Application Note

Food Testing & Agriculture

Direct Analysis of Glyphosate, AMPA, and Other Polar Pesticides in Food

Ion exchange LC/MS/MS with the Agilent 1260 Infinity II bio-inert LC and the Agilent 6495A triple quadrupole LC/MS

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Abstract

A direct method with LC/MS/MS using triple quadrupole technology was developed to provide the sensitivity and selectivity needed for low ppb detection of glyphosate and its metabolites AMPA, HEPA, and MPPA, along with glufosinate and its metabolite N-acetyl glufosinate in food. The direct ion exchange separation was achieved using a quaternary amine bound to a polyvinyl alcohol column.

Introduction

Glyphosate is the active ingredient in the popular herbicide Roundup, and is used throughout the world. Glyphosate is a broad-spectrum systemic herbicide that interferes with the shikimic acid pathway by binding to the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) enzyme[1]. It is an organophosphorus compound, specifically a phosphonate. Recently, its safe use has come into question with a report issued by the IARC[2]. However, the European Commission has approved the use of glyphosate for another five years in a final decision to a European Citizen's Initiative[3]. This decision was made public citing the conclusions of the European Food Safety Authority[4]. Most recently, a USA lawsuit against the producer of Roundup resulted in a large jury award[5]. This has heightened the demand for a sensitive method at the low ppb level for food, and even lower levels for environmental water analysis.

Reliable sample preparation and analysis are needed to routinely analyze glyphosate and its major metabolite, aminomethylphosphonic acid (AMPA). However, glyphosate and its metabolites have high polarity and chelating properties, so they can be challenging for extraction from food and for analysis. Of concern is the affinity of these compounds to stainless steel and other surfaces, making system-to-system reproducibility difficult. This Application Note shows the analysis of glyphosate, its major metabolite, plus six other metabolites and polar pesticides, as shown in Figure 1. All the pesticides are phosphonates. Fosetyl aluminum, a postharvest fungicide, is also important to include, as it can be mistaken for AMPA. We used the Agilent 1260 Infinity II bio-inert LC coupled to the Agilent 6495A triple quadrupole LC/MS for analysis of food samples of plant origin to 10 ppb.

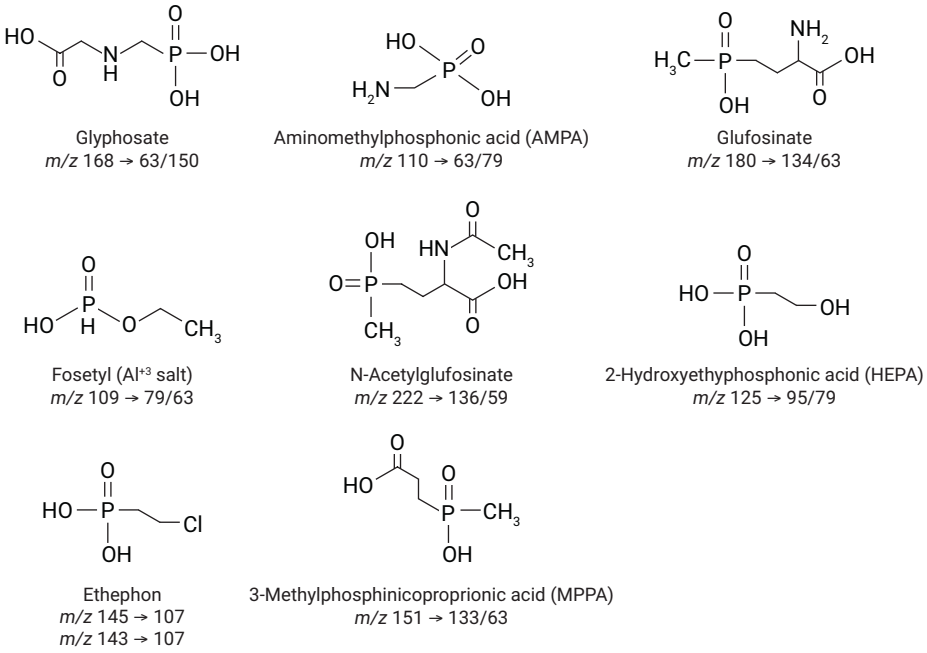

Figure 1. Structure of the polar pesticides analyzed in the application.

