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Direct Determination of Glyphosate, Glufosinate, and AMPA in milk by Liquid chromatography/tandem mass spectrometry

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Abstract

A simple high-throughput liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was developed for the determination of glyphosate, aminomethylphosphonic acid (AMPA) and glufosinate in milk using a reversed-phase liquid chromatography column with weak anion/cation exchange stationary phase. After protein precipitation with diluted acid and Na_2EDTA, the milk extract was passed through an Oasis HLB SPE to retain suspended particulates and phospholipids. The sample was directly injected and analyzed in 6 min, without sample concentration or derivatization steps. Two multiple reaction monitoring (MRM) channels were monitored in the method for each target compound to achieve true positive identification. The linearity of the detector response was demonstrated in the range of 4 to 1000 ng/mL for each analyte, with a minimum coefficient of determination (R^2) value of more than 0.995. Through the use of this internal standard calibration method, the average recovery for all analytes at 0.025, 0.1, 0.5, and 2 μg/mL (n = 7) are between 84-111% with a relative standard deviation of less than 8%.

Keywords:
Glyphosate, milk, LC-MS/MS, direct determination

1. Introduction

Glyphosate (N-phosphonomethyl glycine) and glufosinate (2-amino-4-[hydroxy(methyl)phosphoryl]butanoic acid) are non-selective post emergence herbicides used for the control of a broad spectrum of grasses and broad-leaf weed species in agricultural and industrial fields.

Aminomethylphosphonic acid (AMPA) is the major metabolite of glyphosate and also classified as a toxicologically significant compound [1]. According to recent reports, there has been a dramatic increase in the usage of these herbicides which are of risk to both human health and the environment [2]. In 2005, Battaglin et al., reported that 36% of surface water collected in Midwestern streams contain up to 6.08 μg/mL[3] . Recently, there was a report that glyphosate was found in breast milk (76-166 ng/mL) in 3 of the 10 samples tested using an enzyme-linked immunosorbent assay (ELISA) method with no confirmation method[4] . The European Union maximum residue levels (MRL) of glyphosate in imported milk (code number 102000) is 0.05 ppm [5]. Therefore, there is a need for selective and sensitive methodology to determine trace level residues of these pesticides in milk in response to the public concern.

The polar nature and high water solubility of glyphosate, AMPA, and glufosinate make extraction difficult, especially at residue levels. This has usually required the use of lengthy cleanup procedures that sometimes involve both anion and cation exchange columns[6] . Typical silica based reversed-phase C18 columns experience difficulty with the retention of such polar compounds, and may generate non-resolved, co-eluting peaks with polar analytes eluting in the void volume. The lack of chromophore or fluorophore also necessitates the use of derivatization techniques for the determination of these analyte residues by liquid chromatography and gas chromatography [6, 7, 8]. However, this technique is not highly regarded by analysts as it requires the optimization of a number of parameters (temperature, reaction time, concentration and purity of the reagents, laboratory handling time). Anion exchange, Hydrophilic Interaction Liquid Chromatography (HILIC), Hypercarb, and mixed-mode columns were used with LC-MS/MS to determined underivatized glyphosate and other polar pesticides in food matrixes with limited success[9, 10, 11]. The Acclaim WAX-1 column, which combined reversed-phase and weak anion-exchange properties on one column, provides excellent retention for glyphosate without derivatization. However, the column efficiency degrades over time due to the accumulation of

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trace metal ions on the column during sample analysis. The column must be regenerated for three hours with EDTA solution[10]. Obelisc N, another mixed-mode column, was used with limited success due to the poor retention time reproducibility, the short column life, and its high price[11]. A better column should be evaluated to make the method more rugged and practical regulatory work. In respond to the concern of breast milk contamination, a quick, sensitive, and selective method must be developed to accurately determine glyphosate, glufosinate, and AMPA in milk. Since cows milk has properties similar to breast milk in terms of major ingredients (fat, moisture, protein, minerals, and carbohydrate) and is easier to obtain, it was chosen as the matrix in this study[12].

