (fish), milk (whole cow's milk), eggs (chicken egg), animal fat (butter), oils from oily fruits (olive oil), and difficult/complex (tea).

PSFFL completed a single lab validation (Level 2)¹⁰ of the method in these matrices and determined several quality characteristics for the method including recovery efficiency, precision, LOQ, and linearity. The method covers glyphosate, glufosinate, N-acetyl glyphosate, and AMPA. AMPA is included for identification purposes only and is not quantitated since it is not part of the tolerance expression for glyphosate.

EXPERIMENTAL

Materials and Equipment

Note: suppliers and part numbers provided denote products used in this method validation. Other suitable products are available.

- Extraction solvent: 50 mM Acetic acid and 10 mM EDTA in water with 50 µg/L of isotopically labeled internal standards. Preparation: Mix 2.9 mL of acetic acid and 3.7 g of Na₂EDTA in HPLC Purified Water. Add 2.5 mL of a solution of both internal standards at a concentration of 20 µg/mL and dilute to 1000 mL with HPLC Water.
- Formic Acid, 98%
- Tetrabutylammonium hydroxide (TBA-OH) 0.4 M in water, HPLC grade.
- Acetonitrile, LCMS grade
- Petroleum ether
- Methylene chloride
- DI water
- Standards: AMPA, Glufosinate ammonium, N-acetyl glyphosate, Glyphosate (EPA, #868-713, 1905-223, 1638-745, and 156-726)
- Internal standards: Glyphosate-¹³C₂ ¹⁵N and Glufosinate D₃ hydrochloride (Toronto Research Chemicals, cat. #G765002 and G596952)
- OASIS HLB cartridges (60 mg x 3 cc, 30 µm) (P/N WAT094226)
- Syringe and 25 mm x 0.2 µm nylon syringe filter
- Centrifuge with capacity for 50 mL and 15 mL centrifuge tubes capable of achieving 8000 rpm

- Polypropylene HPLC vials or glass vials with polypropylene inserts
- Phenomenex Luna C8 (150 x 2.0 mm, 5 μ m) HPLC column (P/N 00F-4040-B0)
- Phenomenex Luna C8(2) (150 x 4.6 mm, 5 μ m) HPLC column (P/N 00F-4249-E0) , optional
- HPLC Guard Column: Phenomenex KrudKatcher (P/N AFO-8497)
- MWCO filter: EMD Millipore Amicon Ultra-4 3kDa molecular weight cutoff centrifuge filter (P/N UFC800396)
- Rapid Vap Heater/Evaporator (optional)
- 50 mL centrifuge tube containing built-in 0.45 μ m Nylon filter (optional)
- Thomson 0.2 μ m Nylon filter sample vial (optional)

Extraction Procedure

Notes: the analytes can adhere to glass. Best practice includes using plastic consumables and components whenever possible, especially for long term storage.

1. Weigh sample into 50 mL centrifuge tube (5 g for high moisture or 2 g for dry ("low moisture") or fatty samples(> 2% fat))
2. Add 25 mL extraction solvent to high moisture samples or 10 mL extraction solvent to dry/fatty samples. For fatty samples, also add 6 mL petroleum ether (or methylene chloride).
3. Shake for 10 minutes at 1,000 strokes/minute. Centrifuge for 5 minutes at 5000 rpm.
4. Place Oasis HLB column in a 15 ml centrifuge tube. Add ~2 mL supernatant from extraction tube to cartridge. For fatty samples, take the ~2 mL from the lower aqueous layer. Lightly cap the 15 mL tube.
5. Centrifuge for 2 minutes at 3000 rpm. (For samples consisting of greater than 10% protein, after going through the column, remove the HLB from the tube and heat the sample at 80°C for 20 minutes. Centrifuge again at >5000 rpm for 5 minutes. (An alternative to heating proteinaceous samples is to use MWCO filters which are fast and clean, but can be expensive.)
6. If filtrate remains cloudy, filter with a 0.45 or 0.22 μ m nylon filter into a polypropylene HPLC vial (or a glass HPLC vial fitted with a plastic insert. Thomson 0.2 μ m Nylon Filter vial is a recommended option). The final concentration is 0.2 g/ml.

