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GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID LEVELS IN WATER AND SOIL SAMPLES FROM TRANSYLVANIAN ROMA COMMUNITY ANALYZED BY HPLC-FLD METHOD

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ABSTRACT. This paper purpose was to develop a sensitive and selective method for the determination of glyphosate, and aminomethylphosphonic acid (AMPA) residues in water and glyphosate from soil samples. The method involves a derivatization step with 9-fluorenylmethylchloroformate in borate buffer of these compounds and liquid chromatography separation with fluorescence detection (HPLC-FLD). Separation of derivatized glyphosate and AMPA compounds was performed on an Agilent ZORBAX C18 reversed-phase column. The mobile phase consisted of a mixture of acetonitrile and 0.05 M KH_2PO_4 solution [30:70 v/v]. Limits of detection (LOD) was 0.28 µg L^{-1} for glyphosate, and 0.35 µg L^{-1} for AMPA, and limits of quantification (LOQ) was 0.84 µg L^{-1} for glyphosate and 1.05 µg L^{-1} for AMPA. The method has been validated for surface water and soil by recovery studies with samples spiked at 25 and 5 µg L^{-1}. In water samples, the mean recoveries values ranged between 86.44 - 103.9% for glyphosate, and 71.27 - 99.08% for AMPA. The mean recoveries values for glyphosate ranged from 57 - 81.5% in soil samples. The developed method has been applied for determination of these compounds in water and soil samples from agricultural and rural areas of Roma community from some Transylvania Counties.

Keywords: *Glyphosate, aminomethylphosphonic acid, water, soil, FMOC-Cl, RP-HPLC-FLD.*

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INTRODUCTION

Glyphosate, [N-(phosphonomethyl) glycine] (GLY), is a non-selective herbicide used primarily for weed and vegetation control [1]. GLY degradation in the environment is mainly due to biodegradation and its main metabolite in plants, water and soil is aminomethylphosphonic acid (AMPA) [2]. GLY low toxicity found to mammals and its rapid transport from plant leaves to their underground parts have contributed to the massive use of it, which has become one of the most widely used herbicides in the world [3]. The World Health Organization (WHO) reconsidered glyphosate in 2015 as potential carcinogenic to humans and the European Chemical Agency published in 2017 a scientific opinion as regards the harmonized classification of glyphosate [4,5,6,7].

GLY and AMPA are low molecular weight, low volatility, highly polar and exhibit insolubility in organic solvents and high solubility in water (12 g/L for GLY). Research studies have shown that both compounds, due to their functional groups, behaves as an amphoteric molecule [4], binding strongly to soil particles so that they persist for up to 170 days with a half-life of 45-60 days [8], this period being influenced by temperature and soil moisture [9]. Due to their physico-chemical properties, their analysis using liquid chromatography (LC) are most suitable than gas chromatography (GC) methods. Although, the lack of specific chemical groups of GLY and AMPA, like chromophores, UV absorption, fluorogenics, disturb their measurement by conventional detectors, being necessary pre-column or post-column derivatization procedures [10].

Direct analysis of GLY and AMPA, without the derivatization step, remain an open issue for the analysts. Marek and Koskinen developed a method for the straightforward analysis of GLY and AMPA in soil using for separation a Bio-Rad cation H exchange column coupled to LC–MS/MS [11] Pre-column methods are based mainly on derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) (see Figure 1), fluorogenic labeling with o-phthalaldehyde (OPA) and mercaptoethanol with *N*,*N*-dimethyl-2-mercaptoethylamine. In post-column procedures, the most known reactions are ninhydrin derivatization followed by UV detection [12].

The HPLC methods coupled to a wide variety of detectors were used to analyze these pesticides: fluorescence (FLD) [13,14,15,16,17], ultraviolet (UV) [18]; reversed-phase liquid chromatography–heated electrospray ionization-tandem mass spectrometry (RP-LC-HESI–MS/MS) [19], HPLC coupled to mass spectrometry (MS) 20,21,22,23], HPLC tandem MS (MS/MS) [4,24,25,26], ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [27]; HPLC inductively coupled plasma MS (ICP-MS) [28,29] and time-of-flight MS (TOF-MS) [30].

