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Validation and Application of Liquid Chromatography Coupled with Tandem Mass Spectrometry Method for the Analysis of Glyphosate, Aminomethylphosphonic Acid (AMPA), and Glufosinate in Soil

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Abstract: A method was developed to determine glyphosate, aminomethylphosphonic acid (AMPA), and glufosinate in soil. The worldwide use of this herbicide in agricultural activities, and its known negative effects on both the environment and health, have generated interest in the establishment of methodologies for its determination in several matrices at trace level. The development of analytical methods for the determination of glyphosate, AMPA, and glufosinate is challenging due to its present amphoteric properties, high solubility in water, low molecular weight, high affinity to the ions presents in the soil, and lack of chromophore groups in its structure, making its quantification difficult. The proposed method exhibits a linear range from 5.0 to 600 µg/kg with limits of detection of 1.37, 0.69 and 1.22 µg/kg, limits of quantification of 4.11, 2.08, and 3.66 µg/kg for glyphosate, AMPA, and glufosinate, respectively, and adequate repeatability and reproducibility (coefficients of variation <8.0% and recovery percentages between 93.56% and 99.10%). The matrix effect was calculated for each analyte, proving to be a good alternative for the determination of these contaminants. The described method was applied to 46 soil samples collected from crop fields in Hidalgo, Mexico, with concentrations varying from not detected to 4.358 µg/kg (for AMPA).

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1. Introduction

Approximately two million tons of pesticides are used annually worldwide, of which 47.5% corresponds to herbicides, 29.5% to insecticides, 17.5% to fungicides, and 5.5% to other pesticides [1]. China is the highest contributing country, followed by the United States. However, by 2020, it was estimated that it would increase to 3.5 million tons [2]. Glyphosate ([*N*-phosphonomethyl] glycine) is an herbicide which eliminates undesirable plants (weeds) in agriculture, forestry, and the urban environment. This pesticide presents low toxicity for non-target organisms and its mode of action is achieved by inhibiting the Shikimate pathway, blocking the enzyme enolpyruvylshikimic phosphate (EPSP) synthase, and affecting the production of aromatic amino acids (tryptophan, tyrosine, and phenylalanine) essential for plant growth [3,4]. Glyphosate is a broad-spectrum systemic herbicide with foliar, non-selective, and post-emergent use. Its global consumption has increased over the years. There are records of 126 million kg, in 2014, and estimated sales of 6% by 2024 [5,6]. In Mexico, 17,395,975 kg of formulated glyphosate and 1,323,401 kg of technical glyphosate were used in 2018 for the agricultural management of crops such as maize, beans, wheat, tomato, and vine, amongst others. Recently, its use has been restricted [7,8].

Glyphosate enters target organisms by foliar contact and part of this is strongly absorbed by the cell. The residual glyphosate, without degrading in the plant, is transported to the soil where microorganisms degrade it to the main metabolite, aminomethylphosphonic acid (AMPA). Both compounds exhibit similar characteristics, but a different half-life. Glyphosate remains unchanged from 1 to 197 days, and AMPA has a persistence from 119 to 985 days [9,10]. Glyphosate, glufosinate, and AMPA lead to the significant contamination of environmental compartments, especially groundwater, surface water, air, soil, dust, and food [11]. Until now, maximum residue limits (MRLs) have been established for glyphosate; for example, soybeans 20 μg/g, maize 1–3.5 μg/g, sugar beet 10–15 μg/g, cow's milk 0.05–0.08 μg/mL, and drinking water 0.7 μg/mL [12]. For glyphosate and its metabolites, there is not regulation in soil. The absence of public health policies is probably due to the difficulties of detecting these analytes in biological, food, and environmental matrices, especially in soil [13].

Despite the efficiency of glyphosate as an herbicide, the toxic effects of this molecule and its metabolites on human health and the environment is still discussed. In several studies, glyphosate is classified as toxicologically harmful since it affects non-target species and generates resistance in weeds. In addition, it is a major endocrine disruptor, due to its association with human carcinogenesis, chronic, dermatological, respiratory, neurological, and reproductive disease [14]. However, other studies have stated that it does not generate adverse effects, under the recommended conditions of use [15]. It is worth mentioning that glyphosate has been classified in class III (mildly hazardous pesticides) given the low acute toxicity [16]. The International Agency for Research on Cancer (IARC) defines it as carcinogenic to experimental animals and as probably carcinogenic to humans [17]. According to the regulatory framework, in Mexico, glyphosate is classified as grade IV (slightly toxic). Although there are controversies around this pesticide, global regulatory agencies have established acceptable daily intakes (ADI) to avoid health risk from exposure; ADI in the United States is 1.75 mg/kg bw/day, in the European Union it is 0.5 mg/kg bw/day, and in Mexico it is 0.3 mg/kg bw/day. Notably, some research suggests an ADI of 0.1 mg/kg bw/day or less, due to its widespread and intensive use [18–20].

The analytical method for glyphosate, AMPA, and glufosinate determination should consider the low residue levels, the limited half-life, high polarity, and solubility in water, as well as the high binding affinity with the soil, the volatility, and the lack of chromophore groups in the molecular structure, which makes their detection difficult. On the other hand, isolation and quantification are greater challenges due to the need to eliminate the matrix

effect defined as the influence of one or more co-extracted interference compounds, which can cause an overestimation or underestimation of the concentrations of analytes in the case that they are present [21].