Analysis Method

LC system and conditions: Shimadzu HPLC System (Two LC-20AD pumps, Sil-20A Autosampler, CTO-20AC Column Oven).

Mobile phase A: 4 mM TBA-OH in HPLC grade water, pH 2.8. Preparation: Add 10.0 ml of 0.4 M TBA-OH to approximately 990 mL of HPLC Water. Adjust the pH to 2.80 ± 0.05 with formic acid. Store in a dark bottle.

Mobile phase B: Acetonitrile, LCMS grade.

Flow: 0.3 mL/min

Gradient: 0-1 min: 5% B. 1-5 minutes: from 5%-90% B. Hold at 90% B for 2 minutes. Ramp back to 5% B over 1 min. Re-equilibrate for 6 min.

Injection volume: 10 μ L

Column temperature: 40°C

Divert valve: Divert from 0-1 min and after 6 min

MS system and conditions: Sciex 5500 QTrap in negative mode. Both Q1 and Q3 were operated at unit resolution. See Table 1 for conditions for the electrospray source.

Table 1. Electrospray source conditions

Parameter	Value
Curtain gas	35
Collision Gas	Medium
Ion Spray Voltage	-4000 V
Gas 1	65
Gas 2	65
Temperature	450-650°C

RESULTS AND DISCUSSION

The glyphosate methods utilized aqueous extraction in LIB 4595, 4595, 4604 and 4613 that were very effective for these polar analytes but, when trying to unify the methods, the sensitivity was problematic. The extraction solution contained 50 mM Acetic acid and 10 mM EDTA in water. Acetic acid lowered the pH to precipitate some of the proteins, and the EDTA prevented chelation complex formation between polyvalent metal ions and the analytes. The move to ion pair reverse phase chromatography improved and stabilized the chromatography so that all matrices could be analyzed more successfully.

There were also initial challenges with matrices like eggs. Extracts remained cloudy even after filtration and caused column fouling. It was determined that proteins were not being fully removed during clean up, and another step would be needed to remove the proteins. Heating the extracts after the SPE cleanup step denatures the proteins, and a centrifugation step removes them from solution. If a lab does not want to heat the samples, a MWCO filter can alternatively be used to remove high-molecular weight proteins.

AMPA, the smallest molecule of the four analytes, proved the most challenging to consistently recover. Rather than focus on getting adequate recoveries of AMPA, while potentially lowering the sensitivity of the method for the critical analytes, glyphosate and glufosinate, the choice was made to include AMPA for informational purposes only, since it is not part of the tolerance expression for glyphosate. While AMPA can be recovered using this method, the results are not quantitative. While linearity was acceptable for AMPA across the analytes, recoveries were low or inconsistent leading to high standard deviations, LOQs, and LODs in several commodities.

Table 2 shows the MS transitions used to identify and quantitate the four analytes and related internal standards. Three MRM transitions were used for each analyte and one MRM transition was used for the internal standards. Identification was confirmed per the guidelines for the pesticides program¹¹. Two stable isotopically labeled internal standards were used in this study: glyphosate ¹³C₂ ¹⁵N and glufosinate D₃. Although stable isotopically labeled standards are available for the other analytes and might improve quantitation for AMPA and N-acetyl glyphosate, we chose to only include the above two since it was cost effective for routine use.

Table 2. MRM transitions monitored for the four analytes and related internal standards (IS). Quantitation ions are listed as transition 1 for each of the analytes. Glyphosate ¹³C₂ ¹⁵N was used in the quantitation of AMPA and glyphosate. Glufosinate D₃ was used in the quantitation of glufosinate. No IS was used for the quantitation of N-acetyl glyphosate.

Transition	Q1	Q3	DP	EP	CE	CXP
AMPA 1	110	63	-40	-11	-30	-9
AMPA 2	110	79	-40	-11	-34	-9
AMPA 3	110	81	-40	-11	-34	-9
Glufosinate 1	180	63	-60	-11	-66	-9
Glufosinate 2	180	95	-60	-11	-19	-9
Glufosinate 3	180	85	-60	-11	-25	-9
Glufosinate IS	183	63	-60	-11	-40	-9
Glyphosate 1	168	63	-30	-11	-28	-9
Glyphosate 2	168	79	-30	-11	-56	-9
Glyphosate 3	168	150	-30	-11	-16	-9
Glyphosate IS	171	63	-30	-11	-28	-9
N-acetyl Glyphosate 1	210	150	-40	-11	-20	-9
N-acetyl Glyphosate 2	210	63	-40	-11	-40	-9
N-acetyl Glyphosate 3	210	163	-40	-11	-18	-9

