LC-MS/MS Method for Determination of Glyphosate, AMPA, and Glufosinate in Cereals

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A simplified LC-MS/MS method for the determination of glyphosate, (aminomethyl)phosphonic acid (AMPA), and glufosinate in cereals is described. The method enables the analysis of glyphosate and its metabolites without sample derivatisation. The samples are prepared utilising an extraction method based upon the Quick Polar Pesticides Method and separated by high-performance liquid chromatography with MS detection. A polymer-based, ion-exchange chromatography column allowed retention of the analytes while water, methanol and ammonium carbonate mobile phase system insured proper ionisation under negative ESI conditions. The use of isotopically labelled standards allowed the use of solvent-based calibration curves.

Introduction

Glyphosate is one of the most commonly used herbicides in the world with more than 1.4 billion pounds of glyphosate applied to fields per year [1]. Its usage increased after the introduction of genetically modified, glyphosate tolerant crops such as corn, soybeans and cotton. In the USA, US Environmental Protection Agency (EPA) regulation document Code of Federal Regulations (CFR)-title 40-volume 24 sets the tolerance levels for the occurrence of glyphosate in food commodities and produce [2]. The EPA tolerance for glyphosate residues in cereal grains (also called crop group 15) are set at 30 ppm; this limit excludes rice, soy, and corn. In rice the tolerance is 0.1 ppm whereas in sweet corn it is 3.5 ppm [2]. For glufosinate, a herbicide that is often included with glyphosate in analytical methods, the tolerance values are 0.4 ppm for cereal and 1.0 ppm for rice. These tolerance values include metabolites and degradants. Therefore, a glyphosate metabolite, AMPA, was also included into this study (Figure 1).

Since glyphosate is widely used during production of soybeans and corn, it was expected to be found in these commodities. In this application, the presence of glyphosate in other grains such as oats and wheat used in the production of breakfast cereals, including infant cereal, was explored.

Various methods for glyphosate analysis were developed over the last 30 years. Some required derivatisation of analytes for HPLC with fluorescence detection with o-phthalaldehyde [3]. A method with glyphosate derivatisation using fluorenylmethyloxycarbonyl chloride (FMOC) and fluorescence detection has also been proposed and widely used [4]. Recently, with the advent of modern, more sensitive and rugged LC-MS/MS instruments, it has become possible to analyse glyphosate and its metabolites without derivatisation as illustrated in this work with direct analysis of glyphosate by MS/MS.

Experimental

All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA unless indicated otherwise. Glyphosate, AMPA and ammonium glufosinate were of analytical standard grade. Isotopically labelled internal standards were used including Glyphosate-2-^{13}C,^{15}N, AMPA-^{13}C,^{15}N,D_2. Glufosinate-D_3 was obtained from Toronto Chemicals, North York, ON, Canada. Solutions of internal standards and non-isotopically labelled standards were prepared in water at 1 mg/mL and used for spiking the grain matrices.

Organic instant oatmeal and organic whole wheat flour were selected as test matrices during method development. These foods were scanned for the presence of glyphosate using the methods described below and glyphosate was not found. Whole wheat flour was used as received while the quick oats were ground prior to use. For the method development study, both matrices were spiked to contain 100 ppb of glyphosate and 100 ppb of glufosinate. The oats were spiked in addition to contain 100 ppb of AMPA.