This study describes the single laboratory validation of an LC-MS/MS method under the negative ion-spray ionization mode for the direct determination of glyphosate, glufosinate and AMP-A in milk. It also explains a quick and reliable extraction method that requires small sample size, non-toxic solvent, and an effective sample cleanup procedure to ensure a ruggedness sensitivity, and selectivity.

2. Materials and methods

2.1. Chemicals and Materials

Analytical standards, all 99% purity, were purchased from LGC Standards (Manchester, NH) consisting of glyphosate, AMPA, glufosinate, glyphosate $^{13}C2^{15}N$ (100 μg/mL), AMPA ^{13}C ^{15}N (100 μg/mL), and glufosinate D3. Methanol, acetonitrile, and water of HPLC grade were obtained from Fisher Scientific (Pittsburgh, PA) and used for HPLC mobile phase and standard preparation, and sample extraction. Formic acid was obtained as 98% solution for mass spectrometry from Fluka (Buchs, Switzerland.). Ammonium formate (ACS grade) and Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) were purchased from Fisher Scientific (Pittsburgh, PA). Extracting solvent (50 mM acetic acid/10 mM Na_2EDTA) was prepared by mixing 572 μL of acetic acid and 0.74 g of Na_2EDTA in 200-mL of purified water. Oasis HLB (60 mg) solid phase extraction cartridge was obtained from Waters (Milford, MA). EDP 3 electronic pipettes at different capacities (0-10 μL, 10-100 μL, and 100-1000 μL) were purchased from Rainin Instrument LLC (Oakland, CA) and were used for standard fortification.

A solution of 500 mM ammonium formate/formic acid (pH 2.9) was prepared as follows: 15.76 g were dissolve in approximately 300 mL of water and adjusted with 98% formic acid (approx. 28.3 mL) until the pH was 2.9 (using pH meter), and the solution was diluted to 500 mL with water. The HPLC mobile phase was prepared by mixing 100 mL of the 500 mM buffer solution with 900 mL of purified water so the final concentration was 50 mM.

2.2. Standard preparation

The stock solution of glyphosate, glufosinate, and AMPA at 50, 10, and 1 μg/mL were prepared by dissolving the stock standard in 1:1 water:methanol solution. The solutions were maintained at 4^{o}C in polypropylene tubes to avoid adsorption to glass. The internal standard (IS) solution of glyphosate $^{13}C2^{15}N$, glufosinate D3, and AMPA $^{13}C^{15}N$ at 2 and 10 μg/mL were prepared by dissolving the stock standard in 1:1 water:methanol solution. The calibration standards were prepared in the extracting solvent or blank matrix extract (after the SPE cleanup) with IS solutions for the calibration curves (see supplement data for detail).

2.3. Sample Preparation and Extraction Procedure

Homogenized milk was obtained from a local market. The samples were pipetted at 1 mL each in a 15-mL centrifuge tube (Fisher Scientific, Pittsburgh, PA) and fortified with native standard at 0.025, 0.1, 0.5 and 2 μg/mL (7 replicates). The IS solution (40 μL) at the concentration of 10 μg/mL was added into the sample so the concentration was 0.4 μg/mL for all samples. The samples were mixed for 1 minute on a vortex mixer and allowed to stand for approximately 1 hour. A set of five non-fortified milk sample (blank) without the added IS were also prepared and used for matrix matched standard. The extracting solvent (3 mL) was added to each tube using an automatic pipette. The tubes were capped tightly and shaken for 10 min on a SPEX 2000 Geno grinder (SPEX Sample Prep LLC, Metuchen, NJ) at 2000 stroke/min and then centrifuged at 3,000 rpm for 5 min using a Q-Sep 3000 centrifuge (Restek, Bellefonte, PA). Three milliliters of the supernatant were passed through an Oasis HLB cartridge (60 mg), previously conditioned with 2 mL methanol and 2 mL of the extracting solvent, and the last milliliter of the extract was collected into an autosampler vial. A 10 μL volume of sample was injected into the LC-MS/MS system.