Experimental

Standards

Glufosinate ammonium and fosetyl aluminum were obtained from Chem Service, Inc., West Chester, PA. Aminomethylphosphonic acid, glyphosate, 3-(methylphosphinico) propionic acid, ethephon, 2-hydroxyethyl-(d_4)phosphonic acid, and N-acetyl-d_3-glufosinate were obtained from MilliporeSigma, Saint Louis, MO. Aminomethylphosphonic acid (^{13}C, 99 %; ^{15}N, 98 %) and glyphosate, (2-^{13}C, 99 %; ^{15}N, 98 %+) were obtained from Cambridge Isotope Laboratories, Andover, MI, USA. N-acetyl glufosinate, 3-methylphosphinicopropionic acid-d_3 sodium salt, and ethephon-$^{13}C_2$ were obtained from Toronto Research Chemicals, North York, ON, Canada. All standards were prepared in ultrapure water at 1 mg/mL, then diluted to working standard concentrations. Internal standards were added to all standards and samples at a final concentration of 20 ng/g (20 ppb). All working standards were diluted 1:1 with 0.1 % formic acid in methanol.

Solvents

Methanol was BDH LC/MS grade, and acetonitrile was HiPerSolv CHROMANORM LC/MS grade obtained from VWR International, Pittsburg, PA, USA. Ultrahigh purity (UHP) water was 18 MΩ and 3 ppb TOC from a Milli-Q Integral system (EMD Millipore, Chicago, IL, USA). Formic acid and acetic acid was LC/MS LiChropur from MilliporeSigma. Ammonium bicarbonate, reagent plus grade, was from MilliporeSigma.

Materials

Mobile phase solvent bottles were VWR heavy-duty vacuum bottles HDPE (p/n 89217-522) with a VWR versatile cap, open-top with closed adapter, 50 mm (p/n 89217-536). The open-top caps were fitted with an Agilent InfinityLab Stay Safe cap, GL45, one port, one InfinityLab vent valve, 3.2 mm od fitting PTFE insert. The O-ring from the heavy-duty vacuum bottle cap was used to seal the PTFE insert in the bottle. The standard PTFE solvent line was threaded through the 3.2 mm od fitting PTFE insert before installing an Agilent stainless steel filter, solvent inlet, 12 to 14 µm (p/n 01018-60025), at the solvent line. Even with the sealed containers, the ammonium bicarbonate mobile phase should be made fresh every three days. UHP water should be made fresh every week.

Samples

All food samples were bought at a local store and labeled organic. Wine samples were purchased at a local liquor store and were generically labeled white wine and red wine.

Sample preparation

All sample collection and preparation was done in polyethylene or polypropylene containers. VWR high-performance centrifuge tubes with plug caps, polypropylene, 15 and 50 mL were used throughout. Autosampler vials were Agilent 1 mL polypropylene vials (p/n 5182-0567) with snap caps (p/n 5182-0550). Samples were prepared using the QuPPe method for plant origin[6]. Briefly, internal standard was added to the weighed amount of sample to be extracted. Then, water was added to samples according to Table 24 of the QuPPe method when internal standard is added before extraction. A volume of 0.1 % formic acid in methanol was added that was equal to the total water content. The sample was then vortexed for 10 minutes and centrifuged at 4,000 rpm for 20 minutes. A 1 mL amount of liquid extract was then transferred to the autosampler vial.