The aim of this paper is to develop a sensitive and selective method for the determination of low concentrations of GLY and AMPA in surface water and GLY from soil samples, by RP-HPLC-FLD, after FMOC-CL derivatization. The present study does constitute the first monitoring survey regarding the presence of GLY and AMPA in water sources, and GLY in soil, from the Roma community on the part of Transylvania territory. Samples were collected during the summer -autumn 2021 from agricultural as well as to rural areas, since GLY-containing products are also marketed for non-professional uses.

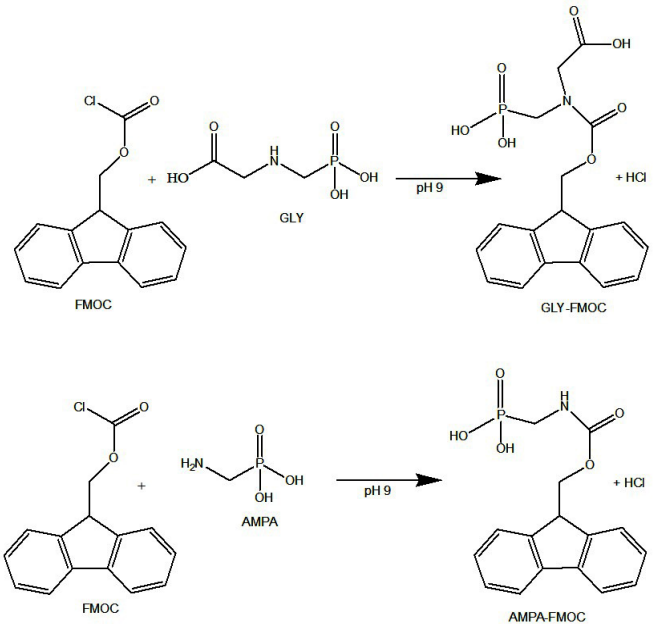

Figure 1. Derivatization reaction of GLY and AMPA with FMOC-Cl

RESULTS AND DISCUSSION

Developing and validation of the RP-HPL-FLD method

An analytical procedure developed by the Garba et. al [17], based on pre-column derivatization with FMOC-Cl followed by high-performance liquid chromatography with fluorometric detection was improved and optimized for the analysis of the GLY herbicide and its metabolite AMPA in water, and GLY in soil. The ultrasound-assisted extraction was used for the isolation of the target compound from soil samples, followed the above-mentioned procedure.

The RP-HPLC-FLD methods were validated on following parameters: selectivity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) [31].

Selectivity was tested by comparing the chromatograms of a standard solution of mixture of GLY and AMPA, with those of a water and soil samples as presented in Figure 2. The chromatograms of the standard solution show the retention times of 2.820 and 4.961 min for GLY and AMPA, respectively. As can be seen from the chromatograms, alongside to the interest peaks, another large peak, which is attributed to FMOC -OH residual, appears at 35 min [17, 32].

