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Article

Method Validation for the Determination of Glyphosate and Aminomethylphosphonic Acid in Water by LC-MS/MS

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Abstract: The massive use of glyphosate as a herbicide compound has led to the ubiquity of AMPA in the environment, especially in water. So, the present work aimed to establish the validation parameters of the LC-MS/MS method for glyphosate and its metabolite AMPA, determination in water. During the extraction process the derivatization of glyphosate as an extreme polar pesticide, was done using FMOC. The validation results show that the method met the criteria given in SANTE/11312/2021 for all the investigated validation parameters as well as estimated measurement uncertainty.

Keywords: Glyphosate; AMPA; LC-MS/MS; validation; water.

1. Introduction

The use of pesticides is one of the most important ways to protect plants and plant products from harmful organisms, including weeds. Because of the possible risks to human health and the environment, pesticides are the most thoroughly tested chemicals in the world. At the international level, the legislative framework is becoming more and more demanding and stricter when approving pesticides, determining maximum permissible amounts (MAQ), and monitoring pesticide residues in food of plant and animal origin [1]. Currently, 526 active pesticide substances with approved status are registered on the European Union market, of which 134 have herbicidal activity, including glyphosate. The authorization for the use of glyphosate is constantly renewed [2]. EU Regulation 2022/2364 is currently in force, approving the use of this herbicide until 15 December 2023 [3]. What its future will be depends on the decision of EFSA (EFSA - European Food Safety Authority) and ECHA (ECHA - European Chemical Agency).

Weeds can significantly affect the yield of cultivated plants by shading and suffocating as well as competition for nutrients and water. In addition, weeds can affect crops by reducing the quality and value of crops, acting as host plants for various pests and diseases of cultivated plants, or affecting the operation of harvesters at harvest. The presence of weeds at harvest can increase the costs of grain drying and cleaning, and some weeds are poisonous to both livestock and humans [4]. Herbicides provide control of weeds and vegetation in many situations where no other method is effective. Herbicide performance is measured by activity, selectivity, and soil residue characteristics.

Since its commercial introduction in 1974, glyphosate has become the dominant herbicide worldwide. The following reasons contributed to its success: it is a very effective broad-spectrum herbicide, it translocates well, and it is the only herbicide whose site of action is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). The most important reason for its success was the low price due to the registration of the generic active substance as well as the introduction of transgenic, glyphosate-resistant crops in 1996 [5].

Aminomethylphosphonic acid (AMPA) is a widespread degradation product of glyphosate and other phosphonates. The massive use of the parent compounds has led to the ubiquity of AMPA in the environment, especially in water [6].

Initially, attention to surface water pollution was focused on the transmission of disease, a problem that arose because rivers and lakes were used as both water sources and waste disposal sites. Then the problem of oxygen consumption began to appear, which had a negative impact on the flora and fauna in the aquatic environment. The water quality of streams, rivers, lakes, and even coastal oceans has often been degraded by the excessive growth of plants and algae caused by anthropogenic input of nutrients, such as nitrogen and phosphorus compounds. In the second half of the twentieth century, the increasing use of antibiotics, hormones, drugs, and pesticides, as well as the excessive use of personal hygiene products, led to new problems. Research shows that although such substances occur in very low concentrations in surface waters, they can have subtle, but harmful, effects [7]. Herbicides are the second most important class of pesticides used in the European Union. The biggest problems when assessing water quality related to herbicide contamination are seasonal changes in application and low maximum allowed concentrations set by European policy [8].

Due to their characteristics such as extremely high polarity, low volatility, low molecular weight, and the lack of chemical groups that would facilitate their detection, most of the developed methods for the determination of glyphosate and AMPA residues in aqueous matrices require a derivatization procedure to enable their analysis by gas or liquid. chromatography (Ibáñez et al., 2005). In our country, no research was conducted to determine the residues of glyphosate and AMPA in water, and there are no data on the presence of these two compounds in water systems.

This paper will cover the validation of the liquid chromatography with tandem mass spectrometry (LC-MS/MS) method as a sensitive and accurate method [9] for the determination of glyphosate and AMPA residues in water samples. The validated LC-MS/MS method will be applied to determine very low levels of glyphosate and AMPA residues in water samples of the Danube-Tisa-Danube hydro system (DTD).