Instrument

The HPLC was a 1260 Infinity II bio-inert LC consisting of an Agilent 1260 Infinity bio-inert quaternary pump (p/n G5611A). The system also featured an Agilent 1260 Infinity bio-inert high-performance autosampler (p/n G5667A) fitted with an Agilent 1200 Series autosampler thermostat (p/n G1330B). The LC included an Agilent 1260 Infinity thermostatted column compartment (p/n G1316A TCC). The mass spectrometer was a 6495A triple quadrupole LC/MS. Table 1 gives the instrument conditions. The mass spectrometer was operated with multireaction monitoring (MRM) with all compound transitions in the negative ion mode. Table 2 gives the precursors and product ions for all analytes and their stable isotopes.

Table 1. LC and MS conditions.

HPLC Conditions	
Column	Strong anion exchange bound to polyvinyl alcohol, 2.1 × 100 mm, HILICpak VT-50 2D
Injection Volume	10 µL
Mobile Phase	A) UHP H_2O B) 50 mM NH_4HCO_3 C) Acetonitrile
Initial	A) 50 % UHP H_2O B) 10 % 50 mM NH_4HCO_3 C) 40 % acetonitrile
Gradient	Time (min) %A %B %C 6.50 40.0 50.0 10.0 10.00 15.0 85.0 0.0 18.00 0.0 100.0 0.0
Flow	0.2 mL/min
MS Conditions	
ESI	Negative
Source Parameters	
Gas Temperature	140 °C
Gas Flow	18 L/min
Nebulizer	30 psi
Sheath Gas Heater	375 °C
Sheath Gas Flow	12
Capillary	3,000 V
V Charging	500
Ion Funnel Parameters	
Negative High-Pressure RF	110
Negative Low-Pressure RF	60
Accelerating Voltage for all Transitions	7
Resolution for MS1 and MS2	Unit

Results and discussion

The studied polar pesticides are difficult to analyze because of their chelating ability due to the phosphinate and carboxylic acid groups many of them possess. Their binding to sodium in glass and iron in stainless steel and other metals causes losses in signal intensity and chromatographic resolution. Numerous procedures have been developed to de-activate the chromatographic system using phosphoric acid, EDTA, or other procedures. These often need to be repeated throughout the analysis. This method uses polypropylene sample containment throughout and high-density polyethylene mobile phase bottles that have been cleaned with LC/MS grade methanol and ultrahigh purity water (UHP). The sample path in the LC is completely PEEK-lined, except for the ceramic injection needle. The only stainless steel in the sample path is the electrospray needle, which did not contribute to any significant loss. In addition, the stainless steel frits used in the solvent bottles did not appear to leach appreciable metal. It was considered that this was a lesser concern compared to adding extra sodium that may have come from the glass frits.

Table 2. Compound transitions, dwell time of each compound, and collision energies (CE).

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell	CE (V)
Glufosinate, N-acetyl-d_3	225.05	62.2	30	16
Glufosinate, N-acetyl	222.05	136.1	30	20
Glufosinate, N-acetyl	222.05	59.2	30	16
Glufosinate	180.04	134	30	16
Glufosinate	180.04	63.1	30	44
Glyphosate $^{13}C_2$, ^{15}N	171	63.1	30	16
Glyphosate	168	150	30	8
Glyphosate	168	63	30	24
3-MPPA-d_3	154	63.1	30	32
MPPA	151	133	30	12
MPPA	151	63.2	30	32
Ethephon $^{13}C_2$	147	109	30	4
Ethephon	145	107	30	4
Ethephon	143	107	30	4
2-HEPA	129	79.1	30	32
HEPA	125	95	30	12
HEPA	125	79.1	30	32
AMPA ^{13}C, ^{15}N	112	63.1	30	16
AMPA	110	79.2	30	28
AMPA	110	63.1	30	20
Fosetyl	109	79	30	12
Fosetyl	109	63.1	30	40
Ethephon	107	79	30	12

Figure 2 shows the chromatographic results obtained using the ion exchange method. Without the addition of acetonitrile at the beginning of the method, AMPA and glufosinate are very broad peaks. The addition of acetonitrile sharpens them to the same efficiency as the other peaks.