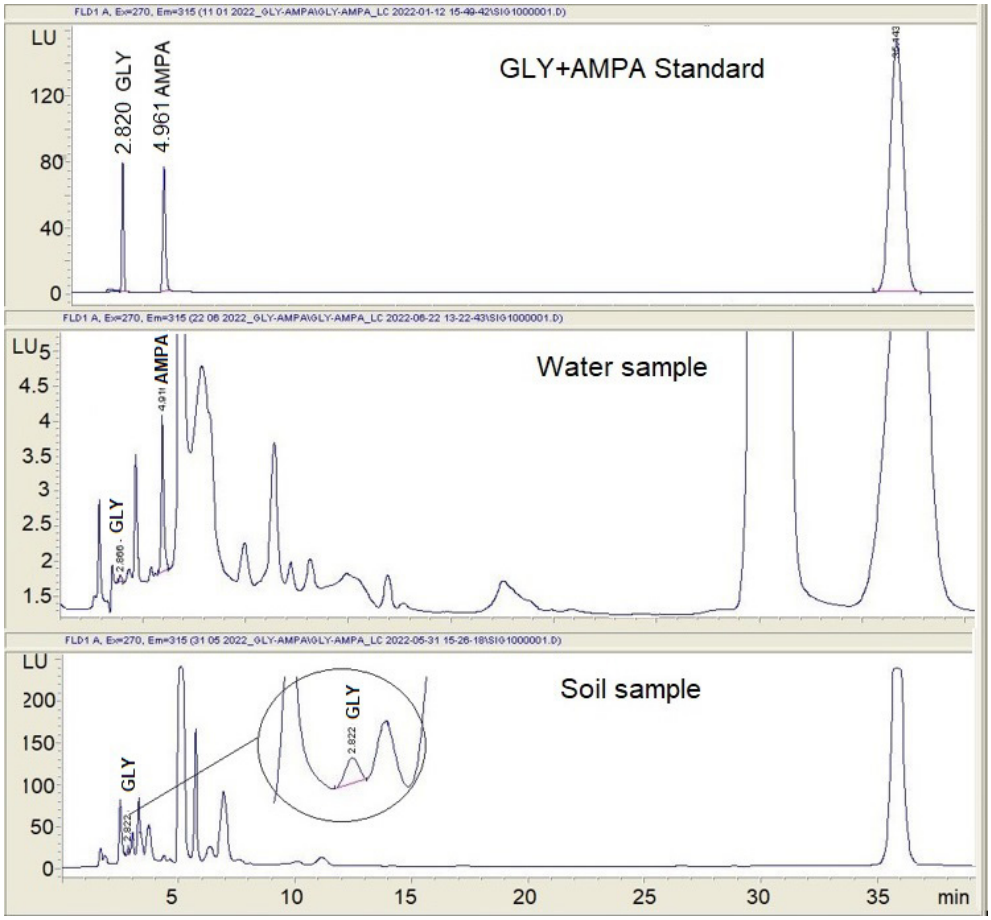

Figure 2. RP-HPLC-FLD chromatograms of GLY and AMPA in standard mixture, water sample and soil sample

Linearity is the method capacity to assure that the laboratory test results that are directly proportional to the concentration of the analyte in a sample. It was established by figured the analyte peak area versus the analyte concentration and was calculated on the basis of the calibration curve. To establish linearity, eight concentrations in the range of 0.195- 25 μg L^{-1} of GLY and AMPA solutions were prepared from the stock solution and analysed in duplicate. The obtained results indicate correlation coefficients (R^2) of 0.99976 for GLY and 0.99945 for AMPA (Table 1). The limits of detection (LOD) reached were 0.28 μg L^{-1} for GLY and for AMPA 0.35 μg L^{-1} and the limits of quantification (LOQ) were 0.84 μg L^{-1} for GLY and 1.05 μg L^{-1} for AMPA respectively. These low values show a good sensitivity of the proposed method. The parameters of the calibration curve are presented in Table 1.

Table 1. Linear regression data, LOD and LOQ of GLY and AMPA compounds

Parameters	Compounds	
	GLY	**AMPA**
RT [min]	2.820	4.961
Equation of calibration	Y=2.34289X-0.406231	Y=3.51385X-0.979488
Linear range [μg L^{-1}]	0.195- 25	0.195- 25
R^2	*0.99976*	*0.99945*
RSD (%)	0.460	1.036
LOD μg L^{-1}	0.28	0.35
LOQ μg L^{-1}	0.84	1.05

RT retention time; R^2 regression coefficient of calibration curve (n=8, 21 points); LOD, the limit of detection (S/N = 3); LOQ, the limit of quantification (S/N = 10)

The data regarding *precision* of the method was determined by measuring repeatability for six independent measurements for each compound, carried out under the same conditions, and the results show there were no significant differences between the test results. The method precision was satisfactory, the data are showed in Table 2.