Several analytical processes have been developed for the determination of glyphosate, AMPA, and glufosinate residues in a wide variety of environmental and food matrices after a derivatization process, to increase their mass and make them more hydrophobic [22–24]. Some of the methods reported for its determination in soil are: liquid extraction (LE) followed by derivatization and subsequent quantification by high performance liquid chromatography, coupled with tandem mass spectrometry (HPLC-MS/MS) [25–27]; solid phase extraction (SPE) followed by derivatization and subsequent quantification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [28,29]; ultraviolet/visible (UV) detector [30,31]; fluorescence (FL) detector [32–34]; diode array [35]; a UV-Vis spectrophotometry [36,37]; an enzyme-linked immunosorbent assay (ELISA) [38]; gas chromatography with a mass spectrometry detector (GC-MS) [39,40] or with a flame photometric detector (GC-FPD) [41–43]; and fluorescence spectroscopy [44], amongst others. Therefore, the objective of this study was to validate a simple, fast, efficient, sensible, selective, and cost-effective analytical method for the determination of glyphosate, aminomethylphosphonic acid (AMPA), and glufosinate in soil by phosphate buffer extraction that involves derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and subsequent quantification by UPLC-MS/MS. The difference between the previously mentioned methods and the one proposed in the present work is a simple derivatization, with a one-step extraction, for the determination at trace levels of glyphosate, AMPA, and glufosinate. At the same time, the method proposed saves resources in the reagents and consumables normally used in the extraction process, as well as provided a shorter execution time. This proposed methodology for the detection and quantification of glyphosate, AMPA, and glufosinate, will help to evaluate the effect of the widespread use of this pesticide on agricultural soils and minimize its application.

2. Materials and Methods

2.1. Chemicals and Reagents

Analytical standard for Glyphosate (purity 98.2%) was supplied by Accustandard Inc. (New Haven, CT, USA). Glufosinate (purity 98%) and aminomethylphosphonic acid (AMPA) (purity 99%) were purchased at Sigma–Aldrich (Toluca, Mexico). The solvents, acetonitrile and methanol, were MS Grade and acetonitrile, and water was HPLC grade. Dichloromethane pesticide grade was provided by Control Técnico y Representaciones (CTR), S.A. of C.V. (Nuevo Léon, Mexico). The following reagents, all ACS grade, were also purchased from Sigma–Aldrich (Toluca, Mexico): 9-fluorenylmethyl chloroformate (FMOC-Cl), ammonium formate, formic acid, monosodium phosphate (NaH_2PO_4), phosphoric acid, and sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$).

2.2. Equipment

The equipment used were as follows: dry bath (JR brand, Model L12), centrifuge (Hettich brand, Model EBA21), ultrasonic bath (Bransonic brand, Model CPX8800H), vortex (Thermo Brand, Model M16715), precision (Mark Sartorius, Model Practum 2102-1S), and analytical balances (Brand Sartorius, Model AX224).

2.3. Instrumental Details, Chromatographic and Mass Spectrometry Conditions

Each sample was automatically injected through a sample manager system—FTN Acquity of Waters—into an Ultra Performance Liquid Chromatography (UPLC Acquity serie H) device. Chromatographic separation was performed on an Acquity UPLC® BEH phenyl column (1.7 µm particle size, 100 × 2.1 mm inner diameter) operated at a flow rate of 0.3 mL/min and at 40 °C. The UPLC system was operated with mobile phase A (ammonium formate 5 mM, pH 3.0) and mobile phase B (acetonitrile + 0.1% formic acid) with a gradient set as follows: (1) 0 min, 10% B; (2) 0−5 min, 90% B; (3) 5−5.1 min,

10% B; and (4) 5.1−8.0 min, 10% B. After reaching the initial conditions, the column was re-equilibrated for 4 min before the next injection. The total run time was 8.0 min, with an injection volume of 10.0 µL.

Ionization was performed in positive electrospray mode (ESI^+) in a tandem mass spectrometry detector (Xevo TQS) using nitrogen as the desolvation and cone gas at 650 L/h and 300 L/h, respectively. The source temperature was set at 150 °C and a capillary voltage of 3.21 kV was applied. The desolvation temperature was 400 °C. Argon, at a pressure of 4.4×10^{-3} mbar and a flow of 0.15 mL/min, was used at the collision cell to produce the respective ion fragments, and acquisition was performed in the multiple reaction monitoring (MRM) mode. The mass/charge ratio (m/z) values for the precursor and fragment ion for each specific analyte, along with their respective cone voltages and collision energy values, are shown in Table 1. A dwell time of 0.032 s was selected. Chromatography and mass spectrometry were acquired, and target compounds were monitored according to the retention time and two ion transitions, and were quantified with workstation MassLynx 4.1. (Waters, Manchester, UK) [45].

Table 1. Optimized MS/MS parameters for the FMOC-Cl derivatives of glyphosate, AMPA, and glufosinate.

Analytes	Precursor (m/z)	Product Ion (m/z)	Cone (V)	Collision (eV)
Glyphosate	392	170	20	15
		214	20	10
Glufosinate	404	136	15	15
		182	15	10
AMPA	334	111.8	20	20
		156	20	15

2.4. Stock Solutions and Spiked Samples Preparations

Stock solution of glyphosate, AMPA, and glufosinate standards were prepared using a 50:50 (v/v) water/acetonitrile mixture acidified 1% with formic acid as solvent, separately, at a concentration of 1.0 mg/mL; subsequently, an intermediate dilution of 10 µg/mL was prepared (a mixture of the three compounds) that was used for the fortification of the blank samples and to prepare the working solutions (system linearity test). All the stock solution and working solutions were stored in amber non-silanized glasses at 4 °C in dark. Before each use, the standard solutions were equilibrated at room temperature and weighed to check for evaporation losses [46].

As there was no reference material or matrix blank available, 5 kg of superficial soil sample (depth of 5 cm) was obtained from a field that manages an organic production scheme located in Culiacan, Sinaloa, Mexico. This was homogenized and dried in sunlight for 24 h, after which the samples were sifted through 2 mm mesh and processed for verification. This was to ensure that they were free of the analytes of interest, and also that there were no co-extractions that could interfere with the determination of the analytes of interest. Once this was confirmed, the samples were used for the validation tests [47]. The percentage content of sand, silt, and clay were evaluated by the Bouyoucos densimeter method and then the soil texture was determined according to the classification proposed by the United States Department of Agriculture (USDA) [47]. The soil used as a blank in the validation corresponded to a loam soil (sand 29.68%, clay 26.32, and silt 44%).

