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Determination of Glyphosate Levels in Breast Milk Samples from Germany by LC-MS/MS and GC-MS/MS

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ABSTRACT: This study describes the validation and application of two independent analytical methods for the determination of glyphosate in breast milk. They are based on liquid chromatography−tandem mass spectrometry (LC-MS/MS) and gas chromatography−tandem mass spectrometry (GC-MS/MS), respectively. For LC-MS/MS, sample preparation involved an ultrafiltration followed by chromatography on an anion exchange column. The analysis by GC-MS/MS involved an extraction step, cleanup on a cation exchange column, and derivatization with heptafluorobutanol and trifluoroacetic acid anhydride. Both methods were newly developed for breast milk and are able to quantify glyphosate residues at concentrations as low as 1 ng/mL. The methods were applied to quantify glyphosate levels in 114 breast milk samples, which had been collected from August to September of 2015 in Germany. The mothers participated at their own request and thus do not form a representative sample. In none of the investigated samples were glyphosate residues above the limit of detection found.

KEYWORDS: *glyphosate, breast milk, residues, LC-MS/MS, GC-MS/MS*

INTRODUCTION

Glyphosate (N-(phosphonomethyl)glycine) is among the most frequently used active ingredients of plant protection products worldwide. It is applied as a nonselective herbicide for pre-emergence weed control as well as for desiccation treatment preharvest. The use of glyphosate might lead to residues in food, especially if applied shortly before harvest. In the European Union (EU), maximum residue levels (MRLs) have been established for glyphosate, which are set for most plant and animal commodities at the limit of quantification (LOQ) of 0.1 mg/kg and 0.05 mg/kg, respectively. MRLs are up to 20 mg/kg for barley, oats, sorghum, sunflower seeds, and soybeans, and 10 mg/kg for wheat, rye, linseed, rapeseed, mustard seed, cotton seed, lentils, peas, and lupins.[1] These food items make up an important part of human and animal diets and thus might lead to intake of small amounts of glyphosate by both humans and livestock. Further exposure of humans to glyphosate might be due to direct exposure during and shortly after its application in agriculture (operator, worker, bystander, and/or resident exposure).

Glyphosate findings in urine have been reported in the literature for farmers and their families as well as for patients in cases of acute intoxication.[2−4] Published data indicated that positive findings of glyphosate in human urine are quite common and result from different exposure or intake pathways.[5,6]

In April 2014, a non-peer-reviewed report was published, in which glyphosate in breast milk of American mothers was detected in 3 out of 10 samples ranging from 76 to 166 ng/mL.[7]

Because of the high media response concerning the potential health risks for breast-fed children, 16 breast milk samples from Germany were analyzed for glyphosate in June 2015. The unpublished and nonpeer reviewed results reported glyphosate levels between 0.2 and 0.4 ng/mL in all 16 samples.[8]

In both surveys, the concentration of glyphosate in milk samples was determined by enzyme-linked immunosorbent assay (ELISA). A 96 well microtiter plate competitive assay was used for detection and quantification of glyphosate levels in breast milk of American mothers. The detection limit of the assay was given as 75 μg/L in milk.[7] Information on suitability of the ELISA for breast milk as well as the documentation of validation results was not provided for the study from Germany.[8] The studies both had methodological shortcomings. The analytical results have not been confirmed by an independent method. The number of samples was quite low and details on study participants were not reported.

The quantification of glyphosate residues is very challenging because of the highly polar and amphoteric nature of the molecule, the low molecular weight, the high water solubility and the lack of chromophores. For these reasons, glyphosate is one of the few pesticides which are not amenable to the multiresidue methods typically employed in pesticide residue analysis.

Several extraction procedures have been reported in the literature involving extraction with water, water−methanol or

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water−acetonitrile mixtures followed by LC-MS/MS measurement.[9−11]

Kacyński and Lozowicka[12] tested different extraction solvents for their ability to extract glyphosate and its metabolite aminomethyl phosphonic acid (AMPA) from rape seed. The recoveries were between 82% and 93% using water or acidified water. For liquid samples, e.g. beer, wine, tap water, surface water, groundwater, direct injection of samples without preliminary extraction steps is also reported.[9,13]

Raina-Fulton[14] reviewed analytical methods for residue analysis of glyphosate and AMPA. Most of the published liquid chromatographic methods for glyphosate are based on derivatization of the molecule followed by reversed phase HPLC separation and mass spectrometric or fluorescence detection. Preferred procedures for derivatization depend on the detection method and include reaction with fluorenylmethoxycarbonyl (FMOC) or o-phthalaldehyde-2-mercaptoethanol.[10,15]

In contrast, the direct chromatographic separation without previous derivatization is possible by using anion-exchange columns or Hydrophilic Interaction Liquid Chromatography (HILIC), since these stationary phases are able to retain polar compounds.[11,16−18]

The EU reference laboratory (EURL) for residues of pesticides, which is responsible for single residue methods (SRM) (CVUA Stuttgart, Germany), developed a method for the analysis of highly polar pesticides.[19] The residues are extracted by an acidified methanol−water mixture. For glyphosate, different detection modules involving liquid chromatographic separation on an anion exchange column or porous graphitic carbon column are described.