In total, twelve matrices were covered in this study: avocado, butter, carrot, corn, egg, fish, honey, milk, olive oil, orange, soybean, and tea. Each matrix was spiked at four fortification levels: 20, 50, 250, and 500 ng/g with two batches of triplicate samples analyzed on different days (total n=6). Blank matrices were also examined for each commodity. No incurred residues were observed in any matrix. Each sample was quantitated against a matrix matched standard curve. A set of representative extracted ion chromatograms is shown in Figure 1 for honey. The analytes show good chromatographic separation. At the 20 ng/g level, it is clear that sensitivity is adequate for glufosinate, glyphosate, and N-acetyl glyphosate, but AMPA has low overall signal and signal-to-noise ratio (S/N).

Spike recoveries were performed at four concentrations (20, 50, 250, and 500 ng/g). Recoveries from 70-120% were considered acceptable. Most recoveries for glufosinate, glyphosate, and N-acetyl glyphosate were within acceptable limits (Table 3). The recovery of N-acetyl glyphosate was high for corn at 20 and 250 ng/g and low for olive oil at 250 ng/g. The recovery of glyphosate in tea was high at the 20 ng/g level. Recoveries were low for AMPA across multiple matrices including avocado, carrot, corn, egg, and tea.

Precision was adequate across most of the matrices and analytes. The glufosinate and glyphosate standard deviations were acceptable (i.e. RSD < 25%) for all matrices and at all levels. Especially at the 20 ng/g level, there were several matrices with high standard deviations for AMPA. Fish, milk, and soybean had high standard deviations at the 20 ng/g level. Tea also had high standard deviations at the 250 ng/g level for AMPA. There were also some high standard deviations seen in the N-acetyl glyphosate results. At the lowest spike level, 20 ng/g, standard deviations were high for olive oil and orange. They were also high for olive oil at the 250 ng/g level. High RSD values for N-acetyl glyphosate in orange and olive oil suggest that there may be some concerns with the partition of this analyte in oil (including surface citrus oil). In the future, the laboratory will operationally monitor this analyte in suspect matrices.

Limits of quantitation (LOQ) were estimated from the S/N of the 20 ng/g replicates as described in ORA-LAB.010¹¹ (Table 4). Briefly, LOQs were calculated by generating the S/N of the quantitation transition using the mass spectrometer's software. The required S/N, 10, was then multiplied by the concentration of the sample and divided by the actual S/N determined by the software. The resulting value is the LOQ estimation (i.e. the concentration required to provide a S/N of 10). LOQs were all below 10 ng/g for glyphosate and N-acetyl glyphosate. LOQs were slightly above 10 ng/g in corn for glufosinate (11 ng/g), but under 10 ng/g for the other matrices. LOQs for AMPA were all under 10 ng/g except for soybean (14 ng/g) and tea (93 ng/g). The LOQ is noticeably high for AMPA in tea. Tea was a difficult matrix in general, and the co-extractants appear to adversely affect AMPA, the smallest and earliest eluting compound.

A similar estimation was made for the limit of detection (LOD) using the same calculation; however, for LOD, a S/N of 3 was used rather than 10 for LOQ. Only AMPA in tea had an estimated LOD above 10 ng/g. For most of the other analytes and matrices, the LOD was estimated to be in the very low parts per billion.

Linearity was determined by creating a response curve for each analyte in each matrix (Table 5). For most of the matrices, a five-level curve was created from 4 ng/ml to 200 ng/ml, and the coefficient of determination value (r^2) was calculated from that curve. Response curves were created for each day of analysis, and the resulting r^2 values were averaged for each matrix. The concentrations in the vial were equivalent to 20-1000 ng/g in the sample. A four-level curve, from 0 to 100 ng/ml (equivalent to 0-500 ng/g in the sample), was used for corn, avocado, and carrot. Coefficient of determination values above 0.990 were considered acceptable¹¹. All curves were above 0.990 and most were above 0.995 except AMPA in orange (0.9944) and tea (0.9938).

