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Matrix solid-phase dispersion extraction and determination by high-performance liquid chromatography with fluorescence detection of residues of glyphosate and aminomethylphosphonic acid in tomato fruit

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Abstract

A method based on matrix solid-phase dispersion (MSPD) is described for the quantitative extraction of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) from tomato fruit. After application of 120 μL of HNO_3 1 M to the sample, the dispersion column was packed with 0.5 g of sample blended into 1 g of NH_2-silica. Two aqueous fractions were obtained. First, AMPA was eluted from the column using deionized water (F1), and then a NaH_2PO_4 0.005 M solution was used for the elution of glyphosate (F2). Cleanup of F1 and F2 was made by ion exchange chromatography on a SAX anion exchange silica. Determination was done by HPLC with fluorescence detection after precolumn derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl). Mean recoveries calculated at fortification levels of 0.5 μg/g for glyphosate and 0.4 μg/g for AMPA were 87% and 78%, respectively. The relative standard deviations ($n = 7$) for the total procedure were 10% and 16%. Detection limits were 0.05 μg/g for glyphosate and 0.03 μg/g for AMPA.

Keywords: Matrix solid-phase dispersion; Anion exchange cleanup; FMOC derivatization; HPLC; Glyphosate; AMPA; Residues; Tomato fruit; Herbicides

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] is a non-selective herbicide mainly used for weed and vegetative control. Its rapid translocation from plant foliage to underground parts and low mammalian toxicity contribute to the popularity of glyphosate which is one of the most widely used herbicides in the world [1]. However, its effects on non-target organisms and overall environmental fate have not been fully evaluated. Very little information about this subject is found in literature. For instance, glyphosate has been involved in a case-control study of non-Hodgkin lymphoma, a kind of human cancer [1], also harmful effects on semen characteristics in rabbits have been shown [2]. Strict control is therefore necessary to protect consumers of agricultural commodities susceptible of contamination. Aminomethylphosphonic acid (AMPA) is the major metabolite in plants, water and soil [3].

Glyphosate and AMPA are very polar compounds ($\log P_{oct} = -3.2$ and -2.36, respectively) and present high solubility in water (12 g/L for glyphosate) and insolubility in organic solvents. They are also very amphoteric compounds, having p*K*a values of 0.78, 2.29, 5.96 and 10.98 for glyphosate and 0.9, 5.6 and 10.2 for AMPA [3–6]. These physicochemical properties make the use of classical organic solvent extractions very difficult and require cleanup procedures with ion exchange and ligand-exchange columns [7]. For this reason, the determination of glyphosate and AMPA in biological matrixes is a challenging task. Various methods have been described in literature for sample preparation of plant matrixes, such as legumes, fruits, vegetables or forage [8]. Most of them are based in the use of water and

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solvents like chloroform and dichloromethane [9–16] or ethylacetate [17] for extraction of glyphosate and AMPA. The protocols involve several reflux extractions or liquid-liquid partitions. Also, they need extensive cleanup of extracts by ion exchange, ligand exchange, gel permeation or adsorption chromatography. Stalikas and Konidari [18] made a very complete review about the analytical methods to determine glyphosate and AMPA. This work compiles some interesting sample pre-treatment applications for crops and fruits. Nevertheless, the lack of information in literature about glyphosate residues in tomato fruit is notable; only one method was found for the trace level determination of glyphosate in tomato plants, not the tomato fruit [15].

Matrix solid-phase dispersion (MSPD) has been successfully applied for the isolation of target molecules from biological matrices considerably reducing the sample size and the solvent consumption [19,20]. The mechanism of MSPD includes sample homogenization, cellular disruption, exhaustive extraction, fractionation and purification in a simple process. Thus, MSPD technology involves blending a small amount of matrix with an appropriate sorbent followed by washing and elution of compounds with a small volume of solvent. Cleanup with adsorbents can be necessary for further purification. At present MSPD has been successfully used to extract several apolar compounds as organochlorine pesticides and polychlorinated biphenyls in animal matrixes, such as aquatic species [21,22] and milk [23]. Likewise, this technique has been applied in plant matrices for residue extraction of medium polarity compounds like some organophosphorus pesticides [8,24–26] and carbamates [27,28]. Some of the pesticides included in these families have been determined in tomato fruit [28–30]. However, no reports about the use of an MSPD method for extraction of glyphosate and its metabolite have been found in literature.