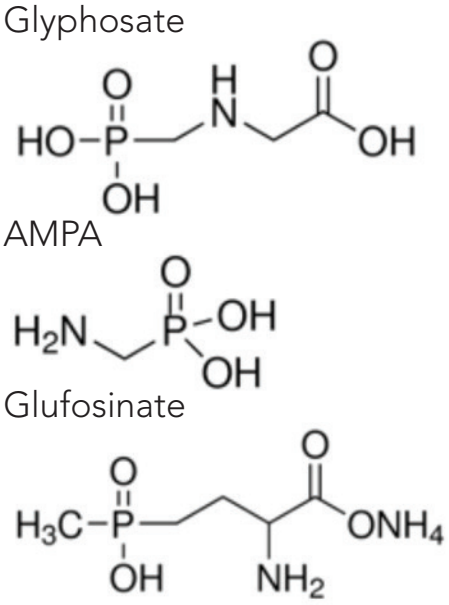

Figure 1. Structures of Glyphosate, AMPA and Glufosinate

For studies of glyphosate occurrence in grain products, the following matrices were tested for glyphosate using the developed methods: wheat white flour, instant oatmeal, infant rice cereal, infant oat cereal, and infant mixed grain cereal. All of these products were purchased in the local grocery store and were not labelled as organic.

Supel™-Select HLB Solid Phase Extraction (SPE) in 1 ml/30 mg size and Amicon® Ultra –0.5 centrifugal filter units with 3kDa Molecular Weight Cut Off (MWCO) were purchased from Sigma-Aldrich (St. Louis, USA).

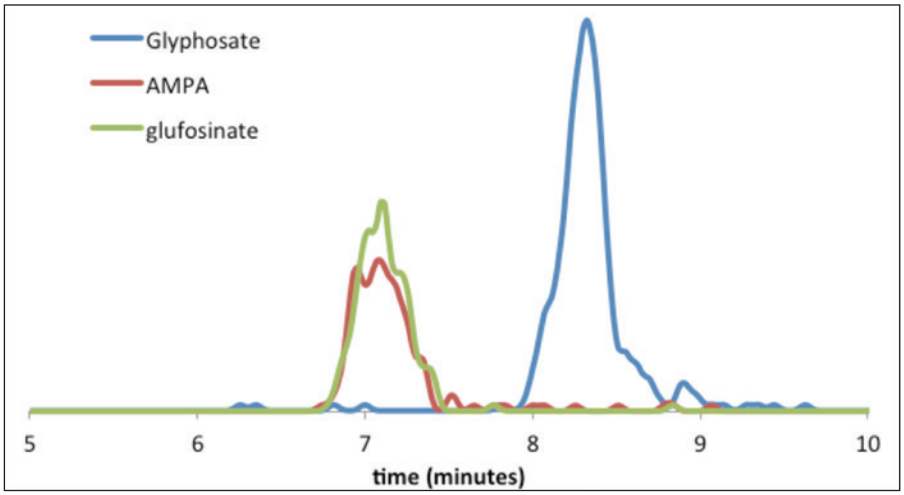

Figure 2. LC/MS Analysis of Glyphosate, AMPA, and Glufosinate using apHera NH2 chromatographic column. Quantitative transitions are shown for each analyte. Conditions are described in the experimental section.

Sample pre-treatment

The extraction method was based on the QuPPe (Quick Polar Pesticides Method) methodology developed in the European Union (EU) for fruits and vegetables, and used water:methanol (50:50) containing 0.5% formic acid as the final extraction solvent [5]. Individual five gram samples of homogenised grain and/or cereal were weighed into 50 mL centrifuge tubes. Water (10 mL) and 100 µL of an internal standard solution (20 µg/mL of each analyte in water) were added to each vessel. The samples were then left to stand for the time period of two hours. At the conclusion of the wait period, 10 mL of methanol containing 1% v/v formic acid was added. The samples were mixed for 15 minutes on a laboratory shaker and then centrifuged. Two grains, oats and wheat, used during method development, gave significantly different extracts when examined visually. The oats produced a clear, yellow extract, while the wheat extract was cloudy and found to be difficult to filter when using syringe filters. As a result, two different sample cleanup procedures were utilised based on the grain sample under study.