Table 2. Intra and inter day precision of GLY and AMPA compounds

Pesticide	Concentration [μg L^{-1}]	Intra-Day Precision (n=6)		Inter-Day Precision (n=9)	
		Concentration Mean±SD	RSD [%]	Concentration Mean±SD	RSD [%]
GLY	-	-	-	24.49±1.21	4.93
	12.5	12.31±0.26	2.08	12.21±0.62	5.08
	-	-	-	1.92±0.13	6.83
AMPA	-	-	--	24.24±1.14	4.72
	12.5	12.26±0.25	2.06	12.10±0.61	5.07
	-	-	-	1.96±0.12	6.02

Mean = Average of *n* determination; SD = Standard deviation;
RSD = Relative standard deviation;

Recovery parameter of the method was measured by addition of 25 and 5 μg L^{-1} GLY and AMPA to a selected analyzed sample of water and 25 and 5 μg Kg^{-1} of these compounds in soils, respectively. This step was taken before the extraction of GLY and AMPA and then the whole procedure was conducted. The obtained results show good accuracy for both compounds, the recoveries ranging between 82-103% for GLY and 71.27-99.08% for AMPA in water samples. In soil samples the recovery degrees were 47.5-81.5% (Table 3). Also, research studies report low recovery of GLY 34-74% from different type of soils in comparison with 80-110% from water samples [17], facts assigned due to high adsorption of GLY by the soil samples.

Table 3. Accuracy of the HPLC-FLD for GLY and AMPA method, recovery degree

Compound [water]	**Amount [μg L^{-1}]**			**Recovery [%]**	**Mean ±SD**
	initial	added	found		
GLY	0	25	23.82	95.28	98.69±4.62
	0	25	25.99	103.96	
	0	25	24.21	96.84	
	0	5	4.51	90.20	91.80±9.90
	0	5	4.14	82.80	
	0	5	5.12	102.40	
AMPA	7.6	25	32.30	99.08	91.03±6.99
	7.6	25	28.55	87.58	
	7.6	25	28.18	86.44	
	7.6	5	11.01	87.38	78.76±8.11
	7.6	5	8.98	71.27	
	7.6	5	9.78	77.62	
Compound [soil]	Amount [μg Kg^{-1}]			Recovery [%]	Mean ±SD
GLY	45.92	25	40.42	57	68.5±12.32
	45.92	25	57.80	81.5	
	45.92	25	47.52	67	
	45.92	5	25.97	51	55.83±11.54
	45.92	5	35.13	69	
	45.92	5	24.19	47.5	

Mean = Average of three determination; SD = Standard deviation;

Aparicio et al., 2013, affirm that the GLY adsorbed into the soil is protected from biological degradation, due to a dynamic process of adsorption/desorption and only the microorganisms can degrade it to his metabolites [27]. Thus, can be a reason we found in almost half of the

samples the presence of GLY in comparison with water samples where GLY was practical absent. The results obtained in studied surface water and soil samples were presented in Table 4.

Table 4. GLY and AMPA found in water and soil samples from localities took studied