The objective of this study was to develop an MSPD method for the sample preparation and quantitative extraction of residues of glyphosate and AMPA in tomato fruit followed by HPLC determination with 9-fluorenylmethylchloroformate (FMOC-Cl) pre-column derivatization and fluorescence detection [4,9,15,18,31,32]. An amino-sorbent was tested for blending the fortified tissue and different desorption solvents were assayed to optimize solute recoveries. Cleanup was made by ion exchange chromatography.

2. Experimental

2.1. Chemical and materials

Glyphosate (99%) was supplied by Chem. Service (West Chester, PA, U.S.A.) and AMPA (99%) by Sigma (St. Louis, MO, U.S.A.). Stock solutions (100 μg/mL) were prepared every 3 months in water and were kept at 4 °C when not in use. No degradation was observed during this period. Working standards of various concentrations were prepared every 2 weeks by appropriate dilutions of stock aliquots in water. HPLC-grade methanol and acetonitrile were purchased from EMSCIENCE (S. Democrat Rb, Gibbstown, NJ). Deionized water (18 ΩM cm resistivity) was obtained from a MilliQ water purification system (Millipore, Bedford, MA, U.S.A.). Analytical grade sodium dihydrogenphosphate, disodium tetraborate decahydrate, sodium hydroxide and ethylether were from Baker (Phillipsburg, NJ, U.S.A.). Nitric acid (analytical reagent grade, 66%) and 9-fluorenylmethyl chloroformate FMOC-Cl were from Merk (S. Plainfield, NJ, U.S.A). Silica based sorbents (particle diameter 40 μm) with aminopropyl (NH_2-normal silica) and quaternary amine (SAX-Cl anionic exchange silica) functional groups were purchased from Varian (Harbor City, CA, U.S.A.).

2.2. Sample preparation

A sample of 0.5 g was placed into an agate mortar (50 mL capacity) and gently blended with 1 g of NH_2-silica to obtain a homogeneous mixture. This mixture was introduced into a 6 mL polypropylene filtration tube with a polyethylene frit in the bottom and tightly compressed and covered with another polyethylene frit. A 30 mL volume of deionized water was percolated through under slight vacuum. This first eluate was collected and rotary evaporated to 10 mL (F1 containing AMPA fraction). Afterwards, a 20 mL volume of NaH_2PO_4 0.005 M (pH 7) water solution was applied to the column and the second eluate was collected (F2 containing glyphosate fraction). Clean up of two collected fractions was made by using SAX exchange silica packed into polypropylene filtrations tubes. F1 was percolated through a 2 g SAX column previously treated with 50 mL of sodium hydroxide solution (pH 8) and rinsed with 5 mL of purified water. AMPA was eluted from this column with 40 mL of 0.01 M nitric acid. On the other hand, F2 was percolated through a 0.5 g SAX column, previously rinsed with a 10 mL volume of deionized water. Glyphosate was eluted from the column with 15 mL of 0.01 M nitric acid. Then, purified F1 and F2 extracts were evaporated to 0.5 mL by rotary evaporation at 40 °C and pH was adjusted to 7–9 with 2 M sodium hydroxide for the derivatization reaction. Fig. 1 shows a global scheme of sample pretreatment.

2.3. HPLC analysis and detection

The instrumental analysis of glyphosate and AMPA in tomato extracts was optimized considering the different approaches developed earlier [4,9,15,31,32]. The HPLC system consisted of a Varian model 9012 pump, a Rheodyne 7125 injector (20 μL loop) and a Varian 9000 fluorescence detector (excitation 270 nm, emission 315 nm). Separations were carried out on a 5 μm ResElut C18 stainless steel column (0.46 cm × 15 cm i.d.) equipped with a guard column (20 mm × 2 mm i.d., same stationary phase), both from Varian. Two different gradient elution programs were developed