Application of liquid chromatographic methods was reported for the quantification of glyphosate residues in environmental matrices, food matrices and human urine and serum samples.

Determination of glyphosate by gas chromatography followed by mass spectrometric detection (GC-MS) requires the derivatization of the phosphorous acid moiety, the carboxyl group and the secondary amine prior to analysis. Two different derivatization approaches are described in the literature involving either trialkysilylation[20] or simultaneous acylation and esterification. The latter method was tested successfully in five laboratories for corn grain, soya forage and walnut meat.[21] The lowest tested concentration was 0.05 mg/kg.

Breast milk is a challenging matrix due to its very complex nature. It is an aqueous mixture of carbohydrates, proteins and fat. The composition varies individually and over the lactation period. Typically, the content of proteins is in the range of 0.8%−0.9%. The fat content is in the range of 3%−5% and the carbohydrate content, mainly present as lactose, is in the range of 6.9%−7.2%.[22] Thus, analytical methods developed for watery matrices cannot be directly transferred to breast milk. An essential step prior to the analysis of glyphosate in breast milk is the separation of the proteins and fat by suitable separation steps.

Recently, Ehling and Reddy[23] published the application of LC-MS/MS method with previous derivatization of the glyphosate with FMOC on different nutritional ingredients derived from herbicide-tolerant soybean, corn and sugar beet as well as breast milk. The authors reported an LOQ of 5 ng/g for milk samples, which is approximately 10-fold higher compared to the reported glyphosate levels of up to 0.4 ng/mL in breast milk samples from Germany.[8]

The aim of the work was the development and validation of two independent analytical principles for the quantification of glyphosate in breast milk samples. The method should validate for the highest sensitivity possible. The first method is based on LC-MS/MS without derivatization. The second method is based on derivatization with trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB) followed by GC-MS/MS determination of derivatives. The two newly developed methodologies were employed to analyze 114 breast milk samples collected from German breast-feeding women for residues of glyphosate. The results of these analyses are reported.

MATERIALS AND METHODS

Collection of Breast Milk Samples. Breast milk samples were collected in August and September 2015 by the Governmental Institute of Public Health of Lower Saxony (Niedersächsisches Landesgesundheitsamt, Hannover, Germany) and by the Bavarian Authority for Health and Food Safety (Bayerisches Landesamt für Gesundheit and Lebensmittelsicherheit, Erlangen, Germany). Since 1999, mothers from Lower Saxony can send in their breast milk for analysis of selected pesticides (e.g., organochlorine pesticides).[24] In the framework of this program, additional samples were collected for a definite period of time for this study. Breast milk samples from Bavaria were collected on a voluntary basis for the analysis of glyphosate. All participants signed a declaration of consent concerning the use of their samples for further scientific purposes. Participating mothers had not been selected by random sampling. Moreover, there are no restrictions for participating in the monitoring program (e.g., relating to age, point of sampling during lactation period, etc.). The milk samples for this study were collected and stored in polypropylene tubes, which remained frozen during storage and shipment. In total 114 milk samples were analyzed. The participants completed a self-administered questionnaire. Information on sample collection, biometric data and self-reported pesticide exposure of the participants is given in Table 1.

Table 1. Biometric Data of Study Participants

parameter	samples from Bavaria, Germany	samples from Lower Saxony, Germany
number of samples	17	97
age of mother (years)		
median	32.1	32.0
range	26−39	22−39
body weight of mother (kg)		
median	63.0	67.0
range	54−90	48−102
duration of lactating period (weeks)		
median	11.0	18
range	3−80	4−52
self-reported exposure to pesticides	6 participants	32 participants

The questionnaire also asked for the place of residence and the jobs practiced in the last 10 years. 38 participants declared the use of chemical insecticides, herbicides or wood preservatives. At least one participant has worked in a residue analytical laboratory and used pesticide standards regularly.

Twenty of the 114 breast milk samples were divided each into two subsamples to allow the parallel analysis by LC-MS/MS and GC-MS/MS.

Fortification of Breast Milk Samples Used for Performance Tests. For performance tests a homogeneous sample of breast milk was prepared and spiked with different amounts of a glyphosate standard solution (LGC Standards, Wesel, Germany) having a concentration of 10 μg/mL in water.