Sampling point	County	Water samples			Soil samples	
		No	GLY range (μg L^{-1}±SD)	AMPA range (μg L^{-1}±SD)	No	GLY (μg Kg^{-1}±SD)
Roșia Montana	Alba	2	<LOD <LOD	<LOQ <LOQ	-	-
Diosig	Bihor	2	<LOD <LOD	<LOQ <LOQ	2	<LOD 2.47±0.02
Țețchea		2	<LOD <LOD	<LOQ <LOD	2	<LOD <LOD
Bața	Bistrița-Năsăud	2	<LOD <LOD	<LOQ <LOQ	-	
Reteag		2	<LOD <LOD	<LOD <LOQ	1	45.92±0.14
Bodoc	Covasna	1	<LOD	<LOQ	1	1.45±0.04
Boroșneu Mare		1	<LOD	<LOQ	2	25.60 ±0.07 14.00±0.09
Avrămești	Harghita	3	<LOD <LOD <LOD	<LOD <LOD 5.83±0.04	-	-
Atid		3	<LOD <LOD <LOD	<LOD <LOQ <LOQ	-	-
Zetea		2	<LOD <LOQ	<LOD <LOQ	-	-
Coltău	Maramureș	4	<LOD <LOD <LOD <LOD	<LOQ 1.02±0.06 <LOQ <LOQ	1	3.33±0.05
	Mureș					
Băgaciu		3	<LOD <LOD <LOD	<LOQ <LOQ <LOQ	2	18.42±0.09 29.45±0.09
Deda		-	-	-	1	2.74±0.03
Deleni		1	<LOD	<LOQ		
Gornești		2	<LOD <LOD	<LOD <LOD	-	-
Gurghiu		2	<LOD	<LOQ		
			<LOD	1.844±0.05		
Saschiz		4	<LOD	<LOD	1	<LOD
			<LOD	5.09±0.04		
			<LOD	<LOD		

Sampling point	County	Water samples			Soil samples	
		No	GLY range (μg L^{-1}±SD)	AMPA range (μg L^{-1}±SD)	No	GLY (μg Kg^{-1}±SD)
			<LOD	<LOQ		
Șaeș		3	<LOD <LOD <LOD	<LOD 2.98±0.03 7.62±0.04	-	-
Terebești		2	<LOD <LOD	<LOD <LOD	-	-
Tiream	Satu Mare	4	<LOD <LOD <LOD <LOD	<LOQ <LOD 3.22±0.03 1.56±0.030	2	12.98±0.06 21.70 ±0.08
Turulung		2	<LOD <LOD	<LOD <LOQ	1	21.41±0.09
Almașu	Sălaj	3	<LOD <LOD <LOD	<LOQ <LOQ <LOQ	1	3.33±0.03
Racovița	Sibiu	2	<LOD <LOD	<LOQ 1.02±0.06	2	7.43±0.04 5.04±0.03
Sebeșu de sus		2	<LOD <LOD	<LOD <LOD	-	-

No - Number of analysed samples

In the studied surface waters, GLY was practically absent, while AMPA was found in almost all samples. In 15 % water samples the AMPA concentration ranging between 1.019 and 7.621 μg L^{-1} and in 75 % samples the found values were under LOQ. In the 79 % of studied soil samples, GLY concentration found ranging between 1.449 and 45.925 μg Kg^{-1}. The studies conducted regarding the presence and concentration of GLY in soil samples by Aparicio et al., 2013 in farms from Buenos Aires, Argentina, presented glyphosate in concentrations between 35 and 1502 μg kg^{-1}. Primost et al., 2017 also from Argentina (Pampas area), measured concentrations of glyphosate between 530 and 4450 μg kg^{-1} in soybean fields treated twice with glyphosate. In studied soils from a public garden in Spain, Ibañez et al., 2005, found concentrations of GLY between 170 and 730 μg kg^{-1} and Karasali et al., 2019 had found in the major basins of the Greek territory GLY presence in concentration levels from 0.026 to 40.6 μg g^{-1}.

CONCLUSIONS

An analytical procedure, RP-HPLC-FLD based on FMOC-Cl derivatization have been developed, for the analysis of GLY pesticide and its AMPA metabolite in water and GLY in soil samples collected from rural Roma communities from 10 counties in Transylvania area.