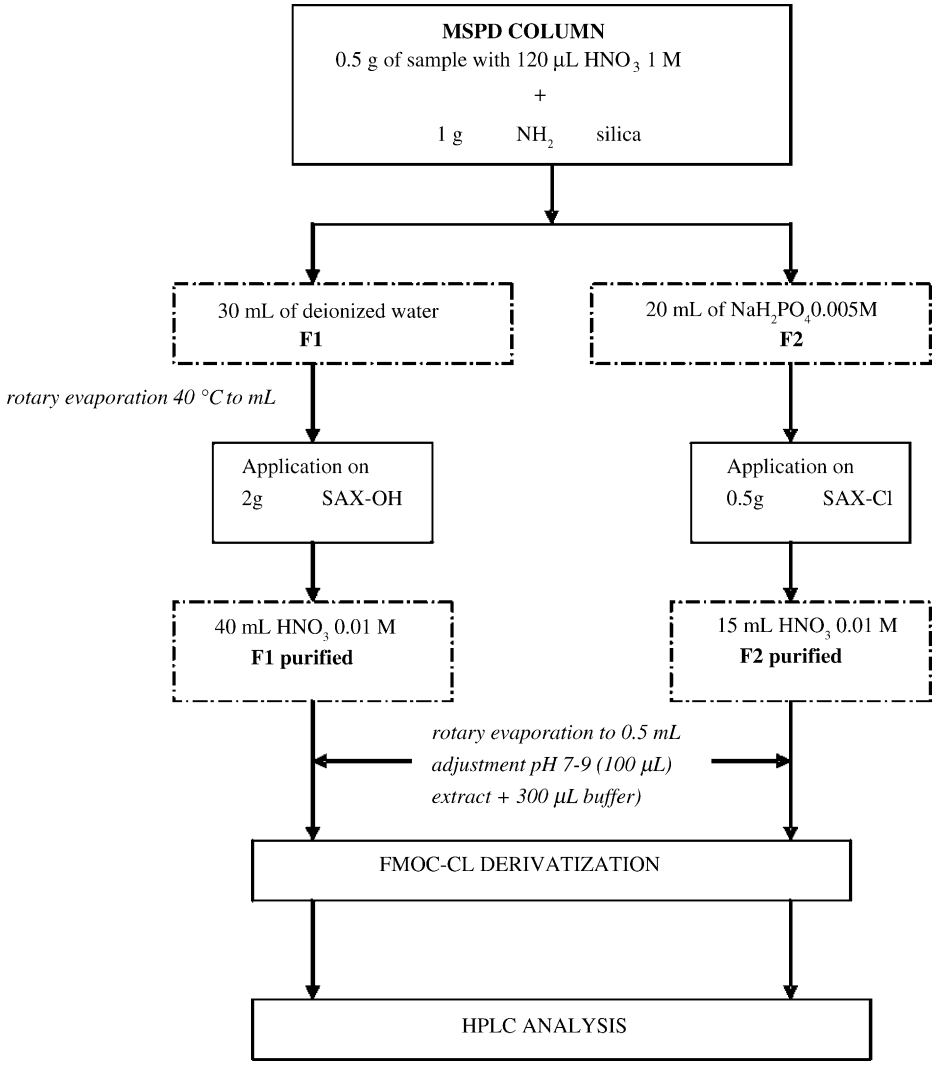

Fig. 1. Scheme of the sample pretreatment.

for the separation of F1 and F2 using acetonitrile and a 0.002 M NaH_2PO_4 (pH 6.4) water solution. Gradient program 1 (for F1) was: acetonitrile 16% (v/v) constant for 7 min, linearly increased to 40% (v/v) in 2 min and finally constant at 40% (v/v) for 20 min. Gradient program 2 (for F2) was: acetonitrile 8% (v/v) constant for 5 min, then linearly increased to 40% (v/v) in 2 min and again constant at 40% (v/v) for 20 min. At the end of each gradient program, a washing program was run by increasing acetonitrile to 80% (v/v) in 2 min, maintained constant for 5 min, and then linearly decreased to initial analysis conditions in 5 min and finally equilibrating the column for 15 min. A flow rate of 1 mL/min was used throughout. Quantitation of pesticides in extracts was calculated by comparing the peak areas for each compound with those obtained from the injection of standard solutions after derivatization.