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Twenty-eight stored breast milk samples from a previous study of the Governmental Institute of Public Health of Lower Saxony were pooled. Using this pooled sample, four aliquots of 100 mL were fortified with glyphosate resulting in concentrations of 0.5 ng/mL, 1 ng/mL, 3 ng/mL, and 5 ng/mL. Moreover, an additional aliquot of the pooled sample served as control sample (blank sample). All five "performance" samples were divided into two subsamples and analyzed in parallel with both methods. These samples served as independent quality control. It has to be noted that analysts were not informed about this additional quality test. The labeling of these "performance" samples did not differ from other samples of this study.

LC-MS/MS analysis. *Chemicals and Apparatus.* Reference compound and internal standard ($^{13}C_2{}^{15}N$ labeled glyphosate) were purchased from LGC Standards. Methanol was obtained from Actu-All Chemicals (Oss, The Netherlands), acetic acid and citric acid from Merck (Darmstadt, Germany) and dimethylamine 40% solution from Sigma-Aldrich (Zwijndrecht, The Netherlands). The water used was purified by a Milli-Q system from Merck Millipore (Tullagreen, Ireland).

The 30 kDa molecular weight cutoff filter used for sample preparation (Amicon Ultra 4 Centrifugal filter, 30000 NMWL) was purchased from Merck Millipore, the LC column (Dionex Ionpac AS 11 (2 × 250 mm) and the AG-11 guard column (2 × 50 mm) from Thermo Fischer Scientific (Breda, The Netherlands). For centrifugation, a Z-513 centrifuge from Hermle Labortechnik (Wehingen, Germany) was used. The syringeless filter devices Mini-Uniprep (PTFE filter, 0.45 μm) from Whatman, (GE Healthcare, Eindhoven, The Netherlands) were used as LC vials. The LC-MS/MS system consisted of a Nexera UHPLC system from Shimadzu (Kyoto, Japan) and a 5500 Q-Trap system from Sciex (Concord, ON, Canada).

Sample Preparation and Measurement. For extraction of glyphosate the use of acidified methanol/water has been described.[19] However, in contrast to published results for plant materials, repeated experiments using this extraction showed no or insufficient findings of both labeled and native glyphosate in milk samples. Spiking of an extract with glyphosate did produce a signal, so matrix effects could be eliminated as the source of the problem. An alternative approach involving removal of fat by centrifugation and proteins by ultrafiltration in one step through centrifugal filtration using a molecular weight cutoff filter (30 kDa) was found to be suitable. For the samples from this study the procedure was as follows: to 3 mL of sample, 30 μL of internal standard solution containing 1000 ng/mL $^{13}C_2{}^{15}N$ glyphosate was added to obtain a level of 10 ng/mL. After mixing, the sample was transferred to the top part of the cutoff filter tube. The filter was centrifuged at 5000 g (corresponding to 3500 rpm) for 20 min. 500 μL of the filtrate was then transferred to the LC filter vial, the solution was filtered and the vial was used for measurement. After this procedure, one mL of final extract contained the glyphosate residue of one mL breast milk.

The LC-MS/MS measurement was based on a method developed by the EURL − SRM.[19] In brief: 25 μL of standard solution or filtrate were injected onto an anion exchange LC column. Glyphosate was eluted from the column using a gradient of (A) water and (B) water containing 1 mM citric acid and brought to a pH of 11 by addition of dimethyl amine solution. Gradient elution was performed: 100% A from 0 to 2 min; linear to 25% B in 5.5 min; then linear to 50% B in 2.5 min; this was held for 4 min; after returning to 100% in 0.5 min the system was re-equilibrated for 7.5 min before the next injection. The total run time (including injection) was 22.5 min. A flow rate of 0.4 mL/min was used. A column temperature of 40 °C was maintained while the temperature of the samples in the autosampler was 12 °C. For detection, the 5500 Qtrap system was used in triple-quad mode. The MS/MS transitions and the transition specific parameters that were used are provided in Table 2.

All three transitions were measured using a declustering potential of −75 V, an entrance potential of −10 V and a dwell time of 50 ms. The Turbospray source was used in negative electrospray mode using the following parameters: Curtain gas 20 arbitrary units; Collision gas Medium; IonSpray −4000 V; Temperature 400 °C; IonSpray gas 1:40 and IonSpray gas 2:50 arbitrary units.

Table 2. Transition Specific Data for the Analytical Methods

method	transition	precursor ion (m/z)	product ion (m/z)	CE[a] (V)	CXP[b] (V)
LC-MS/MS	Glyphosate quantifier	168.2	62.8	−32	−17
	Glyphosate qualifier	168.2	79.0	−52	−19
	$^{13}C_2{}^{15}N$ Glyphosate	171.2	62.8	−32	−17
GC-MS/MS	Glyphosate quantifier	612	213	25	
	Glyphosate qualifier	611	261	25	
	$^{13}C_2{}^{15}N$ Glyphosate	615	213	15	

[a]CE = collision energy. [b]CXP = collision cell exit potential.