2.4. LC-MS/MS Analysis

A Shimadzu HPLC system equipped with two LC-20AD Pumps, a Sil-20AC autosampler, and a CTO-20AC column oven (Shimadzu, Kyoto, Japan), coupled with a 5500 Q-TRAP mass spectrometer from AB SCIEX (Foster City, CA) was used. The Analyst software (version 1.5) was used for instrument control and data acquisition. Nitrogen and air from TriGas Generator (Parker Hannifin Co., Haverhill, MA) were used for nebulizer and collision gas in LC-MS/MS. An Acclaim Trinity Q1 (3 μm, 100 x 3 mm) from Thermo Scientific (Sunnyvale, CA) and a C18 SecurityGuard guard column (4 x 3 mm) from Phenomenex (Torrance, PA) were used for HPLC separation at 35^{o}C with sample injection volume of 10 μL. The mobile phase was 50 mM ammonium formate (pH 2.9) at a flow rate of 0.5 mL/min for a total run time of 6 min. The MS determination was performed in negative electrospray mode with monitoring of the two most abundant MS/MS (precursor/product) ion transitions using a scheduled MRM program of 60 seconds for each analyte. Analyte-specific MS/MS conditions and LC retention time for the analytes were shown in Table 1. The MS source conditions were as follows: curtain gas (CUR) of 30 psi, ion spray voltage (ISV) of -4500 volts, collisionally activated dissociation gas (CAD) was high, nebulizer gas (GS1) of 60 psi, heater gas (GS2) of 60 psi, source temperature (TEM) of 350^{o}C.

Table 1. Retention time (RT) and MRM conditions for LC-MS/MS analysis.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	DP	CE	EP	CXP	Retention Time (min)
AMPA.1	110	63	-60	-24	-10	-10	1.1
AMPA.2	110	79	-60	-26	-10	-10	1.1
AMPA $^{13}C^{15}N$ (IS)	112	63	-60	-24	-10	-10	1.1
Glufosinate.1	180	95	-46	-23	-10	-10	1.65
Glufosinate.2	180	85	-46	-26	-10	-10	1.65
Glufosinate D3 (IS)	183	63	-46	-26	-10	-10	1.65
Glyphosate.1	168.2	63	-110	-30	-10	-10	2.05
Glyphosate.2	168.2	79	-110	-55	-10	-10	2.05
Glyphosate $^{13}C2^{15}N$ (IS)	171	63	-110	-30	-10	-10	2.05

Compound dependent parameters: DP = declustering potential, CE = collision energy, EP = entrance potential, CXP = collision cell exit potential

Table 2. Matrix effect evaluation

	Slope of cal. curve in solvent	Slope of cal. curve in matrix	Matrix effect (%ME)
glyphosate	386.25	373.41	97
glufosinate	745.25	660.26	89
AMPA	1582.7	181.88	11

3. Results and discussion

3.1. Chromatography optimization

Glyphosate, glufosinate, and AMPA possess negative charges in aqueous solution that make retention on a reversed-phase column difficult. Several mixed phase mode columns containing reversed-phase, anion and cation exchange properties were evaluated for use in the study. They are a) Obelisc R (SIELC Technologies, Wheeling, IL), zwitterionic-type mixed mode, b) Scherzo SM-C18 (Imtakt USA, Philadelphia, PA), mixed beads of cation and anion exchange particles, and c) Nanopolymer Silica Hybrid , Acclaim^{TM}(Thermo Scientific, Sunnyvale, CA). Among the Accla^{TM}columns, three different columns were also evaluated. They are Acclaim^{TM} Trinity^{TM} P1 (strong cation, weak anion/reversed-phase), Acclaim^{TM} Trinity P2 (weak cation, strong anion/HILIC), and Acclaim^{TM} Trinity^{TM} Q1 (weak cation, weak anion/reversed-phase). Since these columns have both cation and anion exchange properties, they are the ideal columns for the analysis of both cationic charge pesticides (paraquat, diquat, mepiquat, chlormequat, amitrole, and daminozide) and anionic charge pesticides (glyphosate, AMPA, glufosinate, forsetyl alumina, ethephon, and maleic hydrazide). The idea is to use a single column to determine all these polar pesticides (for future projects) with one LC-MS/MS instrument.