For the derivatization reaction, a 0.5 mL volume of the purified and concentrated extract was placed in a small glass culture tube, adding 0.25 mL of 0.025 M borate buffer and 0.5 mL of 0.004 M FMOC-Cl in acetonitrile. The tube was shaken and allowed to react for 30 min at room temperature. Excess reagent was removed by two 1 mL washes with ethyl ether (top layer). Derivatized extract was diluted with 0.025 M borate buffer (100 μL extract + 300 μL buffer). Extracts were analyzed within 8 h.

2.4. Recovery studies

Recovery studies were carried out on fresh tomato fruit samples (0.5 g) spiked with 100 μL of the working standard solutions and left to stand at room temperature for 3 h. Samples were spiked with glyphosate and AMPA at three concentration levels: high (40–50 μg/g), medium (5–6 μg/g) and low (0.4–0.5 μg/g). Several replicates were analyzed for each level to evaluate the relative standard deviation (%RSD).

3. Results and discussion

3.1. Extraction

Extraction conditions were carefully selected to achieve the highest recovery for the pesticides. NH_2-silica was considered a suitable phase for matrix dispersion because of the high affinity provided for polar compounds. Initial solid-phase extraction studies applying 5 mL of a 10 mg/L standard solution of the herbicides to 0.1, 0.5 and 1 g of this sorbent packed in cartridges and previously conditioned with 5 mL of deionized water, showed a complete retention of both compounds. Subsequent application of a 10 mL volume of 0.005 M NaH_2PO_4 (pH 7) water solution resulted in quantitative elution of pesticides (100%). Unfortunately, although SPE results were satisfactory, the first MSPD test using 0.5 g of sample (fortified at 10 μg/g of pesticides) and 1 g of NH_2-silica, showed no elution of glyphosate neither with 10 nor with 20 mL of the NaH_2PO_4 solution. Therefore, it was necessary to make a previous matrix modification to disrupt the apparently strong interaction of glyphosate with the blended sorbent phase. Small additions of a nitric acid solution were assayed for this purpose. Table 1 shows the recovery of glyphosate from MSPD columns prepared with the fortified sample after addition of different volumes of 1 M HNO_3 solution. The columns were eluted with 4 × 5 mL fractions of the sodium phosphate solution and each fraction was independently analyzed. These results show that an increasing volume of the nitric acid solution in the sample provokes a recovery increase. However, there were not significant differences in recovery between a 120 and a 200 μL addition; so a 120 μL aliquot of the acid solution and a 20 mL elution volume were chosen in the final protocol. A blank tomato fruit sample (non fortified) was treated in the same conditions and no peak of glyphosate or a potential interference was observed in the chromatogram at the same retention time. Fig. 2 shows the HPLC chromatograms obtained from the analysis of the second 5 mL eluent fraction from MSPD columns prepared with a non-fortified tomato sample and a tomato sample fortified at 10 μg/g of glyphosate (test no. 3 of Table 1); both samples were treated with 120 μL of 1 M HNO_3. Although the numerous matrix peaks and the residual derivatization reagent (FMOC-Cl) observed in the chromatogram do not interfere with the target pesticide, it was considered that an additional cleanup of the glyphosate extract (F2) would be necessary to enhance the sensitivity of the method. Interferences were reduced when cleanup of F2 was carried out by ion-exchange chromatography as described later.

The different results obtained with SPE and MSPD cannot be easily explained. The possibility of the blended phase inducing a lot of chemical interactions within the sample components has been mentioned to explain the behavior of other pesticides in MSPD systems [27]. However, further investigation is required to understand the observed behavior of glyphosate and AMPA in these systems; it is evident that sorbent-sample-analyte interactions are particular for each analytical case. Also, the use of alkalis and acids for the glyphosate extraction from soil and plant materials has been reported in literature [11,13,15,33]. Archer and Stokes performed an acid reflux of blackberries to enhance the extraction of glyphosate by hydrolyzing possible conjugates of the pesticide with the plant constituents [11].