Calculations were performed using the ratio of the peak areas of the quantifier transition of glyphosate and the internal standard.

GC-MS/MS analysis. *Chemicals and Apparatus.* Reference compound and internal standard ($^{13}C_2{}^{15}N$ labeled glyphosate) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Methanol HPLC grade, ethyl acetate and dichloromethane were purchased from LGC Standards (Wesel, Germany). Acetic acid, glacial 100% was purchased form Merck (Darmstadt, Germany). Anion exchange resin (Dowex 50WX8 hydrogen form, 200−400 mesh), citral (95%), 2,2,3,3,4,4,4-heptafluoro-1-butanol (98%), trifluoroacetic acid anhydride 99%, water (Chromasolv for HPLC) and hydrochloric acid 37% and 10 N were purchased from Sigma-Aldrich (Seelze, Germany). The solution for cation exchange cleanup (CAX solution) was prepared by mixing of 800 mL HPLC grade water, 13.5 mL 10 N HCl solution and 200 mL methanol.

The derivatization reagent was prepared by mixing 1 volume fraction 2,2,3,3,4,4,4-heptafluoro-1-butanol and 1 volume fractions trifluoroacetic acid anhydride. This solution was prepared fresh on a daily basis.

The columns for anion exchange were empty Poly-Prep columns (Bio Rad Laboratories Inc., Hercules, CA, USA). Vortex mixer REAX from Heidolph Instruments (Schwabach, Germany) was used. Heating block Reacti-Therm III #TS-18824 heating module, evaporator Reacti-Vap III #TS-18826 evaporation unit and vacuum concentrator Express SpeedVac concentrator SC250 from Thermo Fisher Scientific (San Jose, CA, USA) were used. A HS 601 D flatbed shaker was obtained from IKA (Staufen, Germany). A Rotanta 460 centrifuge from Hettich (Tuttlingen, Germany) was used. The 0.45 μm Nylon filter units (Chromafil AO-45/15 MS 15 mm) were purchased from Macherey-Nagel (Düren, Germany).

The GC-MS/MS system consisted of a Thermo Trace GC Ultra equipped with TriPlus liquid Autosampler, split/splitless injector and MS detector TSQ Quantum with triple quadrupole (Thermo Fisher Scientific). The GC column Optima 5HT, 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness was purchased from Macherey-Nagel.

Sample Preparation and Measurement. The extraction procedure was based on the method by Alferness and Iwata[25] and was adapted to reach a lower limit of quantification.

A 2 mL milk sample was extracted with 3.75 mL of 0.6% acetic acid for 10 min on a flatbed shaker with 200 rpm. Taking into account a water content of 87% for breast milk,[26] the obtained volume of aqueous phase containing glyphosate residues is 5.5 mL. Then the resulting mixture was centrifuged for 5 min at 3220 g (4000 rpm). A 2 mL aliquot of the supernatant liquid was removed and transferred in a 15 mL PP tube. Two mL dichloromethane were added. The sample was shaken for 2 min and centrifuged for 5 min at 3220 g (4000 rpm). A 1 mL aliquot of the supernatant liquid was filtered using a 0.45 μm Nylon filter unit.

A cation exchange cleanup was performed using disposable Bio-Rad Poly-Prep columns filled with 1.72 g (equivalent to 2 mL filling volume) of AG 50W-X8 resin (H^+ form). Before use, the columns

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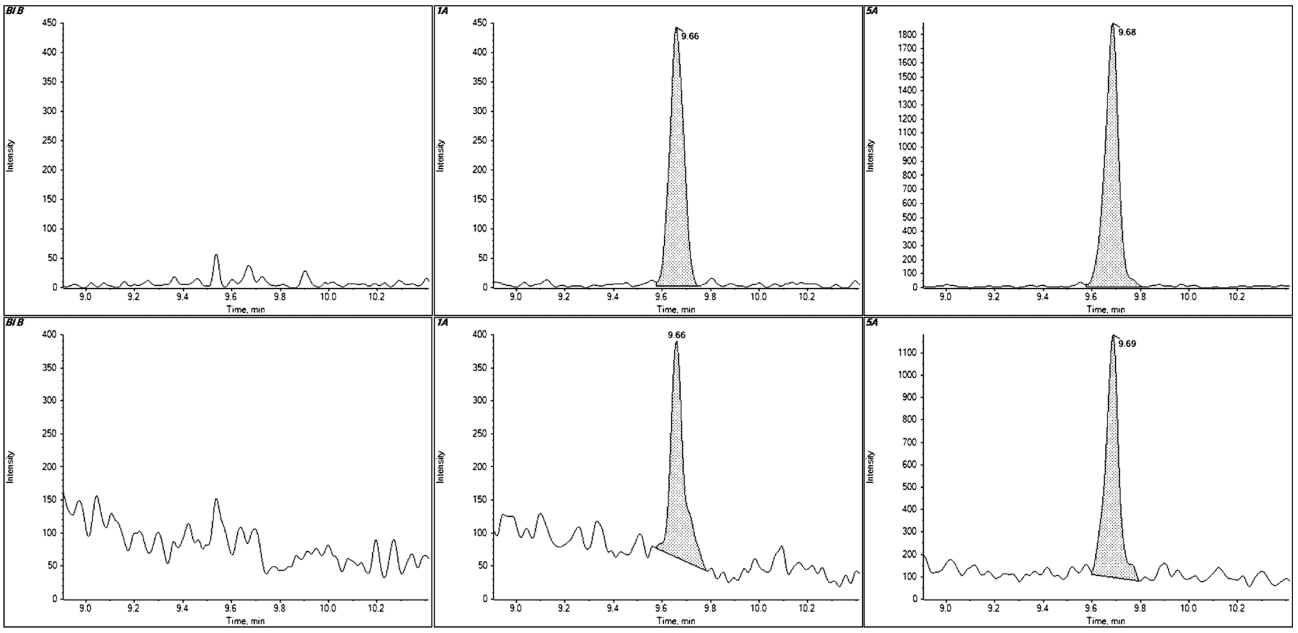