Different mobile phase parameters were evaluated which included pH (2.9 to 5), acetonitrile concentration (0-100%), and salt concentration (0-100 mM). The best column so far was the Acclaim^{TM} Trinity^{TM} Q1 which provided good peak shape and reasonable retention for all analytes. This column possesses a reverse-phase, weak cation and anion exchange properties. It is suitable for the strong anion analyte such as glyphosate and strong cation analyte such as paraquat. The most important parameter for a good analyte retention on column was the pH of the mobile phase. At low pH (2.9), glyphosate eluted well while paraquat and diquat were strongly retained. At higher pH (4.5), glyphosate was a late eluter with a wide and tailing peak shape while paraquat and diquat had good peak shape. Therefore, two analyses on a single column could be performed isocratically with two different mobile phases. Acetonitrile in the mobile phase increased ion-spray efficiency and increased the retention but it resulted in very broad glyphosate peak at pH 2.9. Therefore, acetonitrile was not added in the mobile phase. High salt concentration shortened the retention time of the analytes and decreased analyte response due to ion-suppression. The 50 mM salt concentration was a good compromise between good peak shape and retention time.

It was found that the proposed mobile phase containing 50 mM ammonium formate (pH 2.9) at a flow rate of 0.5 mL/min for the Acclaim^{TM} Trinity^{TM} Q1 (3μm, 100 x 3 mm) produced the optimum condition for peak shape, retention time, and sensitivity for these three analytes. The reversed-phase guard column was used to retained non-polar compounds that may be irreversibly absorbed on the analytical column. After each set of the samples was analyzed, the column was flushed with acetonitrile approximately 20 column volumes to wash out these non-polar compounds. A mobile phase containing 8:2 200 mM ammonium acetate:acetonitrile approximately 20 column volumes may be used wash out ionic interference from the column as needed.

3.2. Optimization of Sample Extraction procedure

For high protein samples such as milk, protein precipitation is a common protocol for rapid sample clean-up and extraction [13, 14, 15]. An organic solvent and/or acid have been used for effecting protein precipitation by exerting specific interactive effects on the protein structure. An organic solvent lowers the dielectric constant of the plasma protein solution and also displaces the ordered water molecules around the hydrophobic regions on the protein surface, the former enhancing electrostatic attractions among charged protein molecules and the latter minimizing hydrophobic interactions among the proteins. Acidic reagents form insoluble salts with the positively charged amino groups of the proteins at pH values below their isoelectric points. Acetonitrile and methanol, with and without 0.1% acetic or formic acid at the sample to solvent ratio of 1:3 were evaluated. The milk samples were fortified with 1 μg/mL of analytes and mixed with the solvent by shaking for 10 min on a Geno Grinder, then centrifuged at 3000 x g. A white precipitate formed at the bottom of the tube indicating effective cleanup. However, the recovery of glyphosate and glufosinate were less than 50%. The low recoveries were investigated and the ion interaction between the analytes and milk components may be the culprit. Milk contains high level of polyvalent metallic cation ions such as calcium, magnesium, and iron. These mineral may form chelates with glyphosate and glufosinate resulting in loss during the protein precipitation process. The chelate formation of tetracycline in milk was previously reported [16]. Extraction solvent containing Na_2EDTA buffered solution was used to improve recovery of tetracycline determination in milk[17, 18]. After adding the Na_2EDTA in the extracting solvent, the recoveries of glyphosate and glufosinate were improved significantly. Na_2EDTA does not dissolve well in either acetonitrile or methanol. The solvent crash method with solvent plus Na_2EDTA is not an appropriate option. Therefore, the proposed extracting solution containing 50 mM acetic acid/10 mM Na_2EDTA was used in the method. Acetic acid lowered the pH of the sample to precipitate the milk protein and Na_2EDTA prevented chelation complex between polyvalent metal ions and the analytes.