The MSPD test for aminomethylphosphonic acid was made with the same sorbent-sample ratio and with 1 M HNO_3 addition, but the sample was fortified at 40 μg/g of the metabolite. However, AMPA elution from the dispersion column could not be made with the NaH_2PO_4 solution as in SPE because in the subsequent cleanup step, phosphate anions strongly compete for ion exchange sites in the SAX exchanger and prevent analyte retention [31]. Moreover, an ion exchanger easily trap phosphorus containing compounds with functional groups ionically similar to glyphosate and its metabolite (peptides, sugars, nucleic acids, etc.) [17]. Thus, in order to obtain the highest recoveries, a 30 mL volume of deionized water was used for the total elution of AMPA. It was verified that glyphosate was not eluted in this fraction. Cleanup of the extract containing AMPA (F1), which will be described in the next section, was imperatively necessary because a large number of interfering peaks, corresponding to other contaminants or endogenous compounds eluted at the same retention time as the studied compound. Table 2 shows the recovery profile of AMPA in four elution fractions (2 × 5 mL + 2 × 10 mL) with deionized water. Each fraction was cleaned up prior to HPLC analysis. Fig. 3 shows the chromatograms obtained from the first 5 mL MSPD elution fraction for a non-fortified tomato sample and for a tomato sample fortified at 40 μg/g of AMPA (fraction 1 in Table 2).

Table 1
Recoveries of glyphosate in four elution fractions (5 mL each) from MSPD columns prepared with tomato fruit samples additioned with different volumes of 1 M HNO_3 solution (sample spiked at 10 μg/g of glyphosate; eluent: NaH_2PO_4 0.005 M)

Test	HNO_3 1 M (μL)	Recovery in 5 mL elution fractions (%)				
		1	2	3	4	Global
1	0	0	0	0	0	0
2	40	6	32	7	0	45
3	120	0	87	5	0	92
4	200	0	93	0	0	93