It is also important to note that various retention times are affected by injection into solvent versus water. For example, there is more than one minute of separation between fosetyl and AMPA for the standard in water (panel A). However, with a 10 µL injection, the effect of the standard solvent, 50 % methanol with 0.1 % formic acid, shows a difference in the retention times. However, the separation between them is still maintained, which is important because they are a critical pair (the ^{13}C isotope of fosetyl aluminum provides the same transitions as AMPA). Fosetyl was weighed as fosetyl aluminum (molecular weight 354.1 g/mol), whereas the instrument measures fosetyl (molecular weight 110.1 g/mol). Therefore, there is 3.2 times more fosetyl in the weighed standard, and the signal appears much stronger.

It was also found that, for some compounds, retention times (and also responses) can be further influenced by matrix type.

Calibration curves in solvent were created, and ranged from 1 to 100 ppb. For those compounds with internal standards, the correlation coefficient was >0.99. When measured before and after analysis of extracts, these curves were consistent, except glufosinate, which showed a loss in response in the end calibration compared to the beginning. However, this was compensated for through the internal calibration process described. The Appendix presents a graphic display of some of the calibrations. Even though calibration was from 1 ppb, ethephon, HEPA, and MPPA showed potentially much lower LOQs in solvent. Indeed, the signals for MPPA indicated that its detection could be at least 20 to 50 times lower.

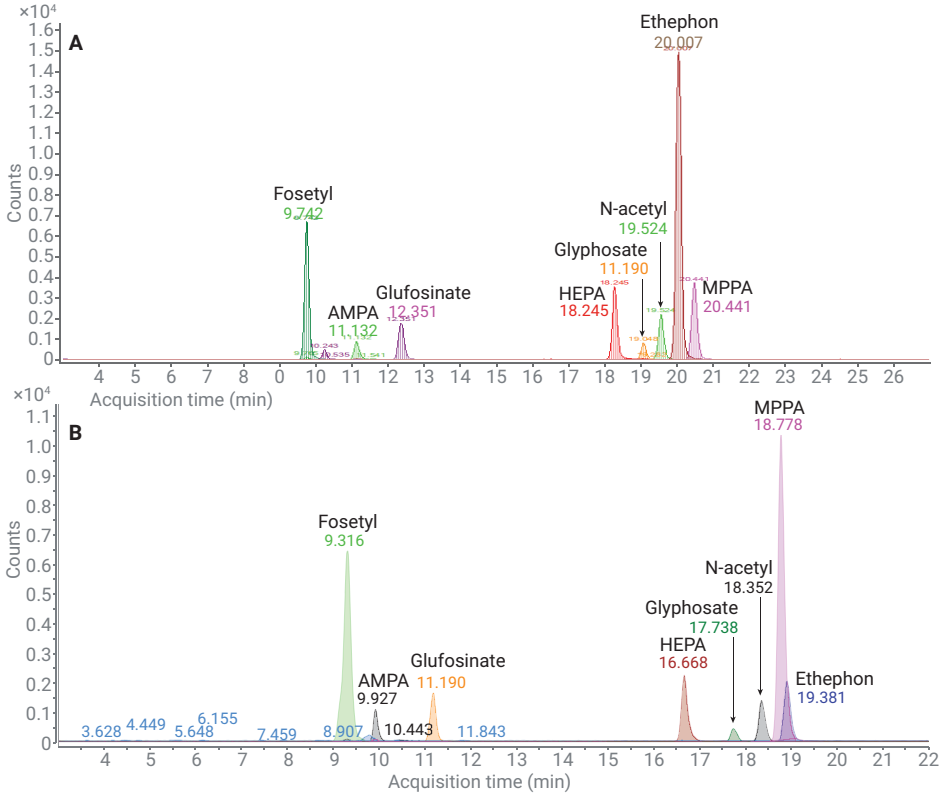

Figure 2. Chromatographic separation achieved with the described method of A) 25 ppb standard in ultrapure water and B) 10 ppb standard in 50:50 water: 0.1 % formic acid/methanol.