Phospholipids are a major component present in milk and could be extracted along with the analytes. They may accumulate at the head of the analytical column under high aqueous mobile phase conditions and degrade column performance. Therefore, the Oasis HLB cartridge cleanup was added to the method to filter the aliquot and trap the phospholipids and other non-polar compounds in the final extract. Special cleanup cartridge specifically designed for phospholipids such as Captiva (Agilent Technology) and HybridSPE-plus (SupelCo) were also evaluated with poor recovery because glyphosate and glufosinate have phosphate functional groups similar to those in phospholipids.

3.3. Evaluation of Matrix effects

Matrix effect (%ME) in the sample extract was calculated as the slope of calibration curve of analyte in sample matrix divided by the slope of calibration curve of analyte in solvent and multiplied by 100. Therefore, a value of 100% means that no matrix effect is present. If the value is less than 100%, it means that there is matrix suppression. If the value is more than 100%, it means that there is matrix enhancement. Glyphosate and glufosinate has acceptable degree of suppression (89 to 97%), while AMPA has severe matrix suppression (11%) (Table 2). Based on this data, IS is not needed for glyphosate and glufosinate analysis (reduces the cost of analysis). However, it is necessary to use IS for AMPA analysis to correct for matrix suppression.

3.4. Method Validation

The calibration standard solutions at concentrations from 4 to 1000 ng/mL were prepared in both milk matrix and extracting solvent with the addition of IS. These solutions were injected along with the fortified samples and sample blank as previously described. For comparison purposes, four different quantification methods were used to determine the accuracy and precision of the recovery results. They were a) standard in matrix with internal standard calibration method, b) standard in matrix with external standard calibration method, c) standard in solvent with internal standard calibration method, and d) standard in solvent with external calibration method. The linearity was evaluated and they showed satisfactory linearity with coefficient of determination (R2) of more the 0.995. The specificity of the method was evaluated by analyzing procedure blank, reagent blank, and blank sample spiked at the lowest concentration (0.025 μg/mL). No relevant signal (above 20% of the lowest concentration) was observed at any of the selected transition in the blank. A reagent blank was injected immediately after the 1000 ng/mL standard and no analyte signals were detected above 10% of the 4 ng/mL standard.

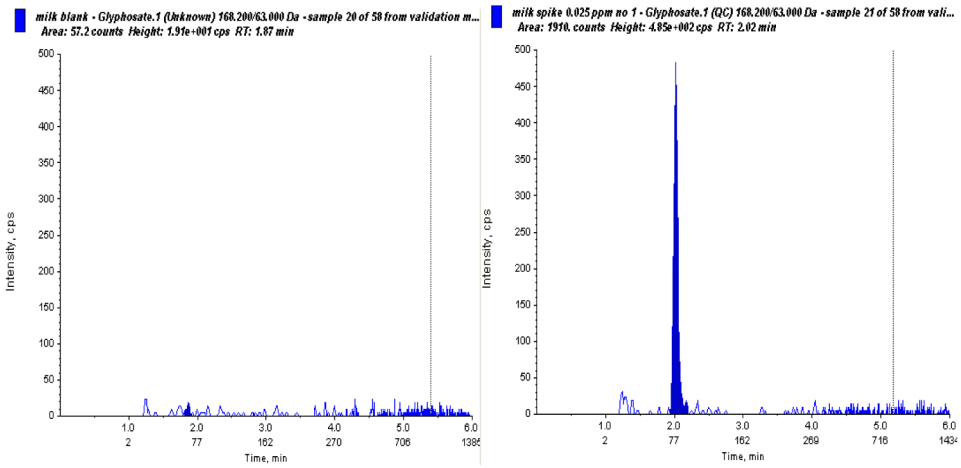
The method detection limit (MDL) for each compound was calculated according to the code of federal regulation title 40 part 136 appendix B guidelines[19] with 7 replicates of the lowest calibration standard (4 ng/mL). The MDL was calculated as standard deviation of 7 replicate x t (Students t value at the 99% confident level value with degree of freedom of 6). The MDL for glyphosate, glufosinate, and AMPA were 0.3, 0.4, and 1.4 ng/mL, respectively. The method quantification limit (MQL) was three times the MDL, and they were 1, 1, and 4 ng/mL respectively.