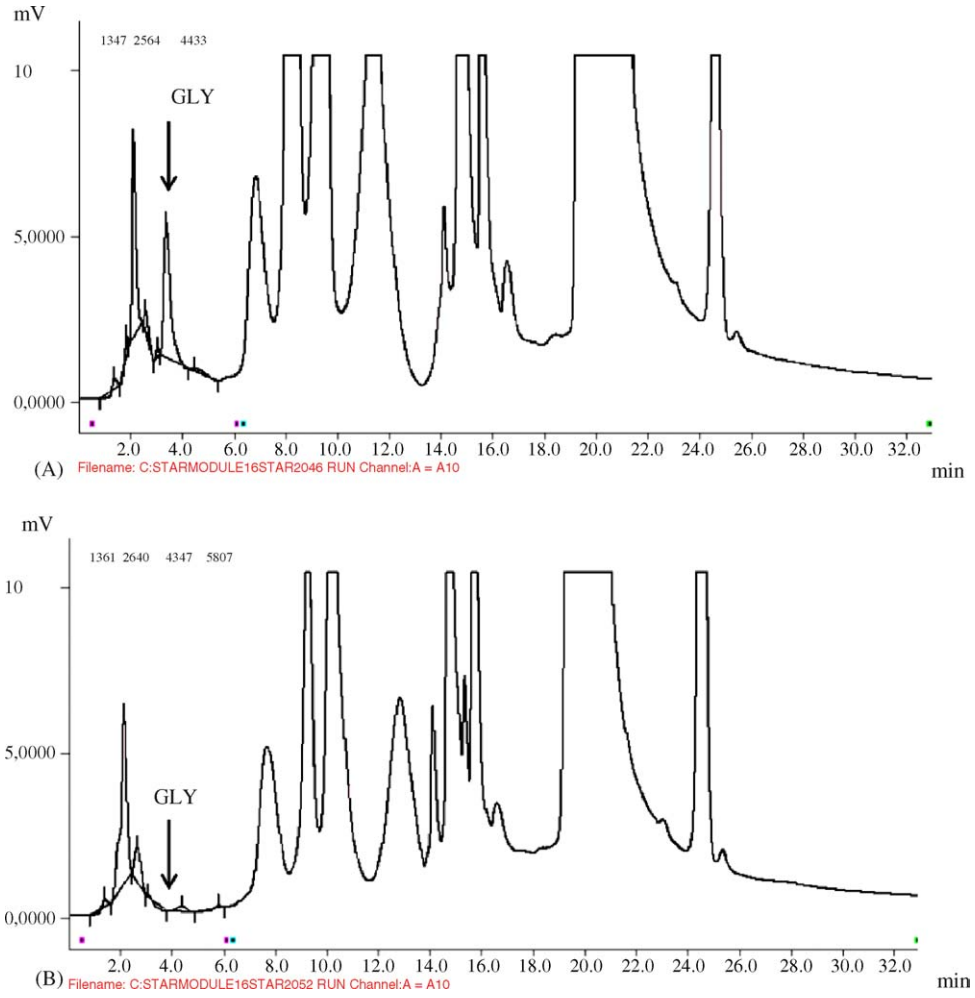

Fig. 2. Chromatograms of the second (5-mL) extract fraction obtained by elution of MSPD columns with aqueous NaH_2PO_4 (0.005 M). Tomato fruit samples (0.5 g) blended with NH_2-silica (1 g) were packed in cartridges and extracted with 4 × 5-mL volumes of eluent. Extracts without cleanup were derivatized with FMOC-Cl prior to HPLC analysis with fluorescence detection. Other chromatographic conditions in the text. (A) Sample fortified at 10 μg/g of glyphosate. (B) Non-fortified sample. Arrows indicate the retention time of glyphosate (GLY).

3.2. Cleanup

Cleanup conditions were selected to eliminate as much as possible the interfering matrix components and the residual derivatization reagent. Nevertheless, the similarity of glyphosate and AMPA to naturally occurring aminoacids and amino sugars further contributes to the difficulty in determining residues of these compounds in biological samples [13].

Table 2
Recoveries of AMPA in different elution fractions from MSPD columns using deionized water as eluent (sample spiked at 40 μg/g of AMPA)

Fraction	Elution volume (mL)	Recovery (%)
1	5	18
2	5	39
3	10	16
4	10	13
Total volume	30	
Global recovery		86

Since glyphosate and AMPA are zwitterionic molecules, they can be retained in cation or anion exchangers depending on pH value. However, adequate retention of these compounds in cation exchangers requires an extremely acidic media, which is not compatible with chemically bonded silica packings (i.e. SCX-silica). Therefore, a cleanup procedure based on the use of anion exchange SAX silica was studied for F1 and F2 separately. Preliminary SPE test applying 5 mL of 1 mg/L standard of glyphosate and AMPA to 0.5 g of SAX-OH packed in cartridges and conditioned with NaOH (pH 8), showed a complete retention of both compounds. It was very important to adjust the pH of the standard solution to ∼8 before application to the anion exchange column for an adequate retention of both compounds. Subsequent application of 0.01 M HNO_3 (pH 2) in water resulted in the quantitative elution of glyphosate and AMPA (∼100%). Although a good retention of glyphosate on SAX-CL (previously rinsed with 10 mL of deionized water) was also observed, AMPA showed a poor retention in these conditions and could only be