Five grams of the soil sample were weighed, and fortified by the addition (in triplicate), directly onto the soil, of the standard solution (mixture of glyphosate, AMPA, and glufosinate standards) at the concentrations to be evaluated (five levels), allowing the solvents to evaporate overnight [47]. The fortification was carried out as follows: control soil samples were added with 27 µL, 53 µL of the intermediate solution, in a mixture at a concentration of 1.0 µg/mL, and with 10, 30, and 300 µL of the intermediate solution, in a mixture at a concentration of 10.0 µg/mL, for the levels 1 (5 µg/kg), 2 (10 µg/kg), 3 (20 µg/kg), 4 (60 µg/kg), and 5 (600 µg/kg), respectively. Subsequently, they were analyzed using the proposed method.

2.5. Methods

2.5.1. Extraction

Subsamples of the control material (5 g ± 0.05 g) were placed in 50 mL centrifuge tubes; the fortification mixture standard was added (at the concentration levels mentioned above). The samples were then shaken to incorporate it into the matrix, and were left to rest for 12 h [48]. Subsequently, samples were extracted with 25 mL of $KH_2PO_4/Na_2B_4O_7$ buffer (0.1 M, pH = 9) and stirred in an ultrasonic bath for 30 min. Then, tubes were centrifuged at 6000 rpm for 10 min.

2.5.2. Derivatization Reaction

Two milliliters of the supernatant were taken and placed in a 10 mL glass vial with a screw cap, and 2 mL of FMOC-Cl reagent in acetonitrile (1 mg/mL) was added. The tubes were sealed, vortexed for 2 min, and incubated at 60 °C for 1 h. After the incubation time was reached, it was allowed to cool, and the reaction was stopped by adding 130 µL of 2% phosphoric acid.

2.5.3. Clean-Up Procedure

To remove excess FMOC-Cl, a liquid–liquid extraction was performed with 5 mL of methylene chloride and centrifugation at 3000 rpm for 10 min. Finally, 1 mL of the aqueous phase was filtered through a nylon syringe filter of 0.22 µm, and 10.0 µL of the final extract was injected into the UPLC-MS/MS chromatographic system.

2.5.4. Quantifications

Pesticide concentrations were calculated by the external standard method [48]:

$$Concentration\ (ppm\ or\ \mathrm{mg/kg}) = \frac{\mathrm{Rm}\ * \mathrm{Cs}}{\mathrm{Rs}\ * \mathrm{Eq}_v} \quad (1)$$

$$Eq_m = W * \frac{Aliquot\ of\ reconcentrated\ dispersed\ extract}{Extraction\ solvent\ volume} * \frac{1}{Final\ volumen} \quad (2)$$

where:

Rm = Response (area) of the sample peak.
Cs = Concentration (ng) of the injected standard in matrix matched.
Rs = Response (area) of the standard peak.
Eq_m = Equivalent mass (mg/L) of injected sample.
W = Sample weight (g).

2.6. Validation Study

The validation of the proposed method was performed under *EURACHEM Guide: A Laboratory Guide to Method Validation and Related Topics* [49]. According to this document, the parameters selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, robustness, uncertainty, and matrix effect, were evaluated.

2.6.1. Selectivity

Selectivity is defined as the degree to which a method can be used to determine specific analytes in mixtures or matrices without interference from other components with similar behavior under stated assay conditions [50]. Selectivity was evaluated by qualitative comparison of the mass spectrum obtained of different blanks of the matrix of interest with those of a standard solution [49].

2.6.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LODs and LOQs were estimated from the repeatability data (n = 10) of the first fortification level, calculating the recovered concentration and obtaining the standard deviation (SD) [49].

2.6.3. Linearity

The linearity of the analytical method was established from 5.0 to 600 µg/kg. To establish the linear range of the system, a matrix calibration curve was constructed. Linearity was assessed using calibration curves (each concentration level of the standard was run under the established operating conditions), in solvent, in matrix matched, and through plotting the peak area versus concentration of each analyte using the software MassLynx 4.1. (Waters, Manchester, UK). The method linearity was based on fortifying control samples at the concentration levels previously mentioned in the Stock Solutions and Spiked Samples Preparations section, and through plotting the average amounts recovered against the amounts of analyte added. Linear regression analysis was performed, and the value of correlation coefficient was noted [45,49].

2.6.4. Precision and Accuracy

Precision (repeatability, expressed as relative standard deviation or coefficient of variation, in %) and accuracy of the developed method (reproducibility, expressed as recovery percentages) were determined within a day at different time points by analyzing fortified blank samples in triplicate. Accuracy was determined by comparing the concentrations found in spiked soil samples with the added concentration [45,47,49]. This experiment was performed at the spiking levels mentioned in the Stock Solutions and Spiked Samples Preparations Section.

2.6.5. Robustness

Robustness was analyzed using the Youden–Steiner test where several parameters were selected based on the probable variability in the analytical method. In this test, an experiment was used for 5 parameters with 8 combinations, and the experiment was carried out using level 4 fortification. For its measurement, eight chromatographic analyses were carried out to evaluate the effect of those parameters. In this study, the selected parameters were: a = pH buffer of 8; b= Volume of dichloromethane of 2.5 mL; c = Stirring time of 15 min; d = Derivatization time of 30 min; and e = Volume of FMOC of 1 mL. The nominal value (under normal method conditions) for each parameter is indicated by capital letters (A–E) and its alternative value is indicated by lowercase letters (a–e). The effect of a variable was calculated by the difference between the mean result obtained with the variable at the nominal value from the mean result reached with it at the alternative value by the equation: Di = Σ upper case − Σ lower case. If a variable influences the results, then the difference of it will be significantly larger than the differences of the other variables. The standard deviation of the differences (S_{Di}) was obtained by $S_{Di} = \sqrt{2\sum \frac{Di^2}{5}}$. If S_{Di} is lower than the standard deviation of the method (RSD of reproducibility), all parameters together do not affect the results and it can be concluded that the method is robust [49,51].