2. Materials and Methods

Chemical and reagents

Glyphosate (GLY), aminomethylphosphonic acid (AMPA), and fluorenylmethoxycarbonyl chloride (FMOC-Cl) were supplied by SIGMA-–Aldrich Chemie GmbH (Steinheim, Germany). Standard solutions of GLY and AMPA were prepared in Milli-Q water (1000 mg/mL) and diluted for their use as working solutions (1 μg/mL).

All the other chemicals and reagents used were analytical grade materials. Hydrochloric acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water (418 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

Validation parameters

The validation parameters were done following the propositions of the EU document SANTE/11312/2021 [10].

Linearity of detector response

During the setting of the validation parameters, the linearity of the method without derivatization was checked at five concentration levels, ie at 0.01; 0.02; 0.05; 0.1, and 0.2 μg/L.

The linearity in which glyphosate and AMPA were determined using FMOC (using derivatization) was checked at four calibration levels ranging from 0.01 to 0.1 μg/L (procedural calibration), spiking sample at the beginning.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ was determined experimentally by enriching blank water samples so that the final concentration of glyphosate and AMPA was 0.01 μg/L.

The LOD was determined using MassHunter software, based on a signal-to-noise ratio = 5. The LOD was calculated based on the ratio of the peak area to the standard deviation of the noise in the chromatogram for the lowest spiked sample concentration.

The LOD represents the lowest concentration that can be determined by a given method but is not quantified with satisfactory reliability.

The LOQ represents the smallest concentration that can be determined with satisfactory accuracy and precision by a given method.

Recovery

The recovery was determined by enriching blank samples in five replicates at two concentration levels. In both methods, the enrichment of samples was at the levels of 0.01 and 0.1 μg/L.

Reproducibility of the method

The reproducibility of the methods was tested by preparing one sample in five repetitions at the same concentration level. The obtained results were statistically processed using Microsoft Excel 2013 and the obtained %RSD value was compared with the criterion using the Horowitz equation. The obtained value of RSDr, RSDR, (%) is compared with the calculated RSD (%), i.e. the theoretical relative standard deviation (AOAC Peer-Verified Methods Program Manual on Policies and Procedures) (Equation 2):

$$RSD_R < 2^{(1-0.5logC)}$$

$$RSD_r < 2^{(1-0.5logC)} * 0.67 \qquad \text{Equation (2)}$$

Where are:

RSDR – Relative standard deviation of interlaboratory reproducibility

RSDr - Relative standard deviation of repeatability

Derivatization procedure

Derivatization of the samples was performed according to the method described by Ibáñez et al. [11] and Hanke et al. [12], as well as Vuković et al. [13] and Agarski et al. [14] for the determination of glyphosate and AMPA in natural waters. Namely, 100 mL of the filtered water sample was acidified with 6 M HCl to pH 1 and immediately transferred to a smaller plastic container with a lid of 250 mL. The samples were left to stand for 1 hour. Then the samples were neutralized with 6 M KOH to pH 6-7, after which 10 mL of borate buffer (pH 9) and 10 mL of 6.5 mM FMOC-Cl were added. Ibáñez et al. [11], applied this step of acidification and neutralization, followed by derivatization of samples with FMOC-Cl to increase the extraction yield. Without this step, extraction yields were around 15%. As a possible explanation, they cite the slow kinetic interaction between glyphosate and some matrix components, which can act as chelating agents, making glyphosate unavailable for derivatization and thus for analysis.

The plastic containers were then placed in an ultrasonic bath for about 10 minutes. The samples prepared in this way were stored in the dark overnight at room temperature. The next day, samples were acidified with formic acid (pH to 3) to stop derivatization and then filtered through filter paper into Erlenmeyer flasks. 100 mL of deionized water and 4 mL of 0.1 M EDTA were added to the filtered derivatized samples and vortexed for about 1 minute.

SPE columns (Bond Elut PLEXA) were mounted on a vacuum manifold (Supelco Manifold) and activated by passing 5 mL of methanol (2x2.5 mL) followed by 5 mL of water. After activating the SPE column, the entire amount of samples was passed under a vacuum of 5 bar. The washing of the column was carried out by passing 3 mL of dichloromethane twice. Elution of the analytes was performed by passing 6 mL of methanol (2x3 mL) with a 15 mL plastic cuvette placed under each column. The obtained extract was evaporated in a stream of nitrogen and re-dissolved in the mobile phase. After filtering through a 45 μm filter, the sample was analyzed by LC-MS/MS.