Figure 1. LC-MS/MS chromatograms of breast milk samples obtained from method validation: From left to right: blank sample, blank spiked at 1 ng/mL glyphosate, and blank spiked at 5 ng/mL glyphosate. Top: Quantifier m/z 168.2 > 62.8 for glyphosate. Bottom: Qualifier m/z 168.2 > 79.0 for glyphosate.

were washed with 10 mL of water (Chromasolv for HPLC). A 0.55 mL aliquot of the filtered extract (corresponding to 0.2 mL breast milk) and 0.100 mL of internal standard (20 ng/mL) were added to the cation exchange column and were eluted until the liquid level reached the top of the resin. Co-extractives were eluted by adding 2.0 mL of CAX solution. Both eluates were discarded. Glyphosate residues were eluted with 12.5 mL of the CAX solution. All elutions were performed using gravity. The eluate was collected in a 15 mL PP tube, and evaporated to dryness using a vacuum concentrator at 60 °C and 60 mbar. The residues were dissolved in 1.0 mL of the CAX solution. It is expected that extraction and cleanup in acidic solvents allows the decomplexation of all cationic glyphosate complexes.[27]

A 1.5 mL aliquot of the derivatization reagent was added to 2 mL vials which were sealed and placed in the heating block. The block was cooled to a temperature of −20 °C before proceeding and after adding sample extract. A 0.05 mL aliquot of the redissolved eluate (corresponding to 0.01 mL breast milk) was drawn into the disposable pipet tip and then dispensed under the surface of the chilled reagent. After 5 min, the derivatization of analyte is started by heating the reaction vial to 92−97 °C for 1 h. After allowing vials to cool, the excess of derivatization reagent was removed by evaporation to dryness. The derivatization step and the structure of the derivative were described by Alferness and Iwata.[25] Briefly, the carboxylic and phosphonic acid group were esterified to the corresponding heptafluorobutyl ester and the amine function was derivatized to the trifluoroacetyl derivative.

The residues were redissolved in 0.2 mL of ethyl acetate containing 0.2 mL/L citral and later concentrated to a final volume of 20 μL. The addition of citral to the injection solvent was made to reduce adsorption of the analytes in the inlet and the GC column. Thus, the peak shape and the sensitivity of the method was improved.[25]

Following this procedure, one mL of final extract contained the glyphosate residue of 0.5 mL breast milk.

These final extracts were analyzed by gas chromatography with tandem mass spectrometric detection (GC-MS/MS). 4.0 μL of the extracts were injected splitless. The injector temperature was 280 °C. Oven temperature program was held at 80 °C for 1.5 min, ramped then with 10 °C/min to 180 °C, ramped with 30 °C/min to 300 °C and was held for 2.8 min. Helium was used as carrier gas with a constant flow rate of 1 mL/min. The expected retention time for the glyphosate derivative was 9.1 min. The temperature of the ion source was 280 °C. Electron impact (EI) energy was 70 eV and emission current was 50 μA. The MS/MS transitions and the transition specific parameters are provided in Table 2.

Calculations were performed using the ratio of the peak areas of the quantifier transition of glyphosate derivative and the internal standard derivative. Calibration solutions were prepared by volumetric dilution of a glyphosate stock solution in a solution containing 20 ng/mL internal standard. The dilutions were made in CAX solution. 0.05 mL aliquots of these calibration solutions were derivatized as described for the breast milk extracts. Concentration of the derivatized calibration solutions ranged from 0.01 to 10 ng/mL. The concentration of the internal standard in the final extract was always 5 ng/mL.