The developed procedure showed good linearities and limits of detection and quantification, being applicable to the analysis of the selected pesticides in studied water and soil samples. The RP-HPLC-FLD procedure has been applied to monitor the target compounds in potable and non-potable surface water samples collected during 2 months in 2021 summer-autumn. The most found compound in water samples was the AMPA metabolite with values between 1.019 and 7.621 μg L^{-1}, while GLY were practical absent. Instead, the GLY was more present in the soil samples collected from agricultural and rural area. GLY concentration found ranging between 1.449 and 45.925 μg Kg^{-1}.

The presence of these compounds in surface water and soils is a current issue for environmental protection, therefore the periodical monitoring is recommended.

EXPERIMENTAL SECTION

Chemicals and reagents

All reagents were of analytical grade. Glyphosate (GLY, 99.7%) standard, amino phosphonic acid (AMPA, 99.0%) and 9-fluorenylmethyl chloroformate standard (FMOC-Cl, 98.0%) (Sigma-Aldrich USA, Germany), $Na_2B_4O_7$ $10{\cdot}H_2O$ and KH_2PO_4 (AnalaR NORMAPUR, VWR Chemicals, Belgium), HPLC grade acetonitrile and KH_2PO_4 (HiperSolv CHROMANORM, Germany). Ultra-pure water, obtained with the Millipore water purification system (Millipore USA). Ethyl ether (AnalaR NORMAPUR, Germany).

Instruments and equipments

Analyzes were performed on an Agilent Technologies 1200 Series high-performance liquid chromatograph (Agilent, USA): Autosampler (Model ALS G 1329 A); Degasser (Model G 1322 A); Quaternary pump (Model G 1311 A; Thermostat (Model TCC SL G 1316 B); Detector FLD (Model FLD 60558084); Data collection and analyzes were performed using Software ChemStation. The samples were prepared using: analytical balance OHAUS (Switzerland), digital pH meter HANNA (Romania), ultrasound bath (ELMA Elmasonic P, Germany) and centrifuge (Eppendorf Centrifuge 5804 R, Germany). Analytical grade water was obtained from Milli-Q Ultrapure water purification system (Millipore, USA).

Chromatographic conditions for determination of GLY and AMPA by RP-HPLC-FLD

Separation of GLY and AMPA, FMOC-Cl derivatives was performed on an Agilent ZORBAX Eclipse Plus C18 reversed-phase column (5 µm particle size, 150 × 4.6 mm i.d.). The fluorescence detector was set at 210 nm (excitation) and 315 nm (emission). The mobile phase consisted of a mixture of ACN and 0.05 M KH_2PO_4 solution [30:70 v/v]. Flow rate of the mobile phase has been selected of 0.7 mL / min, injection volume 20 µL, separation being done at a 40 °C of column temperature.

Samples collection and extraction conditions

The present study was performed on samples collected from 25 rural Roma communities from 10 counties in Transylvania (Figure 3).

Water sampling was done according to SR-ISO-5667-2007, in brown bottles. The soil sampling was performed according to STAS 7184/1-84. The soil samples were collected at a 5 - 20 cm depth, from two-three sampling points. All collected samples (water and soil) were transported in a cooling bag and stored in a freezer until analysis.