While the examination of samples containing incurred glyphosate or glufosinate residues would have been helpful and desirable to demonstrate the performance of the method, it was not possible during the validation process.

CONCLUSIONS

The unified glyphosate method was developed to improve the efficiency of glyphosate analysis across ORR laboratories. It has been successfully used to extract the target analytes from a range of matrices and validated in twelve diverse matrices. Results were especially favorable for glyphosate and glufosinate. Even without a matching internal standard, most of the N-acetyl glyphosate results were also within the acceptable range with outliers seen for difficult matrices like oil. While results for AMPA indicate that the method is not sufficient for quantitation of this analyte, AMPA is not part of the tolerance expression for glyphosate and quantifying this analyte is not necessary. The extraction method is easy to perform, and the analytical method is not cumbersome. Results from this work show that the method is fit-for-purpose for use across the products analyzed by FDA.

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Figure 1. Extracted ion chromatograms of the quantitation ions for AMPA (a), glufosinate (b), glyphosate (c), and N-acetyl glyphosate (d) from a honey extract spiked with 20 ng/g of each analyte.

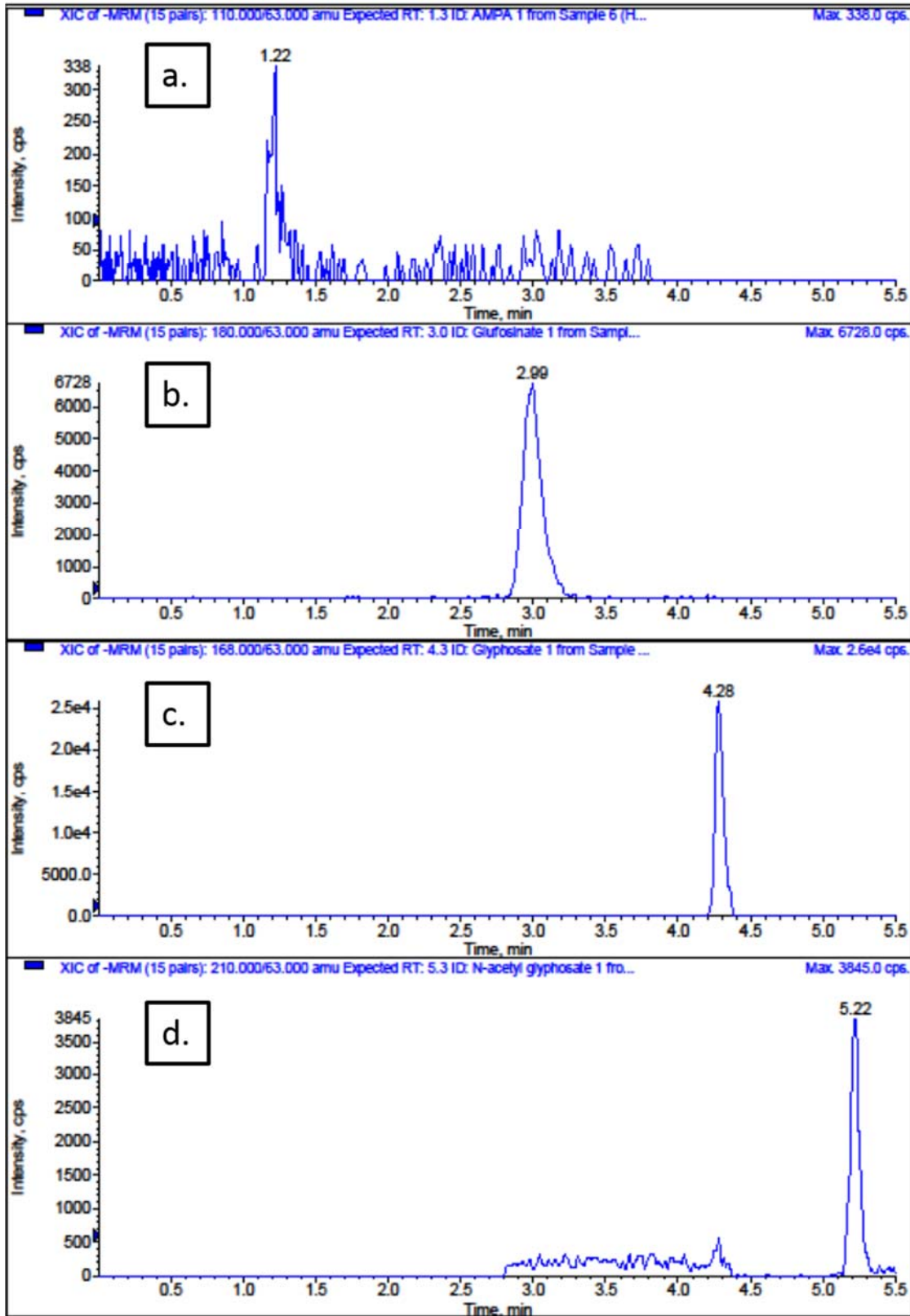


Table 3. Recovery and precision data for the four analytes. Values have been normalized to 100%. Data outside of specifications are italicized. n=6 for each value listed.

AMPA*		Glufosinate		Glyphosate		N-acetyl Glyphosate	
Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)

20 ng/g

Avocado	<i>43.0</i>	3.1	98.0	3.7	106.3	1.8	110.7	3.4
Butter	100.2	6.3	98.3	3.5	98.3	9.9	100.0	17.1