RESULTS AND DISCUSSION

For method development the advantages and disadvantages of different extraction procedures have to be weighed in order to achieve the best method performance. For evaluation of the methods the requirements of the EU guidance document for quality control and validation procedure[28] were considered. The guidance applies to laboratories involved in the official control of pesticide residues in food and feed in the EU. Briefly, a quantitative analytical method has to be validated with respect to sensitivity/linearity, specificity, trueness (bias), precision, and robustness. Matrix effects should be assessed. The average

Table 3. Method Performance Characteristics of LC-MS/MS and GC-MS/MS Methods as Obtained from Spiked Samples Concurrently Analyzed with the Study Samples[a]

method	spiking level (ng/mL)	avg recovery (%)	range (%)	RSD(R)[b] (%)
LC-MS/MS	1	99	85−128	16 (n = 7)
	5	91	83−99	7 (n = 6)
GC-MS/MS	1	84	71−102	13 (n = 6)
	10	83	73−90	8 (n = 6)

[a]For quantifier transition. [b]RSD(R) = within-laboratory reproducibility.

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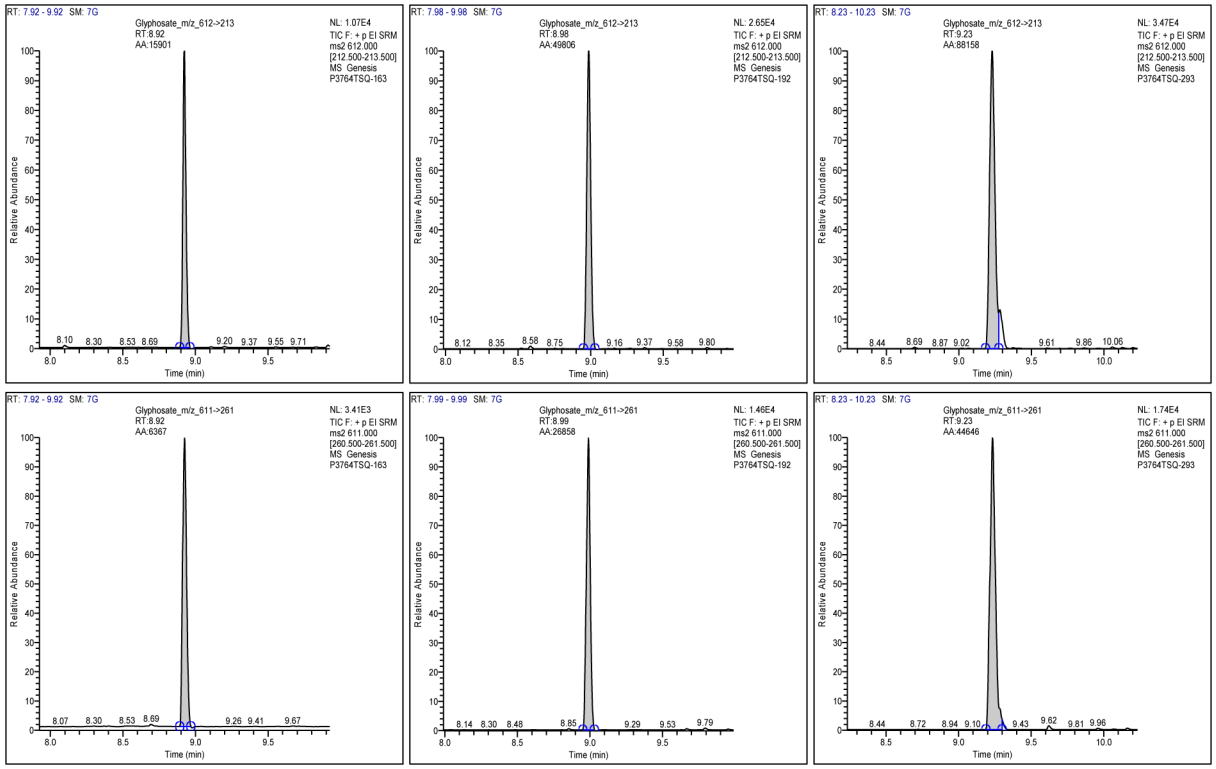

Figure 2. GC-MS/MS chromatograms of breast milk samples obtained from method validation. From left to right: reagent blank sample; breast milk sample spiked at 1 ng/mL glyphosate; breast milk sample fortified at 5 ng/mL glyphosate. Top: Quantifier m/z 612 > 213 for glyphosate derivative. Bottom: Qualifier m/z 611 > 261 for glyphosate derivative.

recovery (trueness) of a minimum of five spiked samples per fortification level should be in a range of 70−120%. The repeatability (relative standard deviation) should be <20% for each analyte. The LOQ is established as the lowest fortification level with an acceptable mean recovery and an acceptable relative standard deviation.