Figure 3 shows the responses for glyphosate at the 10 ppb level in each of the matrices tested. Table 3 provides the method limits of quantification (MLOQ) for each of the compounds in each of the matrices, extrapolated from the quantifier signal at 10 ppb.

Because of matrix interferences and mostly suppression unique to each food commodity, the MLOQs are unique to each matrix. Any other matrix analyzed must have its MLOQ determined.

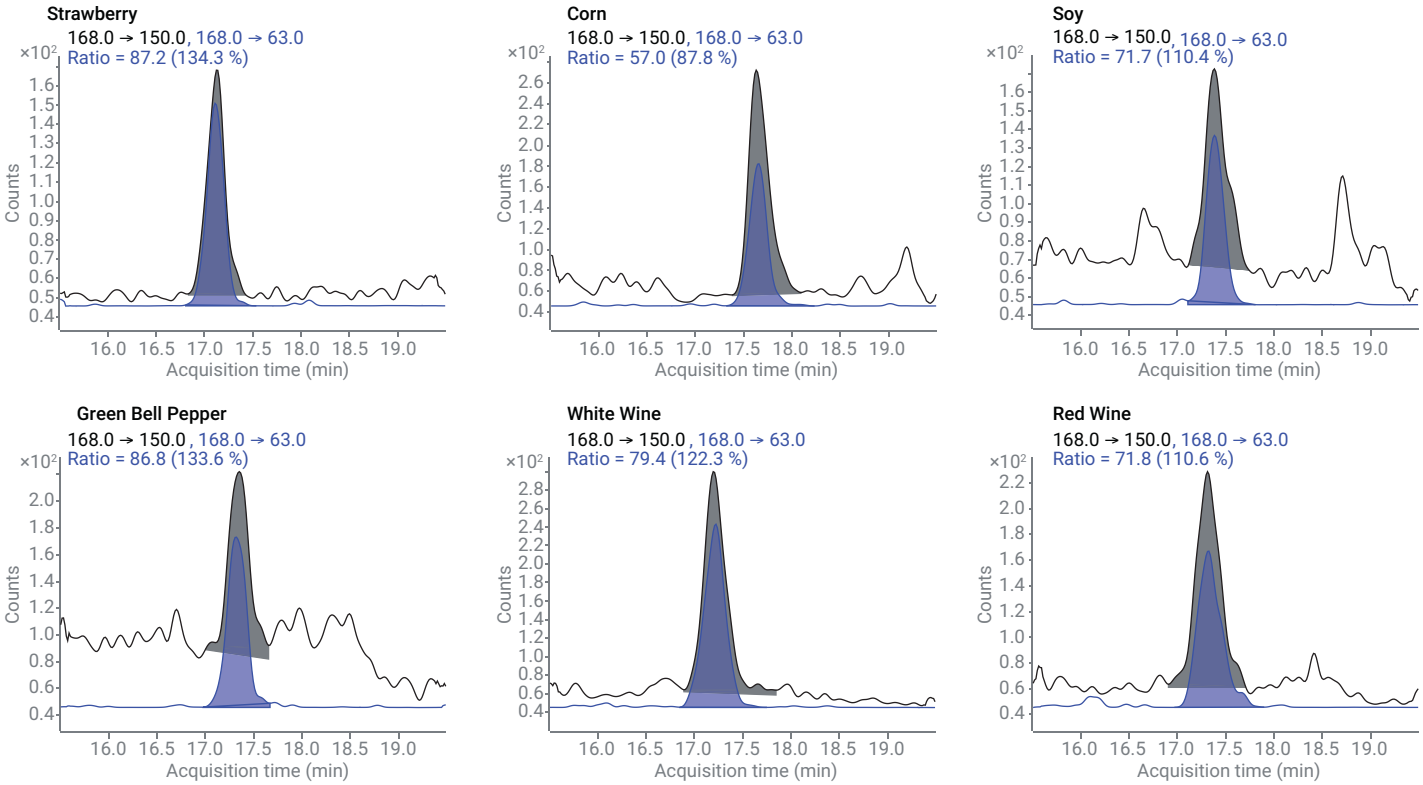

Figure 3. Chromatograms of glyphosate at 10 ppb in each of the matrices that were spiked. The quantifier ion is in gray, and the qualifier ion is in blue. Although the quantifier transition gives a greater peak height signal, the S/N is greater for the qualifier ion, and the two could be reversed.