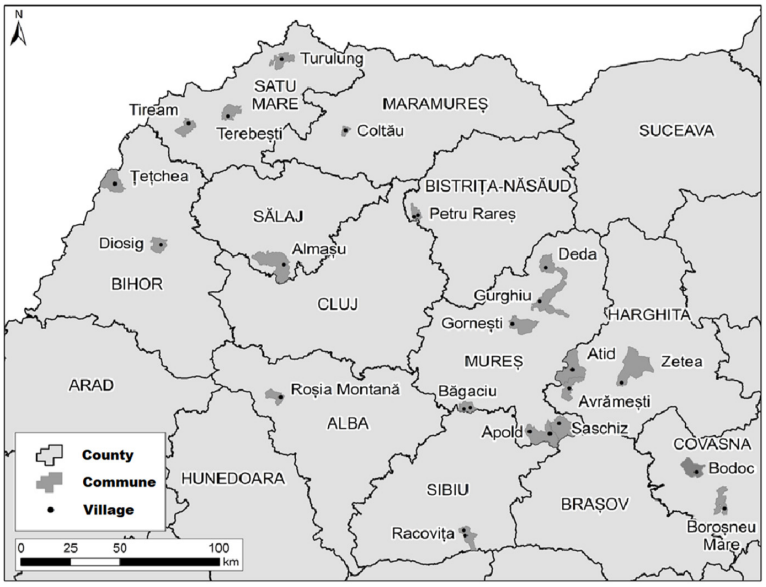

Figure 3. Geographic location of the studied area, regarding the locations of water and soil sampling from 10 counties in Transylvania.

Derivatization procedure of GLY and AMPA

Water samples were thawed, brought to room temperature, homogenized and filtered through a syringe 0.45 µm filter (Teknokroma, Spain)

To 500 µL aliquot of the water sample was added 500 µL of 0.02 M FMOC-Cl and 1 ml of 0.05 M borate buffer solutions. The mixture was vigorously stirred for 10 minutes, and then allowed to react in the darkness for 60 minutes. The excess FMOC-Cl reagent was extracted, by stirring for 3 minutes, with 1 mL of ethyl ether, twice. The upper layer of ether was removed by suction with a pipette. The aqueous layer was then transferred to a 1.5 ml vial for HPLC analysis. Samples were done in duplicate.

Soil samples were thawed, air dried, sieved for pebbles removal and weeds, and homogenized in a mortar. An approximately amount of 15 g of the soil sample was weighed and treated with 15 ml of 0.1 M KH_2PO_4. The homogenized sample was ultra-sonicated for 30 minutes (100% power, 87 KHz), centrifuged for 15 min at 4000 rpm, and the supernatant was collected. The operation was repeated twice. The reunited supernatants were filtered by syringe 0.45 µm filter and further an aliquot of 0.5 mL was subjected to derivatization with FMOC-Cl as in the method described above. Samples were done in duplicate.

Preparation of the standard solution

The stock standard solution of 200 mg·L-1 GLY and AMPA each was prepared with Millipore water. Working standard solutions were prepared in water by appropriate dilution of stock solution, in the range of 0.192–25 µg mL^{-1}. Solutions of 0.05 M $Na_2B_4O_7 \cdot 10\ H_2O$ (pH= 9), 0. 1 M and 0.05 M KH_2PO_4 were prepared in water. The 0.02 M FMOC-Cl solution was prepared in acetonitrile. The solutions were kept in the refrigerator at 4°C and were stable for two weeks.

Method validation

The selectivity was tested by comparing the chromatogram of a standard solution of GLY and AMPA and the same compounds present in the studied samples. *Linearity* was assessed based on a plot of the analyte peak area against analyte concentration. Calibration range was between 0.192 – 25 µg·L-1 from each standard.

Accuracy of the method was studying the recovery degree. Three different concentration levels of 2 $\mu g \cdot L^{-1}$ (low level), 12.5 $\mu g \cdot L^{-1}$ (intermediate level) and 25 $\mu g \cdot L^{-1}$ (high level) of standard mixtures were added to the water

and soil sample. Spiked samples were prepared in triplicate. The recovery was calculated as follows equation:

$$Recovery\ [\%] = found\ amount - initial\ amount\ /\ added\ amount\ x\ 100$$

The *intra-day precision* was obtained from the data of the 5 replicates analysis of 12.5 μg L^{-1}, standard solution;

Inter-day precision (three replicate for three consecutive days) determinations were performed on the three different standard solutions of 25, 12.5 and 2 μg L^{-1}; the precision was expressed as percentage of relative standard deviation (% RSD).

LOD and *LOQ* parameters were calculated as the concentration corresponding to three and ten times respectively, of the background noise of the blank (signal-to-noise ratio, S/N).

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