In our work, the method development is clearly focused on reaching the lowest quantification limit in breast milk with still sufficient method performance. From farm animal metabolism studies with radiolabeled glyphosate a very low transfer into muscle, milk and fat was observed.[29] Consequently, if glyphosate concentrations occur at all in breast milk, they are expected to be low. Therefore, the LOQ of the method should achieve at least the published LOQ of Ehling and Redding[23] of 5 ng/g for milk measured by LC-MS/MS. Unpublished results of the analysis of glyphosate in urine by GC-MS/MS indicated that this method might be at least similarly sensitive.

According to published results, a derivatization of glyphosate residues should improve the detection in LC-MS/MS. On the other hand, after derivatization several additional cleanup steps might be required to remove the excess of derivatization chemicals. Considering this aspect, it was preferred to forego a derivatization step for the determination by LC-MS/MS.

Prior to the LC-MS/MS analysis removal of fat by centrifugation and proteins by using a 30 kDa cutoff filter was necessary to prevent contamination of the system. The LC-MS/MS method was validated for glyphosate in accordance with the requirements of the EU guidance document for quality control and validation procedure.[28] Recovery and precision of glyphosate were determined for 6 or 7 replicates at two fortification levels. The linearity of the system was tested by injecting eight standards in water in a concentration range from 0 to 50 ng/mL. A linear relationship between concentration and the ratio of the peak area of glyphosate and its internal standard was observed. The coefficient of determination was greater than 0.99. All calibration points were within 20% of the theoretical value. The quantification was performed using single-point calibration which is acceptable if the response of the analyte in the samples is close to the response in the standard.[28]

The lower level (1 ng/mL) demonstrated sufficient recovery and precision. This level is considered as the LOQ of the LC-MS/MS method. Possible matrix effects were corrected by use of the stable isotope labeled internal standard $^{13}C_2{}^{15}N$ glyphosate. At a concentration of 0.5 ng/mL, a signal-to-noise ratio of approximately 4 is obtained. This concentration is considered as the limit of detection of the LC-MS/MS method. Chromatograms of blank milk samples and fortified samples are provided in Figure 1. It is clearly visible at the 1 ng/mL level that the signal-to-noise ratios for both quantifier and qualifier transitions are well above three.

For GC-MS/MS determination, extraction with acidified water was combined with cleanup on a cation exchange column to remove interfering natural compounds present in breast milk. Since glyphosate is too polar for gas chromatography, a derivatization of all polar groups (the phosphorous acid moiety, the carboxyl group and the secondary amine) prior to analysis by heptafluoro-1-butanol and trifluoroacetic acid anhydride was chosen. The validation data for the GC-MS/MS method determined in accordance with the requirements of the relevant EU guidance document[28] are given in Table 3.

The calibration was performed with freshly prepared derivatives of eight glyphosate standard solutions in the concentration range from 0.01 to 10 ng/mL. All standard

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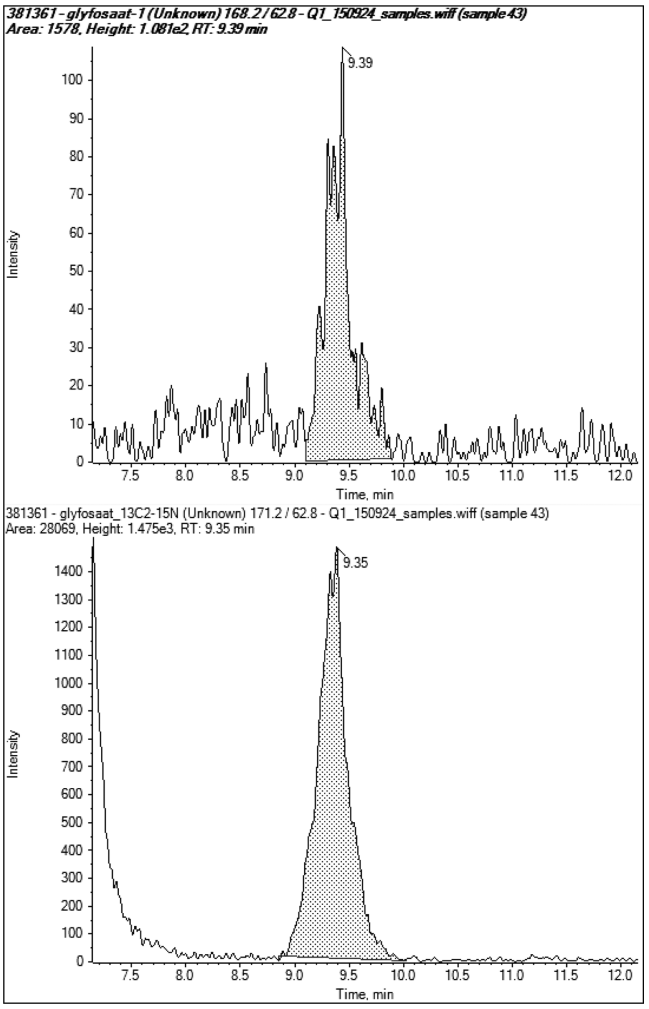