Accuracy (recovery%) and precision (relative standard deviation or RSD%) were evaluated at the fortification level of 0.025, 0.1, 0.5, and 2 μg/mL in seven replicates (Table 3) using all 4 calibration methods. For glyphosate and glufosinate, the average recovery was in the range from 90 to 115% with the RSD of less than 10% for the first three methods. The calibration of standard in solvent without IS had average recovery ranged from 92 to 106% with the RSD of less than 5% for glyphosate and glufosinate at all levels. On the other hand, very low recovery of AMPA (15-42%) was observed when the same calibration standard was used. This result was not surprising because of the matrix suppression effect near the area of solvent front. Therefore, IS of AMPA must be used to correct for this. Based on this data, it was concluded that the calibration standard in solvent with IS could be used to accurately quantify

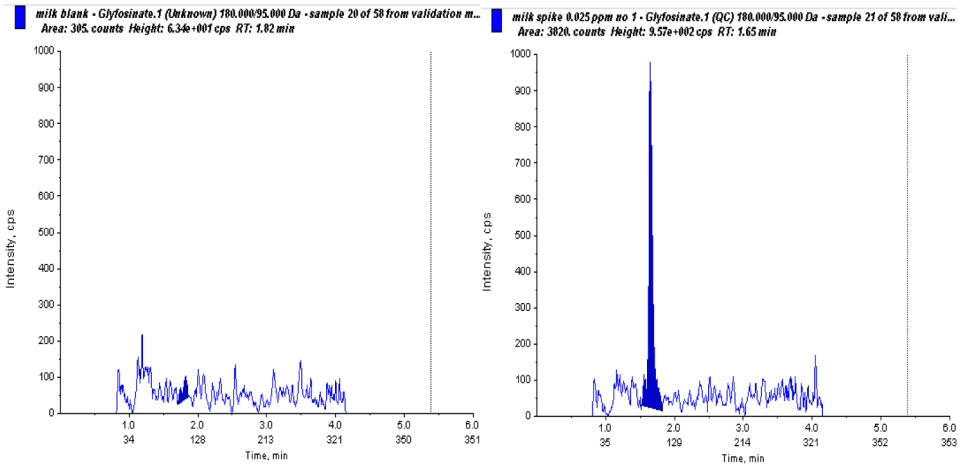
Table 3. Recovery (%) and RSD (%) data obtained in the validation experiments (n = 7).

	Fortification level		Matrix with IS	Matrix without IS	Solvent with IS	Solvent without IS
Glyphosate	0.025 µg/mL	Recovery (%)	81	85	85	123
		RSD (%)	8.36	9.94	7.62	7.30
	0.1 µg/mL	Recovery (%)	107	110	111	106
		RSD (%)	2.78	2.76	2.19	3.25
	0.5 µg/mL	Recovery (%)	93	99	100	98
		RSD (%)	2.04	2.41	2.05	2.37
	2 µg/mL	Recovery (%)	91	97	99	97
		RSD (%)	3.36	3.15	4.54	3.16
Glufosinate	0.025 µg/mL	Recovery (%)	86	89	90	123
		RSD (%)	5.43	6.83	7.66	8.00
	0.1 µg/mL	Recovery (%)	106	106	105	96
		RSD (%)	3.62	3.62	4.00	4.94
	0.5 µg/mL	Recovery (%)	94	101	96	93
		RSD (%)	6.01	1.64	6.16	1.52
	2 µg/mL	Recovery (%)	94	99	97	92
		RSD (%)	7.07	1.31	7.04	1.38
AMPA	0.025 µg/mL	Recovery (%)	83	89	84	43
		RSD (%)	2.73	5.12	3.86	2.35
	0.1 µg/mL	Recovery (%)	104	115	106	28
		RSD (%)	6.65	2.98	7.06	3.28
	0.5 µg/mL	Recovery (%)	90	113	97	17
		RSD (%)	4.35	2.53	5.44	2.67
	2 µg/mL	Recovery (%)	93	113	100	15
		RSD (%)	5.17	2.26	6.06	2.62