Conditions for LC-MS/MS analysis after derivatization

The LC-MS/MS conditions were given in Table 1.

Table 1. LC-MS/MS conditions.

Liquid chromatography	
Instrument	Agilent 1260
Autosampler	1260 ALS, model G1329B
The volume of the injected sample	$V_{inj,pest}$= 10 μL
Type of injection	With flushing
Binary pump	1260 QuatPump, model G1311B
Mobile phase	A: 10 mM ammonium formate MeOH B: 10 mM ammonium formate in water
Flow rate	0.3 mL/min
Gradient	0 min – 70% B 5 min – 70% B 10 min – 10% B 15 min – 5% B 18 min – 5% B 20 min – 70% B
Duration of analysis	20 min
Return time to initial conditions	5 min
Column thermostat	1260 TCC, model G1316A
Column temperature	35 °C
Mass spectrometer	
Instrument	Agilent 6410B Triple Quad LC/MS
Ion source	Agilent ESI
Type of ionization	(-)ESI
Drying gas flow	10 L/min
Gas temperature	350 °C
Nebulizer	40 psi
Mass measurement range	m/z 15 – 1650
Capillary voltage	negative 4000 V
Frag (V)	100 V
CE (V)	100 V

3. Results and Discussion

The chromatographic analysis included optimization of the mass spectrometer, i.e. tuning the fragmentation and collision energies to obtain ions with the strongest response. Ions that gave the strongest signals were taken for quantification (Q), while ions with weaker intensity were used for confirmation.

MRM transitions (m/z) for glyphosate and AMPA, as well as fragmentation and collision energies, are shown in Table 2.

Table 2. MRM transitions of glyphosate and AMPA.

Anlyte	**Transition (m/z)**	**Frag (V)**	**CE (V)**
Glyphosate	390.2 → 167.8	100	6
	390.2 → 150.0	100	6
AMPA	332.0 → 110.0	100	5
	332.0 → 109.8	100	6
	332.0 → 136.0	100	6

Validation parameters

Chromatograms of the selective ion monitoring reaction (MRM) using the derivatization method are shown in Figure 1.