2.6.6. Uncertainty

To calculate the expanded uncertainty, the relative standard deviation of control samples run through all method steps during validation was used [49], following the steps below:

1. Perform spiked determinations at different concentration levels evaluated.
2. Calculate concentration and percent recovery.
3. Calculate the standard deviation (SD) and relative standard deviation (RSD) of results where the process is under statistical control (no outliers or out-of-control results).
4. Calculate the measurement uncertainty at the 95% confidence level as follows:

$$U = k * RSD \quad (3)$$

where:

U = uncertainty;
RSD = relative standard deviation;

k = coverage factor (for 95% = 2).

5. Calculate the measurement uncertainty interval for a measured value as follows:

$$\text{Interval} = U * c \quad (4)$$

where:

U = uncertainty;

c = concentration.

2.6.7. Matrix Effect (ME)

To calculate this parameter, the curves of standards in solvent and in matrix matched were compared [49]. Matrix matched curves are commonly used to compensate the matrix effect. An important aspect of this parameter is that, for each type of matrix, the effect produced should be studied independently. Once the calibration curves were obtained, the matrix effect was carried out by comparing the external calibration with that of standard fortification. Subsequently, the quotient (R) between the two slopes and the associated standard deviation (S_R) were calculated (Equations (5) and (6)):

$$\boldsymbol{R} = \frac{\boldsymbol{S_{EC}}}{\boldsymbol{S_{MM}}} \quad (5)$$

$$S_R = R\sqrt{\left(\left(\frac{SD_{EC}^2}{S_{EC}^2}\right) + \left(\frac{SD_{MM}^2}{S_{MM}^2}\right)\right)} \quad (6)$$

where S_{EC} and S_{MM} are the slopes of the external calibration and matrix matched curves, respectively, and SD_{EC} and SD_{MM} are their corresponding standard deviations.

If both slopes are nearly equal, their ratio, R, should not be significantly different to 1. Student's t was calculated as follows:

$$\boldsymbol{t_{cal}} = \frac{|\boldsymbol{R} - \boldsymbol{1}|}{\boldsymbol{S_R}} \quad (7)$$

The calculated value (t_{cal}) is compared to the coverage factor k = 2 for a 95% confidence level. If t_{cal} is greater than 2, the two slopes will not be comparable, and the analysis must be carried out by the standard addition method.

The ME is equal to (1 − solvent slope/matrix slope) multiplied by 100 to express the result as a percentage. A positive percentage indicates an increase while a negative percentage indicates a decrease in the signal. As far as the ME is known, a correction is made in the calculations if necessary [49].

2.7. Method Application to Agricultural Soil Analysis

To check the applicability of the proposed method to real matrices. The sampling plan included 46 agricultural soils taken from the Alto Mezquital region located in Hidalgo, Mexico. The agricultural activities in this region include maize crop production, and most of them are based on technology packages that demand a moderate loading of glyphosate and other agrichemicals [52].

The soil samples were taken from the 0 to 15 cm topsoil layer using a soil sampler [47,53]. Each soil sample consisted of 1.5 to 2.0 kg stored in labelled clean plastic bags and were sent for analysis to the National Laboratory for Food Safety Research (LANIIA, by its acronym in Spanish) in the Food and Development Research Center, A.C. Culiacan Unit (CIAD, by its acronym in Spanish) in portable containers under low-temperature conditions and constant darkness. Once in the laboratory, the type of soil was determined and, if it did not correspond to the same as the one used in the method validation, the matrix effect was determined [47,49]. The samples were kept at −20 °C until analysis.

2.8. Quality Assurance of the Method

Internal quality control was performed with each analytical batch to verify that the established continuous system suitability specifications were met, as well as to demonstrate the accuracy and precision or other parameters determined for the method used in the determination. Two blank samples prepared as mentioned in the Stock Solutions and Spiked Samples Preparations section were analyzed; in the first, the determination was carried out directly while, in the second, a fortification was carried out with the analytes of interest at a concentration of 250 µg/kg. Blanks were evaluated and had to be below the LOD. In the case of the fortified blanks, the recovery of the glyphosate, AMPA, and glufosinate analytes was established. Accuracy, expressed as a percentage of recovery, should have been between 70 and 120% to ensure good quality control [49]. In this sense, the fortified blank samples showed recoveries of 95, 98, and 92% for Glyphosate, AMPA, and Glufosinate, respectively; in addition, the sample blank showed no interferences, demonstrating good performance.

3. Results and Discussion

3.1. Analytical Method Validation

3.1.1. Selectivity

Under the optimized conditions, the proposed method demonstrated excellent selectivity for the target compounds. The UPLC-MS/MS can easily detect and quantify after derivatization, owing to the high selectivity of the technique of the resolution, rapid analysis, and low injection volume towards the organic compounds such as glyphosate, AMPA, and glufosinate. This was demonstrated with minimum signals or interference peaks in the chromatograms for the injected soil blanks; this indicates that the method was selective for the compounds of interest (Figures 1–3 and S1–S3).