Sample cleanup using SPE

For the samples that did not have particulates after extraction and centrifugation, such as oats and corn, a solid phase extraction (SPE) cleanup using Supel-Select HLB cartridges was applied, similarly to a method reported by Chamkasem and Harmon [6]. Polymeric hydrophilic-lipophilic balance or HLB SPE can be applied to broad range of analytes and used under reversed phase methodology. The SPE cleanup method in the present work is based on the chemical 'filtration' of the extract through the HLB cartridge when the impurities that are more hydrophobic in comparison to the analytes are retained on the SPE phase while the more polar analytes do not retain and come through in the eluate. The HLB SPE cartridges were conditioned using 100% methanol followed by water:methanol (50:50) containing 0.5% v/v formic acid. For 1 mL SPE cartridges, 0.5 mL of the sample extract was used to pre-wet the cartridge. The eluate from this step was discarded. A second aliquot of the sample extract (0.5 mL) was loaded into the HLB cartridge. This eluate was collected and filtered through 0.2 micron filter vials with polypropylene membrane.

Sample cleanup using ultrafiltration

Ultrafiltration devices were used for cleanup of sample extracts that had particulates, such as wheat. 1 mL of the sample extract was loaded onto the Amicon ultrafiltration devices. The ultrafiltration step was performed by centrifugation for 45 minutes at 4000 rpm. The clear sample that passed through the membrane was collected and analysed. Low-absorption vials were used for this analysis to prevent loss of analytes through adsorption to the glass surface.

Sample preparation for beer

Beer samples were also analysed using the same methodology. First, a thorough degassing of beer was undertaken by placing the beer sample in the ultrasonic bath for 15 minutes. Then 5 mL of the beer sample was mixed with 5 mL of methanol with 1% formic acid and internal standard. This sample was briefly mixed and cleaned using the SPE procedure.

LC-MS/MS method

The analysis was done using Agilent 1200 HPLC stacked with AB Sciex QTrap 3200. The HPLC column used for this analysis was the polymer-based ion-exchange apHera™ NH2 column, which can provide stable and robust LC separations in the wise range of pH values. The column's size was 15 cm x 4.6 mm packed with 5-micron size particles. The aqueous mobile phase used water and ammonium carbonate at pH 9 with 5% methanol. This mobile phase ensured the proper ionisation of glyphosate, which has a phosphate group in its structure, with detection under negative ESI conditions. In addition, ammonium carbonate buffer is volatile and is fully compatible with LC/MS instrumentation. The flow through HPLC column at 0.5 mL/min started with water for 2 minutes, the change to 95% 20 mM ammonium carbonate buffer (pH 9) and 5% methanol occurred at 2.1 minutes, continued for the next 10 minutes, and the column was flushed with pure water for 5 minutes at the end of the run. Column temperature was 35°C. 60 µL injection volume was used. Table 1 lists the MS source conditions for all analytes. In addition, voltage was at -4000 V, curtain gas was at 45, source temperature at 500 °C, Gas 1 at 50 and Gas 2 was at 60. Figure 2 presents a chromatogram of a standard injection.

Calibration curves used internal standards and were made in 50-50 methanol-water with 0.5% formic acid. Concentration range of calibration curves was from 10 ng/mL to 300 ng/mL.

Table 1. MS parameters for analytes.

Compound		Q1	Q3	DP	EP	CEP	CE	CXP
Glyphosate	Quant	167.8	63	-30	-5.5-8	-32	-4	
	Qual	167.8	79	-30	-5.5	-8	-58	-3
Glyphosate-2-$^{13}C,^{15}N$	Quant	19.8	62.9	-30	-4.5	-10	-28	-12
AMPA	Quant	110	63	-45	-10	-10	-28	-6
	Qual	110	81	-40	-10	-10	-18	-1
AMPA-$^{13}C,^{15}N,D_2$	Quant	113.9	63	-40	-5	-10	-38	0
Glufosinate	Quant	180	63	-35	-6	-12	-56	0
	Qual	180	136	-40	-4	-12	-25	-2
Glufosinate-D_3	Quant	182.9	63	-30	-7.5	-12	-70	0