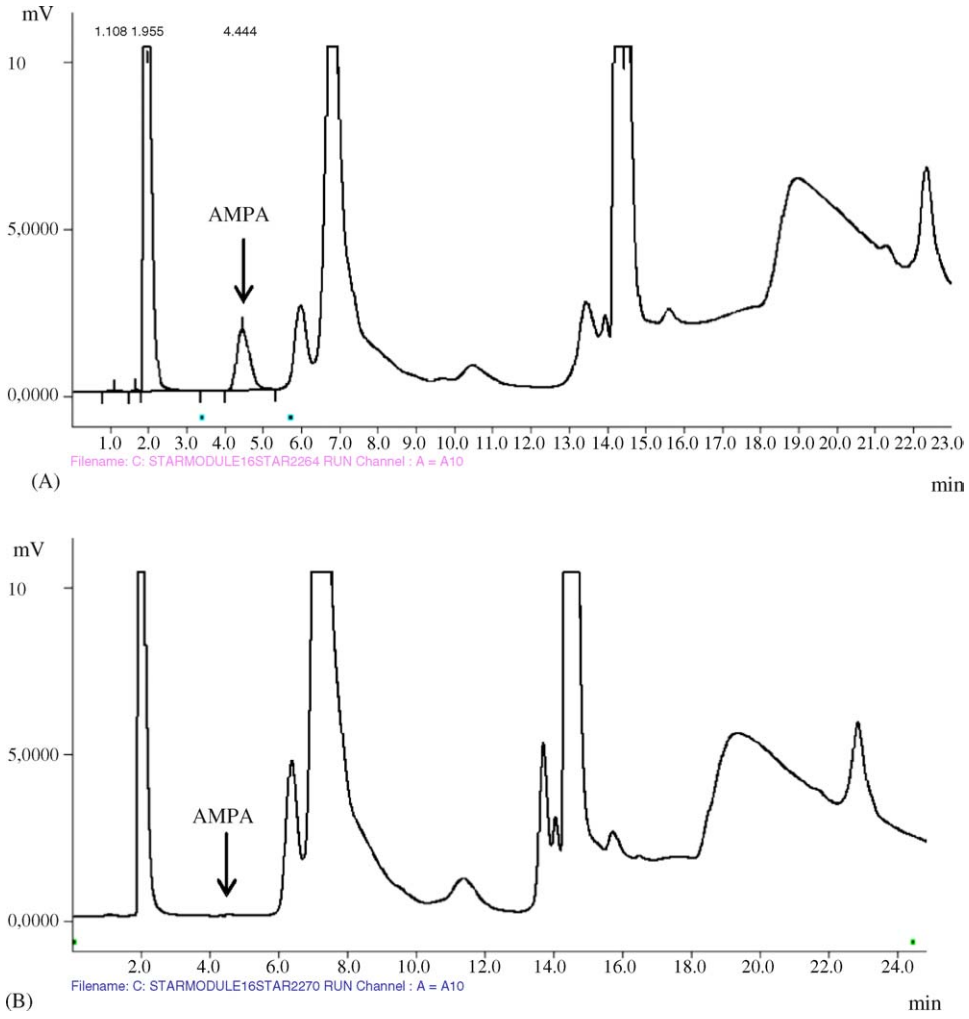

Fig. 3. Chromatograms of the first (5-mL) extract fraction obtained by elution of MSPD columns with deionized water. Extraction with 2 × 5-mL + 2 × 10-mL volumes of eluent. Extracts analyzed after cleanup on SAX-OH and derivatization with FMOC-Cl. Other conditions in Fig. 2. (A) Sample fortified at 40 μg/g of AMPA. (B) Non-fortified sample. Arrows indicate the retention time of AMPA.

retained on SAX-OH. Higher retention at pH 8 of glyphosate is explained by its two net negative charges whereas the metabolite only have one. For this reason, glyphosate can compete more effectively with phosphate ions and other coeluted components present in the MSPD extract, such as chlorophylls, triglycerides and phytosterals, common components in vegetables [34].

On the basis of the previous results, the following cleanup procedure was established for the handling of F1 and F2 extracts obtained from the MSPD column. Clean up of F1, corresponding to AMPA elution with a 30 mL volume of water was made in a cartridge packed with 2 g of SAX-OH. The amount of phase was increased because breakthrough of the pesticide occurred very rapidly when only 0.5 g of exchanger was used. Besides, the 30 mL extract had to be reduced to 10 mL by rotary evaporation at 40 °C to avoid analyte losses. The measured pH of the evaporated F1 extract was 7–8. Elution of AMPA from the SAX-OH cartridge required a 40 mL volume of 0.01 M nitric acid solution. Cleanup of F2, corresponding to the glyphosate elution with 20 mL of 0.005 M NaH_2PO_4 (pH 7) solution, was made in a cartridge packed with 0.5 g of SAX-Cl. The measured pH of F2 extract was 7–8. Elution from SAX-Cl column was made with a 15 mL volume of a 0.01 M nitric acid solution. Finally, purified F1 and F2 extracts were evaporated to 0.5 mL and the pH was adjusted to 7–9 with sodium hydroxide for the derivatization reaction.