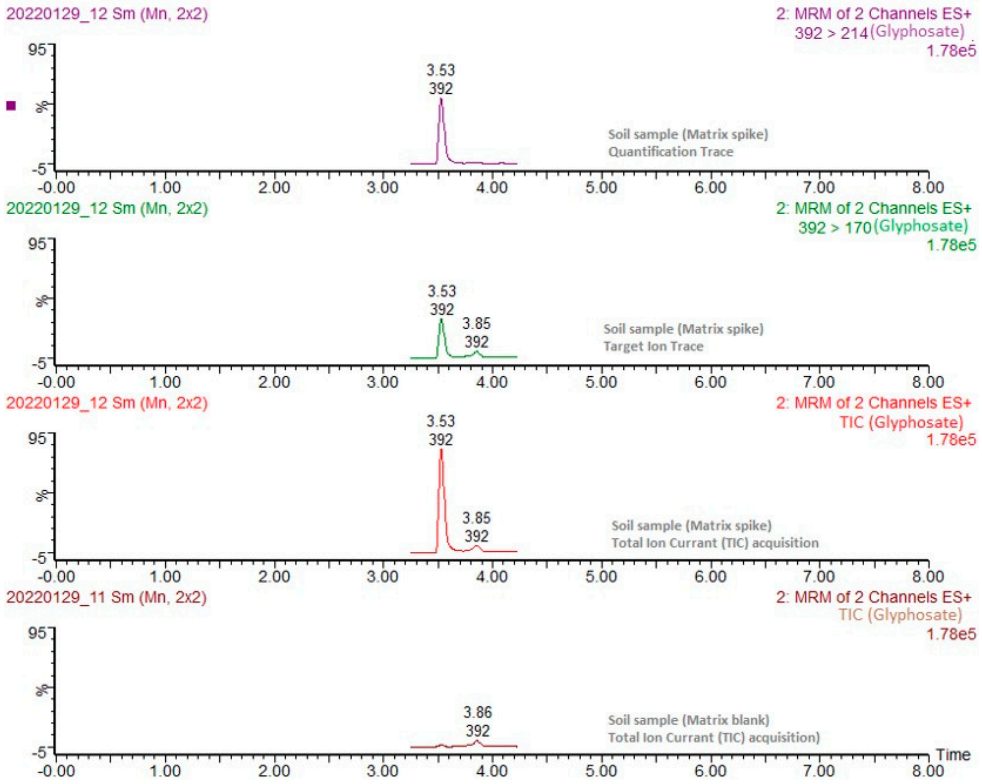

Figure 1. MRM acquisition chromatogram for glyphosate (blank reagents vs. fortified samples at spike level of 60 µg/kg).

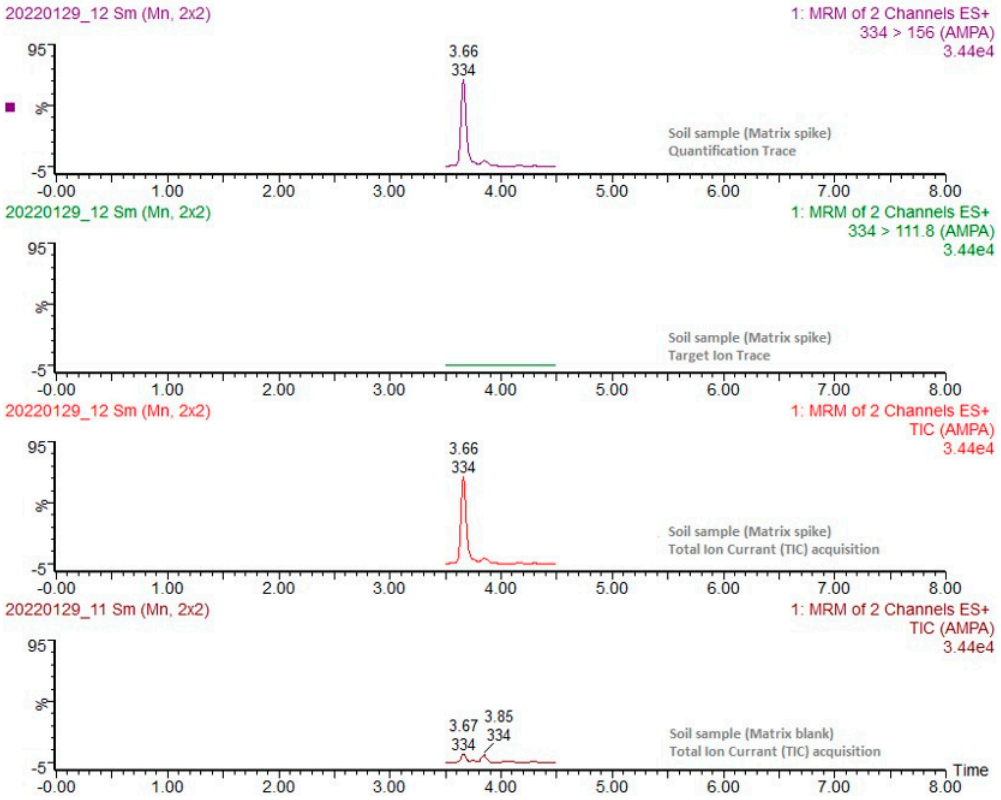

Figure 2. MRM acquisition chromatogram for AMPA (blank reagents vs. fortified samples at spike level of 60 μg/kg).