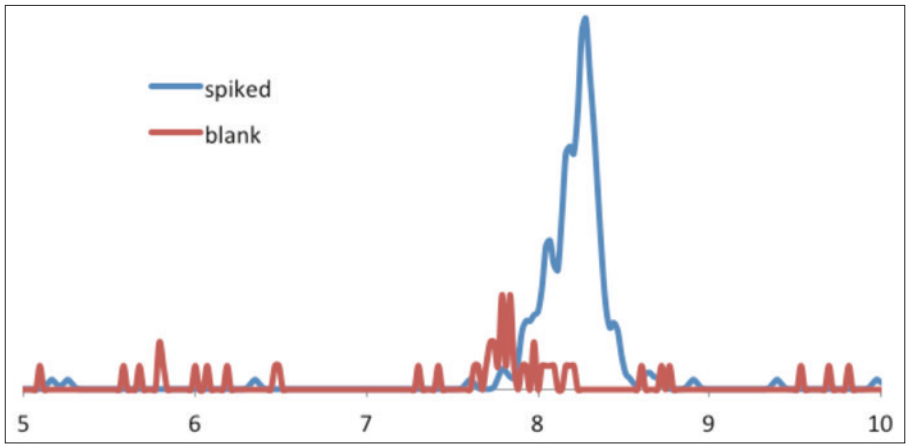

Figure 3. Organic Oatmeal Sample, Blank and Spiked at 100 ppb Glyphosate.

Results

Method development

While multiple columns and mobile phase conditions were tested during method development for detection of underivatised glyphosate using LC-MS/MS, the final chromatographic method used an anion exchange column. It allowed for retention of compounds under the basic mobile phase conditions after direct injection of samples in 50-50 water-methanol extraction solvent. No further dilution or solvent exchange was required for the samples which resulted in convenient and fast analysis method. The elution off the HPLC column was performed by increasing the pH of the mobile phase to 9.2 using 20 mM ammonium carbonate buffer and introducing small amount of methanol (up to 5% methanol in the mobile phase). Multiple injections of samples extracted from multiple matrices did not result in significant shifts in the retention time indicating the ruggedness of this LC method.

Sample preparation was performed using fast extraction with methanol. SPE was the first choice for sample cleanup as the sample can be simply passed through the cartridge. Since difficulty was encountered with undissolved co-extracted particulates in wheat samples, ultrafiltration cleanup was employed. The sample cleanup by both methods was acceptable for LC/MS analysis, injection of multiple extracted samples did not result in the shift of retention times or decrease in the analyte's signals.

Results from spiked grain samples

Oatmeal and wheat samples that were labelled 'organic' were used during method development. The samples were analysed for glyphosate and related compounds. The compounds were not found to be present in these samples (Figure 3). Consequently, the samples were spiked with analytes and analysed using the proposed method. The results of method development using spiked organic samples are shown in Table 2. In the spiked oatmeal samples, all three analytes were detected and quantified at 100 ppb, and in the wheat flour samples glyphosate and glufosinate were detected and quantified at 100 ppb. Wheat flour was not spiked with AMPA. Accuracy of the method was measured as the % recovery of the known spiked amounts. For glyphosate, the recovery values ranged from 118% to 125%, while for glufosinate they were between 105% and 112%. For AMPA in oatmeal sample recovery was at 118%. The method for wheat samples produced slightly higher uncertainties for both glyphosate and glufosinate, up to 19% RSD. The sample preparation method for wheat used only ultrafiltration and SPE cleanup was not employed. The resulting signals were found to have higher variability in comparison to the samples cleaned using SPE.

Glyphosate in beer

No analytes were found in the tested beer. Thus, the beer sample was spiked at 50 ppb with glyphosate and glufosinate and analysed. The results are presented in Table 2. In this work further analysis of beer samples was not attempted due to the limited instrumental detection limit.