Carrot	<i>68.1</i>	5.0	98.2	4.1	108.2	3.8	109.6	3.0
Corn	<i>29.1</i>	2.0	96.1	5.0	101.8	2.6	<i>123.3</i>	2.3
Egg	75.5	12.4	100.5	2.6	112.5	7.3	88.5	5.3
Fish	110.3	<i>26.3</i>	97.9	8.5	104.5	10.4	112.1	20.1
Honey	<i>57.8</i>	6.8	91.2	3.6	90.2	5.9	100.1	6.0
Milk	116.1	<i>26.0</i>	103.1	7.4	109.4	9.5	97.3	17.8
Olive Oil	105.5	11.4	100.4	3.8	112.5	5.6	82.3	<i>40.9</i>
Orange	86.8	19.6	98.1	7.0	84.1	10.1	82.5	<i>30.6</i>
Soybean	<i>123.4</i>	<i>72.1</i>	103.7	4.9	102.3	8.9	95.6	17.4
Tea	<i>68.6</i>	21.4	88.3	8.1	<i>124.9</i>	16.7	103.6	7.5

50 ng/g

Avocado	<i>18.0</i>	4.9	112.6	0.9	101.6	0.6	109.0	3.2
Butter	98.2	4.6	97.8	0.8	100.4	10.2	97.0	11.1
Carrot	<i>26.2</i>	2.3	108.1	2.7	102.2	2.0	106.5	0.0
Corn	<i>27.0</i>	0.7	95.6	0.4	104.3	0.3	118.6	0.3
Egg	106.3	16.5	101.0	2.3	100.8	3.8	99.0	2.3
Fish	105.8	14.5	102.1	1.5	104.9	7.5	112.7	16.8
Honey	97.5	6.6	105.3	3.6	101.5	4.7	115.5	16.5
Milk	108.0	6.3	103.5	4.2	107.2	3.2	113.6	15.6
Olive Oil	102.6	5.5	99.1	1.1	102.6	3.7	92.1	7.5
Orange	116.5	18.6	99.2	3.2	98.4	4.8	106.3	17.7
Soybean	92.1	14.6	98.1	4.1	98.0	3.5	95.1	17.1
Tea	<i>68.3</i>	23.4	79.7	9.5	112.9	9.2	91.3	7.2

Table 3 (continued)

AMPA		Glufosinate		Glyphosate		N-acetyl Glyphosate	
Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)

250 ng/g

Avocado	8.3	1.7	98.8	2.8	109.6	1.8	105.4	0.9
Butter	100.4	8.2	98.9	0.6	99.5	3.3	101.7	12.9
Carrot	24.1	0.4	105.2	0.8	106.4	1.1	99.7	3.4
Corn	21.7	1.4	95.8	1.0	107.8	0.2	120.3	1.4
Egg	71.1	15.2	101.6	3.9	105.9	2.8	94.7	8.0
Fish	115.5	20.4	100.1	0.9	100.7	1.2	102.1	6.9
Honey	102.6	19.8	96.8	5.9	94.9	7.7	95.7	22.7
Milk	97.6	4.6	99.9	2.2	91.2	8.7	91.8	5.0
Olive Oil	106.9	13.4	99.4	3.6	98.6	4.4	63.8	34.8
Orange	87.5	11.7	94.5	6.1	91.3	7.8	104.4	3.5
Soybean	96.4	7.7	100.4	1.7	98.7	2.5	104.9	6.9
Tea	70.1	32.7	95.9	7.3	108.9	7.4	104.2	10.1