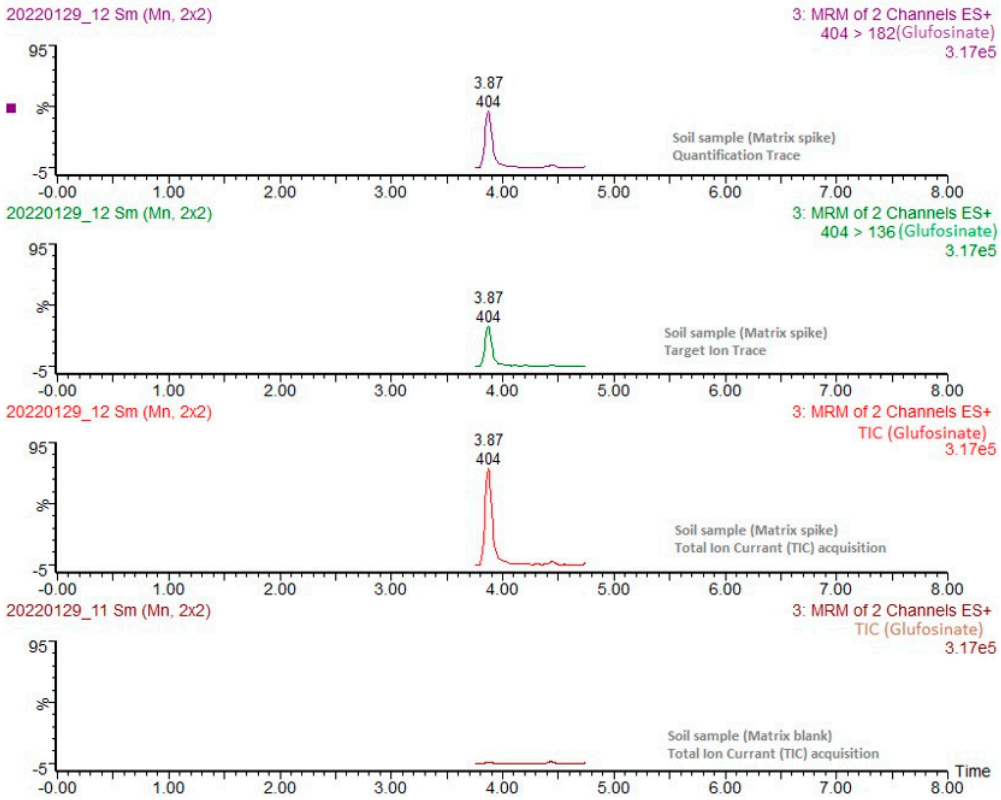

Figure 3. MRM acquisition chromatogram for glufosinate (blank reagents vs. fortified samples at spike level of 60 μg/kg).

The enhanced sensitivity and selectivity of the UPLC-MS/MS method make this technique perfect for the residue level determination of polar and/or ionic compounds (glyphosate, AMPA, and glufosinate) in soil [54].

3.1.2. LODs and LOQs

The developed method exhibited high sensitivity, which can be clearly guaranteed at the LODs of 1.37, 0.69, and 1.22 µg/kg, and LOQs of 4.11, 2.08, and 3.66 µg/kg for glyphosate, AMPA, and glufosinate, respectively (Table 2).

Table 2. Limits of detection (LOD), limits of quantification (LOQ), linearity (Criterion $R^2 \geq 0.99$), accuracy (criterion: recovery percentage between 70 and 120%) and precision (criterion coefficient of variation (CV) $\leq$ 20%) for the evaluated pesticides.

Pesticides	LOD (µg/kg)	LOQ (µg/kg)	Linearity (R^2)	Accuracy (Average Recovery %)	Precision (% CV)	Expanded Uncertainty (%)
Glyphosate	1.368	4.105	0.996	97.45 ± 7.17	7.78	15.88
AMPA	0.692	2.076	0.992	99.10 ± 7.58	7.23	14.76
Glufosinate	1.219	3.658	0.999	93.56 ± 7.76	8.29	16.93

The LODs in the proposed method for glyphosate were better than those determined by Sun et al. [20], Ibañez et al. [25], Delhome et al. [28], Druart et al. [33], Börjesson and Torstensson [39], Silva et al. [27], and Peruzzo et al. [55], who reported LODs of 10, 5, 9, 103, 3, 20, and 100 µg/kg, respectively. The same was demonstrated for the case of AMPA, since authors have reported LODs higher than the one proposed here, for example Ibañez et al. [25] (3 µg/kg), Delhome et al. [28] (7 µg/kg), Druart et al. [33] (16 µg/kg), Börjesson and Torstensson [39] (3 µg/kg), and Silva et al. [27] (30 µg/kg). While for glufosinate, the same was observed with LODs of 5 µg/kg [25], 6 µg/kg [28], and 15 µg/kg [33].

A similar behavior was observed regarding the LOQs, which were higher for all the evaluated compounds compared to those reported for other methods. For example, in the case of glyphosate, values of 40 µg/kg [20], 50 µg/kg [25], 30 µg/kg [28], 6 µg/kg [39], 12 µg/kg [41], 10 µg/kg [46], and 250 µg/kg [55] have been reported. In the case of the AMPA, the LOQs vary compared to those reported in the literature, in the range of 6 to 50 µg/kg [27,28,39,41,46], being, in all cases, better than those reported in our proposed method. Finally, for Glufosinate, LOQs higher than those proposed here have been reported, with values ranging between 20 and 50 µg kg [25,28,41].

3.1.3. Linearity

The equipment and method linearities were good, in the concentration ranges evaluated, with coefficients of determination above 0.99, which was confirmed by a linear regression analysis of the data with a plot between the pesticide concentrations versus peak area (Table 2).

The concentration range evaluated in this work is broader than some previously reported. For example, Karanasios et al. [46] report a linear response for a glyphosate and AMPA concentration range between 10 and 500 µg/kg similar to that reported by Sun et al. [21] for glyphosate, who established an adequate linear response (coefficient of determination >0.99) for glyphosate in a concentration range between 100 and 1000 µg/kg.

3.1.4. Accuracy and Precision

The method showed a good recovery percentage for the analytes evaluated with recovery percentages of 97.45 ± 7.17, 99.10 ± 7.58, and 93.56 ± 7.76% for glyphosate, AMPA, and glufosinate, respectively. Furthermore, the relative standard deviation (RSD) or coefficient variation (CV) was less than 8.3% in all cases (Table 2). These data agree with the criteria of the EURACHEM guide, that recommends general recovery limits of 70–120%

within laboratory and CV $\leq$ 20% [49]. Therefore, the method could be considered accurate and precise for this purpose.