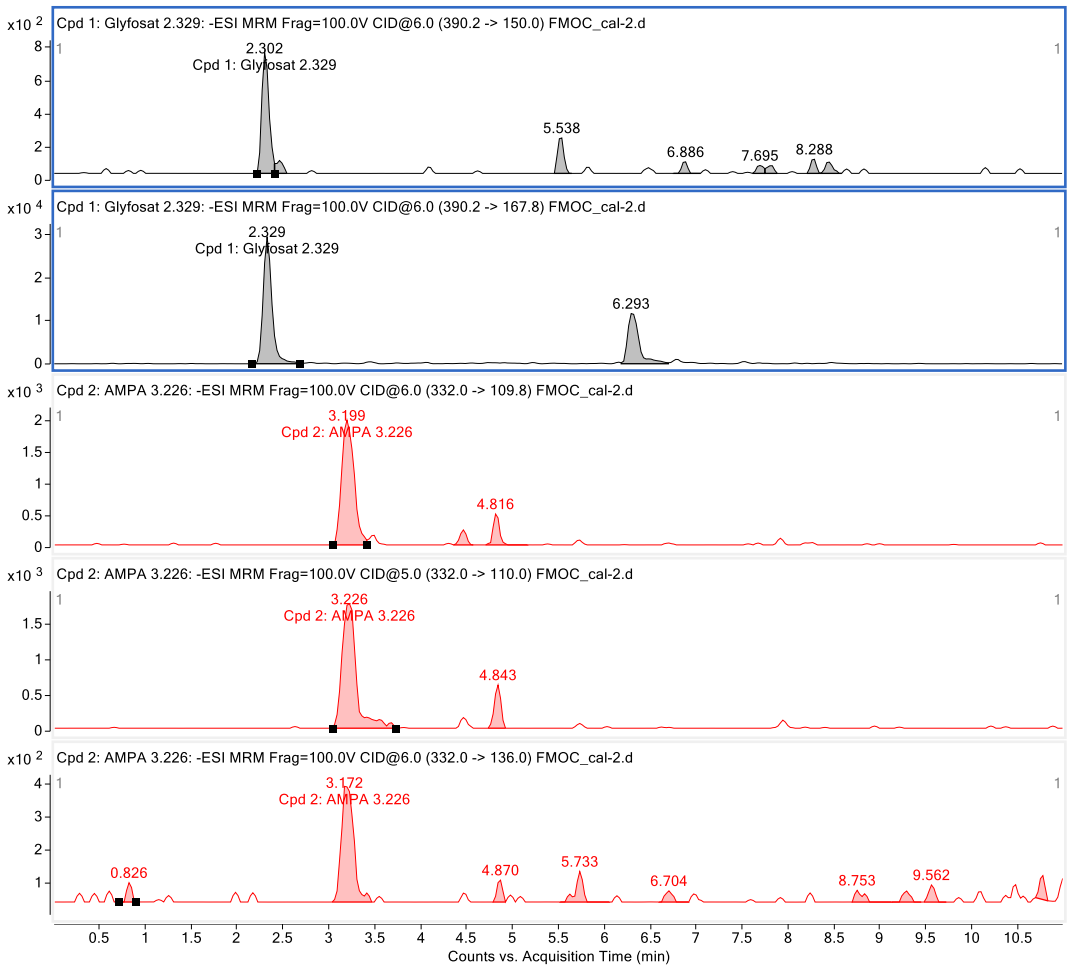

Figure 1. MRM chromatograms of the enriched water sample (glyphosate and AMPA at a concentration level of 0.025 µg/L.

The calibration curve of the method for determining glyphosate and its metabolite was tested for the concentration interval in the range of 0.01 - 0.1 µg/L and is shown in Figures 2 and 3.

The coefficient of correlation for glyphosate (R^2=0.9968) and for AMPA (R^2=0.9936) met the linearity requirement for the given test range (R^2>0.99) for mass spectrometry. Calibration curves with associated equations for glyphosate and AMPA were y=2219535.060*x - 21578.655 and AMPA y=429904.924x-5105.060, respectively.