Glyphosate channel



Glufosinate channel



AMPA channel

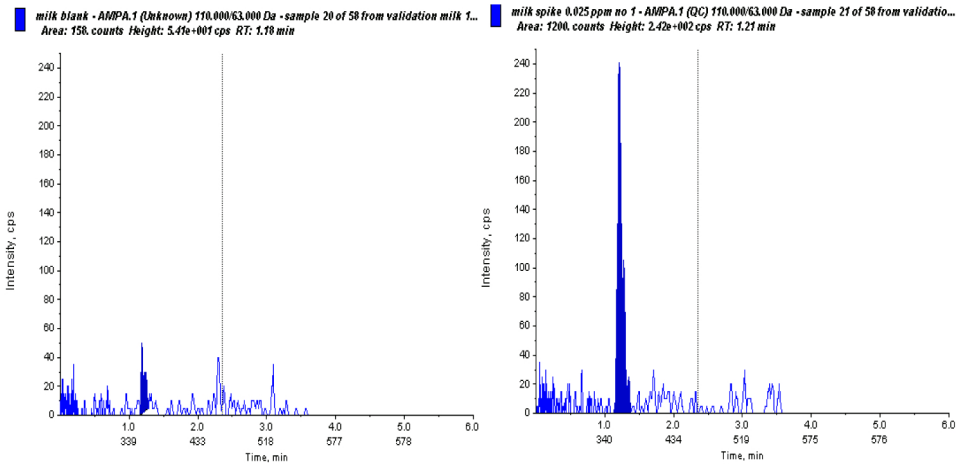


Figure 1. Chromatograms of milk blank (left) and milk blank fortified at 0.025 μg/mL of glyphosate, glufosinate and AMPA (right)

these analytes with good accuracy and precision. There is no need to prepare blank matrix to construct the calibration curve in order to reduce the cost of analysis. If only glyphosate and glufosinate were targeted, standard in solvent with external calibration method could be used with good accuracy and precision at the level above 0.1 μg/mL. Chromatograms of glyphosate, glufosinate, and AMPA in milk sample fortified at 0.025 μg/mL and in milk blank were shown in Figure 1.

The Acclaim^{TM} Trinity^{TM} Q1 combined reverse-phase, weak anion, and weak cation exchange properties in one column. This column retained glyphosate, glufosinate, and AMPA by the ion-exchange mechanism similar to the previous work done by Hao et al., on the Acclaim^{TM} WAX-1 column[10]. However, a lower concentration of salt in the mobile phase (50 mM ammonium formate) at a much lower pH, significantly improved peak shape and sensitivity with simple isocratic elution. The column was rugged and gave good peak shape and reproducible retention time over 100 injections of sample matrix without the need of column regeneration from trace metal contamination as previously suggested by Hao and others [10].

4. Conclusion

This the first paper of the kind that describes a quick, easy and reliable 6-min LC-MS/MS method for the measurement of glyphosate, glufosinate and AMPA in milk sample with the lowest fortification level of 0.025 μg/mL. One milliliter of milk sample was extracted with aqueous solution, passed thru an SPE cartridge, and directly injected and analyzed without going through tedious and time-consuming derivatization and concentration steps. Negative mode ion-spray with MS/MS measurement gives excellent sensitivity and selectivity that produce distinct chromatographic peaks with minimal interference. This is also a significant improvement over the ELISA method in term of sensitivity and selectivity.

5. Declaration of conflicting interest

The authors declare that there is no conflict of interest. Research was funded by U.S. Food and Drug Administration.

6. Disclaimer

The views expressed are those of the authors and should not be construed to represent the views or policies of the U.S. Food and Drug Administration. Any reference to a specific commercial product, manufacturer, or otherwise, is for the information and convenience of the public and does not constitute an endorsement, recommendation or favoring by the U.S. Food and Drug Administration.

7. Article information

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