Compared with other methods reported in the literature, the proposed method proved to be more accurate and precise. For example, Ibañez et al. [25] reported recovery values between 90–92%, 88–89%, and 83–86%, for glyphosate, AMPA, and glufosinate, respectively. Karanasios et al. [46] reported a range of recovery between 89.6 and 118.8% for glyphosate and between 67.9 and 94.6% for AMPA, whereas the CV was 15.35% for glyphosate and 11.9% for AMPA. El-Gendy et al. [38] reported recovery values of the spiked soil samples with different concentrations of glyphosate in the range of 87.4–97.2%, and Sun et al. [21] reported for glyphosate a relative standard deviation (RSD) of 15% and recovery values ranging from 75% to 110%.

3.1.5. Robustness

In Table 3, the parameters variation for the Youden–Steiner test is shown. The results display the difference (Di) ranges from −35.22 to 28.28, with the higher and lower values corresponding to the "Volume of dichloromethane" and "pH" variables, respectively, both being for glufosinate. The variable pH 8 significantly affects glyphosate, while the variables volume of dichloromethane and derivatization time affect all analytes evaluated (Table 3).

Table 3. Results obtained in eight runs performed for robustness for glyphosate, AMPA, and glufosinate method extraction.

Factor			AMPA (%)	Glyphosate (%)	Glufosinate (%)
a = pH Buffer of 8	A	A-a	−3.33	14.91	−35.22
b = Volume of dichloromethane of 2.5 mL	B	B-b	25.77	25.01	28.28
c = Stirring time of 15 min	C	C-c	−30.36	−25.06	−17.91
d = Derivatization time of 30 min	D	D-d	8.98	7.260	8.41
e = Volume of FMOC of 1 mL	E	E-e	−7.86	−10.25	−14.56
Mean			94.35	90.32	96.79
Standard deviation (s)			12.09	12.27	16.42
$\sqrt{2}$*s			4.92	4.95	5.73
Criterion (X-x) < $\sqrt{2}$*s					

Although the significance of the differences cannot be determined, this robustness test indicates that it is necessary to control the variables identified, and that they influence the results of the analysis.

Karageorgou and Samanidou [56] indicate that the main advantage of the Youden test is the fact that it entails the required time and minimal effort, since only a limited number of determinations must be made using combinations of the chosen investigated factors.

In this regard, Da Costa César and Antônio Pianetti [57] mention that the measurements are susceptible to variations in analytical conditions; therefore, the conditions identified as possibly altering the result must be adequately controlled and monitored.

3.1.6. Uncertainty

The calculated expanded uncertainty was 15.88, 14.76, and 16.93% for glyphosate, AMPA, and glufosinate, respectively (Table 2). To calculate the measurement interval of the uncertainty of a real sample, the expanded uncertainty will have to be multiplied by the analyte concentration found [58]. For example, a soil sample containing a glufosinate concentration of 2.10 µg/kg the interval will be equal to (16.93/100) × 2.10 = ±0.36 µg/kg, and the result will be reported as glufosinate = 2.10 ± 0.36 µg/kg.

Goon et al. [59] developed an analytical procedure for the determination of glyphosate in soil samples extracted with aqueous sodium hydroxide solution followed by pre-column

derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and LC-ESI-MS/MS analysis using multiple reaction monitoring (MRM), reporting an expanded uncertainty of 6%. In the case of AMPA and glufosinate, no studies were found that report the uncertainty of their measurement in soil.

3.1.7. Matrix Effect (ME)

A matrix effect was observed for all evaluated analytes and the type of soil used in the validation; glyphosate presented a negative matrix effect (−3.21%), while AMPA and glufosinate presented a positive effect with 15.97 and 35.29%, respectively (Figure 4, Table S1). Therefore, glyphosate presents a signal suppression, while AMPA and glufosinate show an enhancement. According to the EURACHEM guide [49], if the matrix effect is equal to 0%, there is no matrix effect. On the other hand, if it is less than or equal to ±20%, the matrix effect is considered mild, it is considered medium if it is found with percentages greater than ±20% and less than or equal to ±50%, and strong, in the case of finding values greater than or equal to ±50%. In this sense, glyphosate and AMPA had a mild matrix effect, while glufosinate had a medium effect (Figure 4, Table S1).

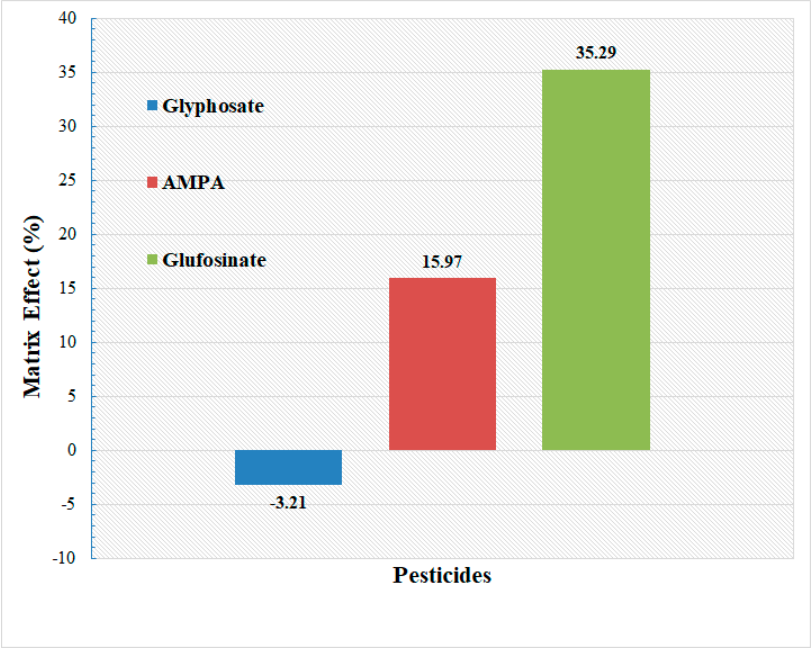

Figure 4. Matrix effect (ME %) for glyphosate, AMPA, and glufosinate by validated method.