Table 3. MLOQs for each matrix calculated as the concentration giving an S/N of 10:1 for the quantifier transition.

MLOQ (ppb)	Strawberry	Corn	Pepper	Soy	White Wine	Red Wine
AMPA	1.6	5	7.5	7	3.2	3.8
Glyphosate	0.42	7	1.7	7	0.34	7.1
N-Acetylglufosinate	1.3	3	5.2	9	4.3	4.7
HEPA	0.14	3	2.7	4.4	5	6.7
MPPA	0.34	3	2	0.8	0.36	0.5
Ethephon	0.3	2.5	0.7	0.4	0.67	0.33
Fosetyl Al	0.4	4	3.1	1.6	0.27	0.09
Glufosinate	7	5	18	5	1	1

Table 4 gives the recoveries for each of the foods tested at the 10 ppb level. For the first six compounds in the table, internal standards were used, and added before extraction. In general, recoveries are good for these compounds, except for the first duplicate of glyphosate and AMPA in corn and soy. Grains can be more problematic than higher water content fruits and vegetables. However, even in these foods, the detection of these compounds at 10 ppb is easy. For compounds where internal standards were not used, recoveries were low. Post spike samples (not shown) demonstrate that this is due mostly to matrix effects and not poor extraction. Given dilution factors and matrix effects, the data show that internal standards added before extraction are necessary for reliable results.

The spike results for each matrix at 100 ppb in Table 5 show good recoveries for all the compounds with internal standards.

Matrix-matched standards added before extraction could be used as an alternative to internal standards. However, this would burden the analyst with obtaining a zero matrix (no analytes found) and preparing a calibration curve for every matrix analyzed. The addition of internal standards before sample extraction removes these needs and eliminates concerns over not being able to source zero food samples.

Both double blanks and zero blanks (blanks with and without internal standard) were run for all the food matrices. White and red wines showed a consistent response for glyphosate (in both blanks). The white wine's determined concentration of glyphosate in the zero blank was 7.9 ppb, and 9.3 ppb for the red wine. None of the other polar pesticides was detected in both blanks in the food and wine samples. Correcting for the incurred amount of glyphosate in both wine samples, the recoveries for both the 10 and 100 ppb spikes are acceptable.

Table 4. Spike recovery data at 10 ppb for food samples. The two columns of values for each compound are the duplicate results of the spiked samples.

Spike 10 ppb	Glyphosate		AMPA		N-Acetyl Glufosinate		Ethephon		HEPA		MPPA		Glufosinate		Fosetyl-Al	
Strawberry	11.5	12.5	9.9	11.2	9.9	9.6	10.5	11.7	8.8	10.8	9	9.4	1.61	1.59	1.98	1.77
Corn	35.4	13.3	57.2	11.4	11.8	10.6	11.9	9.77	9.21	10.5	10.4	9.86	1.26	1.14	7.32	2.02
Soy	17.9	8.78	17.8	12.5	22.1	18	16	13.1	13	12.1	10	10.4	1.56	1.59	2.94	2.34
Green Bell Pepper	10.5	11.1	11.3	10.9	**	14.3	42.4	10.6	13.8	13.6	9.9	9.85	2.62	1.98	1.94	1.75
White Wine	20	22.5	12.2	11.3	10.8	11.5	11.5	11	15.8	14.6	9.71	10.3	2.1	2.05	4.74	4.94
Red Wine	19.6	20.2	12.4	10.9	11.2	10.6	11.5	10.2	11.5	12.6	10.6	10.7	1.48	1.8	1.78	1.68

Table 5. Spike recovery data at 100 ppb for food samples. The two columns of values for each compound are the duplicate results of the spiked samples.