Identification and quantitation of glyphosate in cereals

The results of glyphosate analysis in cereals using the proposed methods are presented in Table 3. These samples were purchased in the grocery store, were not labelled 'organic' and were not spiked with standards. Internal standards were used as described in the experimental section. The samples of instant oatmeal (Figure 4) and wheat white flour contained significant incurred amounts of glyphosate, 1.2 and 0.8 ppm, respectively. Infant rice cereal had very low levels of glyphosate. The levels were close to the limit of instrument sensitivity in that matrix and the resulting RSD was high. Infant oat cereal contained glyphosate at 1.1 ppm and infant mixed cereal was found to contain glyphosate at 0.25 ppm. Glufosinate was not found in any cereal products. AMPA was found only in instant oatmeal at low levels.

Table 2. Method development results after spiking 100 ppb into cereal/grains labelled 'organic' and 50 ppb into beer.

Analyte/Matrix	Glyphosate		AMPA		Glufosinate	
N=6	Recovery (%)	% RSD	Recovery (%)	% RSD	Recovery (%)	% RSD
Oatmeal	125	5	118	15	105	5
Whole wheat	125	19	-	-	105	16
Beer	118	21	-	-	112	7

Table 3. Analysis results in non-organic grains and cereals

Analyte/Matrix	Glyphosate		AMPA		Glufosinate
N=3	Found, ppm	% RSD	Found, ppm	% RSD	Found, ppm
Oatmeal	1.2	8	0.04	27	ND
White wheat flour	0.8	12	NT		ND
Infant rice cereal	0.06	12	ND		ND
Infant oat cereal	1.1	4	NT		ND
Infant mixed cereal	0.25	5	NT		ND
Beer (Lager)	ND		ND		ND

NT=not tested, ND=not detected

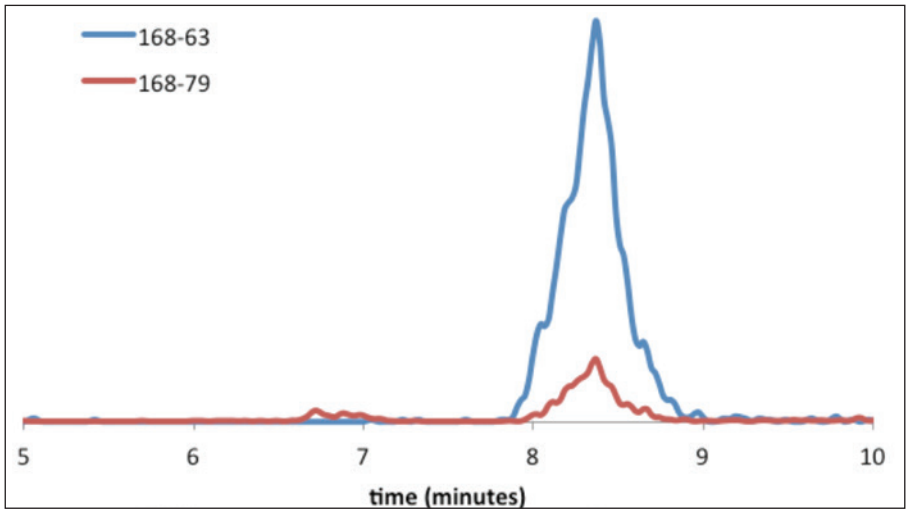

Figure 4. Glyphosate in Instant Oatmeal. Two MRM transitions are shown: 168/83 in blue and 168/79 in red.

Conclusions

The proposed method developed for glyphosate and related compounds uses LC-MS/MS detection and an ion-exchange polymer-based apHera NH2 column that is stable under higher pH conditions. Mobile phase contained 20 mM carbonate buffer with 5% methanol, was fully compatible with mass spectrometry and allowed efficient ionisation of analytes. Sample preparation methodology included cleanup using polymeric SPE, which was successfully applied to samples of cereals including oatmeal and infant cereal products. Cleanup that included ultrafiltration was developed for wheat-containing products and was successfully applied to wheat flour samples. Use of isotopically labelled standards resulted in good accuracy for glyphosate determination and allowed the use of solvent-based calibration curves. The method was applied to multiple grain products; significant levels of glyphosate higher than 0.8 ppm were found in oatmeal and wheat flour products.

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