In the case of the soil samples analyzed under the proposed method, three types of soil, different from the one used in the validation of the method, were observed: sand (10.87%), clay (67.39%), and sandy clay loam (21.74%) textures, the observing matrix effect in all cases. In this sense, a suppression effect was observed in all the types of soil analyzed: in the sandy soil the matrix effect was −37.29, −31.96, and −28.71% for glyphosate, AMPA, and glufosinate, respectively; in the clay soil the matrix effect was of −44.85, −30.36, and −13.80% for glyphosate, AMPA, and glufosinate, respectively; and finally, in sandy clay loam soil, the matrix effect was of −35.83, −20.99, and −10.31% for glyphosate, AMPA, and glufosinate, respectively (Table S1).

The results observed in the present study are different, at least for AMPA and glufosinate, from those reported by other authors, who indicate that, when UPLC-MS/MS determinations are made using ESI^+, it is common to find ion suppression [60,61]. Among the possible causes of this negative matrix effect could be the presence of co-extracted compounds that improve the properties of the drops and therefore, the ionization efficiency of the analyzer.

Łozowicka et al. [62] evaluated the matrix effect in an analysis of multi-residue pesticides in soil (216 pesticides), finding mainly a matrix effect of signal improvement with a range between −25 to 74%. Of the pesticides evaluated, 87% exhibited a mild ME, 10.6% showed a medium ME, and only 2.4% showed a strong ME.

On the other hand, Acosta-Dacal et al. [61] analyzed pesticide residues in clay loam soil, observing a matrix effect with a negative trend (signal suppression) being non-significant for most of the compounds evaluated (95.7%) and the rest (4.3%) presented a medium or strong effect.

3.2. Method Application

The analyses of the agricultural soil samples from Alto Mezquital (Hidalgo, Mexico) showed trace values (below LOD), with a significant number of samples not showing the presence of the analytes of interest. Only the soils of two sampling sites showed residues above the LOQ for AMPA, with concentrations of 3.78 and 4.358 μg/kg.

In Mexico, there are no regulations that establish the maximum limits allowed for glyphosate, AMPA, and glufosinate in soil [63], and studies that report the presence of these analytes in soil are limited. Muñoz et al. [44] reported the presence of glyphosate in soils collected in crop fields in the state of Hidalgo, Mexico that had a concentration of glyphosate between 320,600 and 607,700 μg/kg, being superior in several orders of magnitude to that which was found in the present study.

On the other hand, Pastrana Cervantes [29] analyzed the presence of glyphosate and AMPA in agricultural soil with different crops (soybean and maize) from Hopelchen, Campeche, Mexico, reporting mean concentrations of glyphosate of 210 ± 100 and 80 ± 10 μg/kg, for soybean and maize crops, respectively.

In all cases, the concentrations observed in the abovementioned studies were higher than those found in the Alto Mezquital region located in Hidalgo, Mexico that were sampled in the present study.

3.3. Comparative Advantages of the Validated Method

According to Kocadal et al. [64] "the main advantages of LC-MS/MS are (i) easy application to low molecular weight compounds that act as a limitation for immunoassays, (ii) relatively simpler workflow and higher output than its alternatives, and (iii) relatively lower cost of equipment in comparison to the alternatives".

Furthermore, since mass spectrometry is based on the identification of specific ions of each of the analytes evaluated, it allows the unequivocal differentiation of each one of them, especially for MRM methods using two transitions (for quantification and confirmation of the analyte of interest). Even though a clear chromatographic separation is not observed, this is something that cannot be observed when other types of detectors are used [18].

Some of the disadvantages presented by the use of the ELISA technique in the determination of the analytes evaluated here are as follows: (1) the preparation of the antibodies used in the detection has a high cost; (2) it presents a high risk of both false positives and false negatives, so it is recommended to normally use another analytical technique as a confirmation method; (3) the assays must be handled and maintained under special conditions (e.g., low temperatures); and (4) it is highly dependent on the availability of commercial kits which are usually very expensive [18,64]. It is difficult to monitor several pesticides at the same time with ELISA due to the need to have specific tests available and present in a mixture [10].

Finally, in the case of LC with UV or FL detectors, it has been reported that the results obtained with these techniques, although satisfactory, present a reduced sensitivity and selectivity. In addition, when these detectors are used coupled with GC, multiple steps are required during the sample preparation [18].

4. Conclusions

An economical, simple, and fast method for the quantification of glyphosate, AMPA, and glufosinate in soil samples, using derivatization with FMOC-Cl and UPLC-MS/MS, was developed. The present method showed good linearity ($R^2 > 0.99$), selectivity, precision, accuracy (recovery percentage), robustness, uncertainty, matrix effect, and sensitivity (LODs and LOQs). This guarantees that the proposed method is suitable for the proposed purpose because the evaluated parameters are within the recommended values for an analytical method according to the validation guidelines used. However, since soil is a complex matrix that presents both a negative and a positive matrix effect, it is recommended that the analysis be performed using the matrix-matched calibrations samples.

Finally, the method was successfully applied to agricultural soils, showing it is better than the other methods reported in the literature for the quantification of the analytes evaluated in these types of samples.

In terms of future perspectives, the method proposed here can be applied to other types of comparable matrices, such as sediments, and the scope can be broadened to other analytes that have similar properties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13061131/s1, Figure S1: Monitored transitions for glyphosate; Figure S2: Monitored transitions for AMPA; Figure S3: Monitored transitions for glufosinate; Figure S4: Method linearity for glyphosate; Figure S5: Method linearity for AMPA; Figure S6: Method linearity for glufosinate; Table S1: Variables used in the determination of the matrix effect by each evaluated analyte and soil type.

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