Spike 100 ppb	Glyphosate		AMPA		N-Acetyl Glufosinate		Ethephon		HEPA		MPPA		Glufosinate		Fosetyl-Al	
Strawberry	102	97	101	80	97	92	99	96	92	97	102	93	19	12	19	16
Corn	104	133	96	141	100	116	97	115	97	124	101	114	17	18	19	20
Soy	110	106	143	120	108	121	106	106	106	105	100	102	25	14	20	16
Green Bell Pepper	111	111	81	89	109	110	109	105	114	109	104	106	21	18	15	17
White Wine	117	125	106	103	111	108	116	106	112	109	103	104	25	24	11	12
Red Wine	103	118	111	113	102	104	99	107	104	113	103	109	24	23	10	10

Conclusions

A method for the detection of glyphosate and its metabolite AMPA was developed and shown to provide detection at 10 ppb in foods of plant origin. The method requires all surfaces in contact with the sample to be either plastic or PEEK, including the use of polypropylene sample containers. A PEEK and ceramic sample path through the instrument was achieved with the 1260 Infinity II bio-inert LC. High-density polyethylene mobile phase containers were used to reduce sodium in the analytical method. Using the 1260 Infinity II bio-inert LC coupled to the 6495A triple quadrupole LC/MS enables routine analysis. All food matrices must be validated for the method, and the use of stable isotope internal standards added to the samples before extraction enables accurate quantitation against a solvent standard. Results could be improved with a sample cleanup that removes more interferences without removing the analytes.

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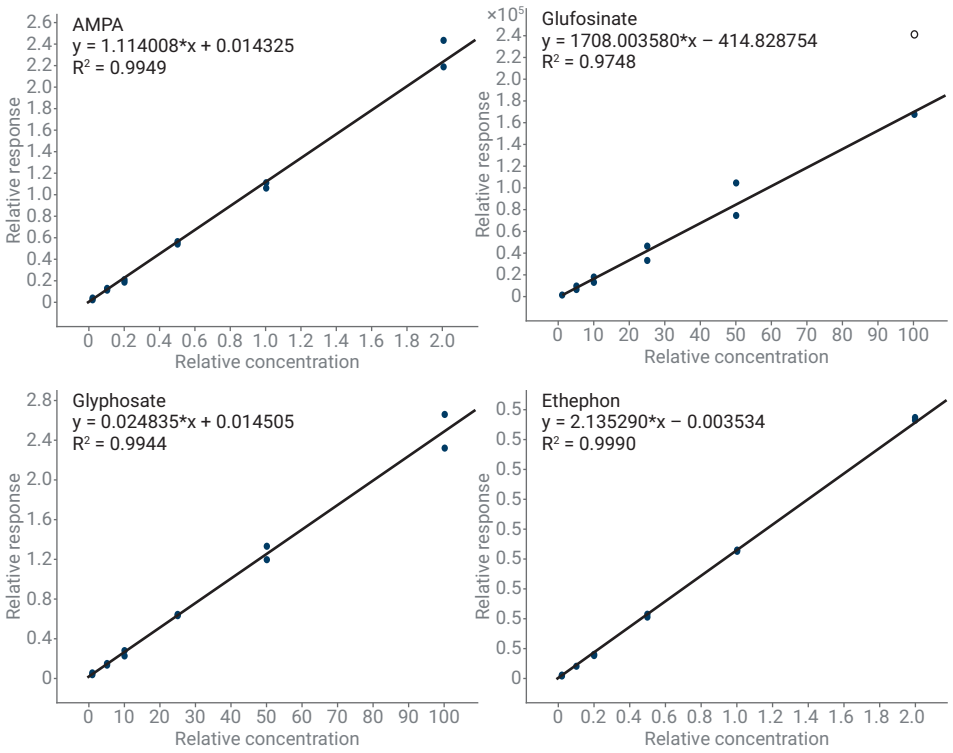
Appendix

Calibration curves for polar pesticides run before and after samples



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Printed in the USA, March 14, 2019
5994-0796EN

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