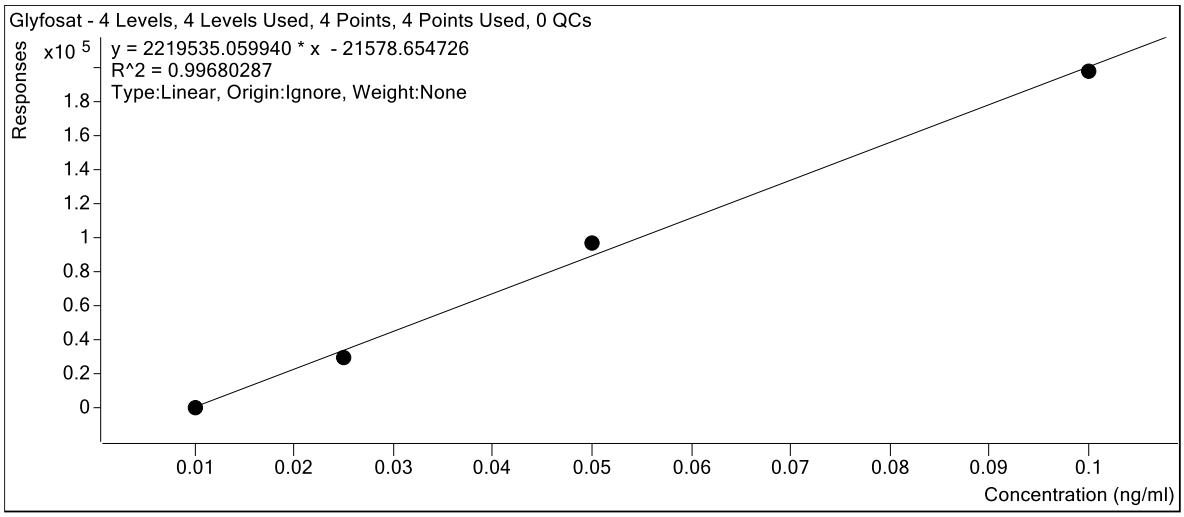

Figure 2. Calibration curve of glyphosate

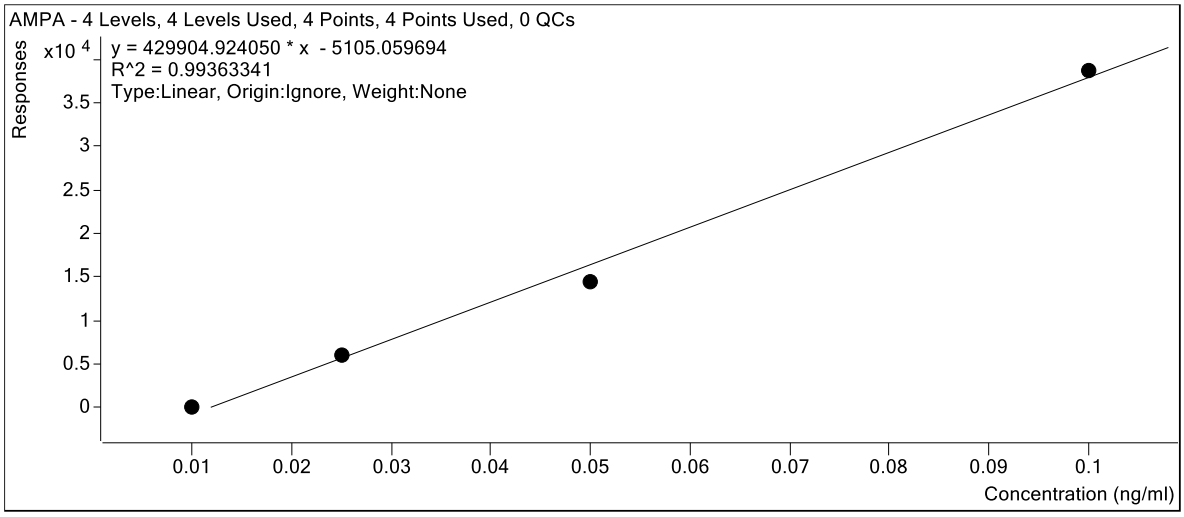

Figure 3. Calibration curve of AMPA

Recovery

The recovery (Re, %) was determined by enriching blank samples in five replicates at the levels of 0.01 and 0.1 μg/L. The table (Table 3), shows Re values by levels and repetitions, their average values as well as the achieved recovery. The relative standard deviation of the individual enrichment level (%RSD1,2), the average relative standard deviation (%RSDr), and the estimated measurement uncertainty (U©2, %) are also tabulated.

Table 3. Recovery, RSD, and U©2.

Analyt	Level μg/L	Re1	Re2	Re3	Re4	Re5	$Re_{1,2}$	Re,% average	$\%RSD_{1,2}$	%RSDr	U©2
AMPA	0.01	121.5	95.8	122.5	127.8	122.2	**118.0**		10.71		
	0.10	109.3	111.9	109.6	120.9	109.0	**112.1**	**115.1**	4.50	**13.60**	**42.14**
GLY	0.01	82.4	100.2	90.1	79.1	84.1	**87.2**		9.49		
	0.10	89.9	69.6	79.8	69.3	81.8	**78.1**	**82.6**	11.20	**12.04**	**48.80**

*Concentration spiking levels of blank water sample (μg/L), Re – recovery (%), PE1,2 – recovery mean value (%), Re , average - mean value of recovery for both spiking levels (%), %RSD1,2 - relative standard deviation of individual spiking level, %RSDr - intercept relative standard deviation, U©2 - estimated measurement uncertainty (%) for confidence level 95% (k=2)

4. Conclusions

The validation results show that the method met the criteria given in SANTE/11312/2021 for accuracy (recovery between 70 and 120%) and precision (%RSDr<20%). Also, the estimated measurement uncertainty was lower or around 50%, which is another requirement of the SANTE/11312/2021 document. The recovery of AMPA was greater than 100%, which may be a consequence of the matrix influence which affected the increase of the signal. The result for glyphosate was lower than 100%, which may indicate that during the derivatization procedure, glyphosate was not completely derivatized.

Conflicts of Interest: The authors declare no conflict of interest.

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