500 ng/g

Avocado	8.7	0.9	95.3	0.5	104.2	0.8	104.3	3.1
Butter	100.7	8.7	99.8	1.5	97.8	4.7	110.5	8.6
Carrot	25.9	0.4	101.3	0.7	101.7	1.3	87.3	0.4
Corn	26.9	0.4	94.1	0.1	103.3	0.7	114.4	3.4
Egg	78.0	5.7	100.1	3.2	95.7	6.7	102.4	22.9
Fish	101.3	11.9	98.3	2.8	96.9	1.0	92.7	8.8
Honey	88.6	10.2	92.9	7.7	95.3	10.7	104.3	5.8
Milk	97.7	4.3	99.8	0.8	97.4	3.8	106.1	12.3
Olive Oil	100.5	4.9	97.9	0.7	99.3	3.4	94.9	4.3
Orange	101.2	1.6	100.5	1.7	99.3	1.6	98.3	3.2
Soybean	97.3	8.0	100.4	1.1	98.9	2.3	100.1	2.1
Tea	63.9	18.5	89.0	10.5	87.4	8.6	107.2	10.2

*AMPA results are not quantitative and included for informational purposes only.

Table 4. Estimated LOD and LOQ values (in ng/g) based on SNR data from the spiked samples. Values above 10 ng/g are in italics.

	AMPA		Glufosinate		Glyphosate		N-acetyl Glyphosate	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Avocado	1.4	4.8	2.6	8.6	1.6	5.2	1.6	5.3
Butter	0.4	1.2	0.4	1.3	0.2	0.6	0.4	1.4
Carrot	2.3	7.6	2.6	8.8	2.3	7.7	1.8	5.8
Corn	2.2	7.4	3.2	<i>10.8</i>	2.2	7.5	0.2	0.6
Egg	2.3	7.5	1.9	6.4	0.2	0.8	1.7	5.8
Fish	0.9	3.0	0.7	2.2	1.2	4.1	2.8	9.2
Honey	0.7	2.8	0.8	2.8	0.3	1.0	0.2	0.6
Milk	1.7	5.6	0.9	3.1	1	3.3	2.1	7.1
Olive Oil	0.5	1.5	0.4	1.2	0.2	0.5	0.7	2.4
Orange	2.2	7.3	0.7	2.3	0.9	2.9	0.2	0.8
Soybean	4.2	<i>14.1</i>	1.1	3.8	0.8	2.6	1.4	4.8
Tea	<i>27.9</i>	<i>92.8</i>	1.6	5.3	0.3	1.1	0.3	1.1

Table 5. Coefficient of determination (r^2) values from linear regression models from each of the matrices.

	AMPA	Glufosinate	Glyphosate	N-acetyl Glyphosate
Avocado*	0.9978	0.9998	0.9998	0.9998
Butter	0.9960	0.9960	0.9986	0.9984
Carrot*	0.9968	0.9998	0.9998	1.0000
Corn*	0.9990	1.0000	1.0000	0.9994
Egg	0.9994	1.0000	0.9990	0.9996
Fish	0.9994	0.9996	0.9996	0.9996
Honey	0.9997	0.9994	0.9994	0.9980
Milk	0.9996	0.9996	0.9992	0.9998
Olive Oil	0.9980	0.9998	0.9986	0.9990
Orange	0.9944	0.9996	0.9994	0.9988
Soybean	0.9992	0.9998	0.9998	0.9994
Tea	0.9938	0.9986	0.9970	0.9984

* Linearity data encompasses the blank, 10, 50, and 100 ng/ml levels (corresponding to 0-500 ng/g in the sample). If not noted, regression was performed on results from spiking at 5 concentration levels: 4, 10, 50, 100, and 200 ng/ml (corresponding to 20-1000 ng/g in the sample). All values were averaged over 2 days of analyses.

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