Figure 3. Extracted ion chromatograms of a breast milk sample spiked with glyphosate at 0.5 ng/mL (limit of detection). Top: Quantifier m/z 168.2 > 62.8 for glyphosate. Bottom: Internal standard $C_2{}^{15}N$-glyphosate m/z 171.2 > 62.8.

solutions contained the internal standard at a level of 5 ng/mL. The coefficient of determination was always equal to or greater than 0.9980.

Considering the sensitivity of the instrument, the method would have allowed the detection of glyphosate residues at a level as low as 0.02 ng/mL (instrumental detection limit). However, significant blank values were detected in all samples, the reagent blank and all calibration standards. To identify the source of this glyphosate interference, all solvents and most reagents were tested (i.e., ultrapure water from three different sources, all components of CAX solution and extraction solution). However, a clear origin of the interference was not detected. Therefore, it is assumed that one of the derivatization agents produced the blank signal. It was not possible to check this hypothesis within the scope of this study. It is noteworthy that there was no detectable interference of the mass transition monitored for the internal standard in all derivatization blanks.

Since this interference could not be eliminated, all results obtained by GC-MS/MS had to be corrected for (reagent) blank interferences. A set of reagent blanks (at least 4 samples) were analyzed within each set of breast milk samples. The average measured blank values ranged from 0.2 to 0.6 ng/mL. The relative standard deviations of blank values in the sample sets ranged from 19% to 33%. Considering the blank values from the derivatization reagent, the LOQ of the GC-MS/MS method is 1 ng/mL. Chromatograms of reagent blank and spiked milk samples are given in Figure 2.

The recovery and precision data of both methods obtained during method validation are provided in Table 3.

Notwithstanding the interference problem of the GC-MS/MS method, both analytical methods were able to measure the occurrence and level of glyphosate residues in breast milk from German women with an LOQ of 1 ng/mL. The availability of two validated methods offered the chance to confirm positive results, if this would be required.

In total, 114 different breast milk samples were analyzed for glyphosate. 75 samples were analyzed by LC-MS/MS only. Because of the lower performance of the second method, only 19 samples were analyzed exclusively by GC-MS/MS. Further 20 milk samples were analyzed by both methods.

In addition to these 114 samples, five samples for the performance test were analyzed by both LC-MS/MS and GC-MS/MS: four breast milk samples which were spiked in advance with glyphosate and one control sample. Glyphosate was identified by LC-MS/MS in all samples containing glyphosate. The recoveries for the LC-MS/MS method were 110%, 97% and 102% for the spiking level of 1 ng/mL, 3 ng/mL and 5 ng/mL, respectively.

In the sample spiked at 0.5 ng/mL, glyphosate could still be detected by the LC-MS/MS method. An ion chromatogram of this sample is shown in Figure 3. Due to the interference problem in GC-MS/MS, no clear detection of glyphosate was possible at this level.

The recoveries for the GC-MS/MS method were 70%, 70%, and 54% for the spiking levels 1 ng/mL, 3 ng/mL, and 5 ng/mL, respectively. Generally, the GC-MS/MS method tended to result in lower concentrations, probably due to the correction for the procedural reagent blank values. The bias of the GC-MS/MS method is higher compared to the LC-MS/MS method. This might be due to dilution steps with very small volume. The concentration step to yield the final volume might result in a partial loss of the glyphosate derivative.

Nevertheless, both methods are able to quantify glyphosate residues in breast milk at or above a concentration of 1 ng/mL. Because of the lack of significant blank values in the LC-MS/MS method, residues of glyphosate higher than 0.5 ng/mL are still detectable by this method. In none of the 114 analyzed breast milk samples, apart from the spiked samples, was glyphosate detected.

An LC-MS/MS and a GC-MS/MS method have been newly developed for the detection of glyphosate in breast milk. Both methods have been fully validated and are suitable for the determination of glyphosate with an LOQ of 1 ng/mL. The LC-MS/MS method, furthermore, allows detection of glyphosate at or above a level of 0.5 ng/mL. The LC-MS/MS method is much faster than the GC-MS/MS method, thus making it suitable for higher sample throughput.

Summarizing the results, the positive findings of glyphosate in breast milk of American women[7] could not be confirmed by our results. In none of the 114 breast milk samples collected from German women in August and September 2015 was glyphosate found within the detection limitations of the analytical methods.

Available data from farm animal studies on glyphosate with nonlabeled material support these results. They provide no

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indication of a significant carry-over into fatty tissues or milk even at high dosing levels.[29]

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