3.3. Method evaluation

Method linearity was verified with four concentration levels (triplicate analysis): 50, 20, 10 and 5 μg/g for glyphosate and 40, 25, 12 and 6 μg/g for AMPA. The regression coefficients were 0.9994 and 0.9968 and the relative standard deviation (RSD) ranged between 2–8% and 7–10%, respectively. Accuracy, calculated as the percentage of recovery, and reproducibility expressed as %RSD at three fortification levels are shown in Table 3. Method detections limits

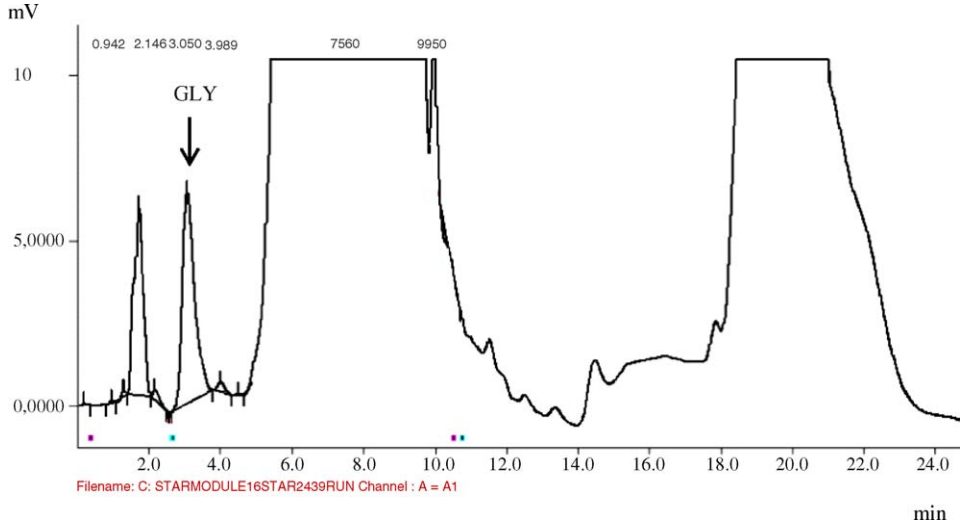

Fig. 4. Chromatogram of a fortified tomato sample (1 μg/g of glyphosate).

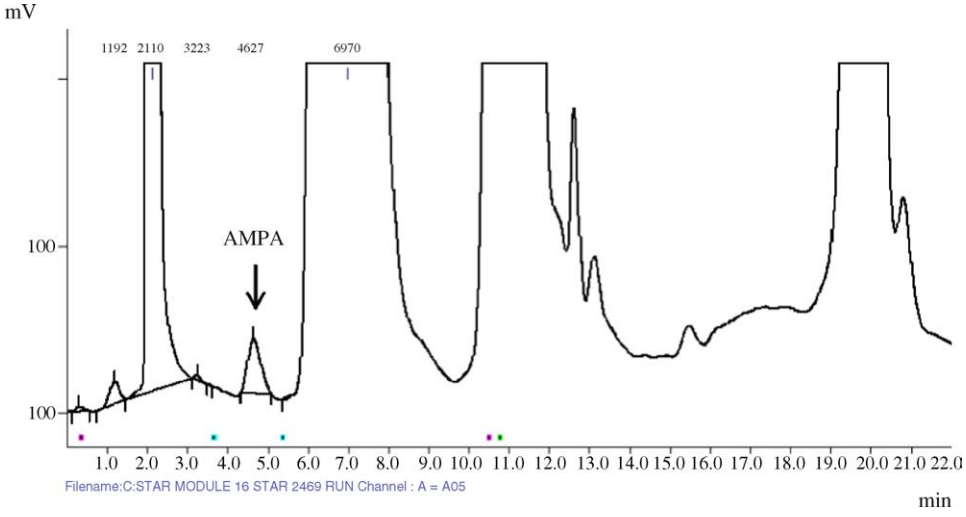

Fig. 5. Chromatogram of a fortified tomato sample (1 μg/g of AMPA).

at signal to noise ratio of 3 were 0.05 μg/g for glyphosate and 0.03 μg/g for AMPA. Instrument detection limits were 0.08 ng for glyphosate and 0.04 ng for AMPA. Figs. 4 and 5 show the chromatograms obtained from the analysis of a tomato sample fortified at 1 μg/g of the analytes using the whole procedure.

Table 3
Overall percent recoveries and RSD for pesticides in fortified tomato fruit using the whole procedure illustrated in Fig. 1

Level of spike (μg/g)	Average recovery (RSD)	
	Glyphosate	AMPA
50 (n = 3)	94 (2%)	
40 (n = 3)		88 (7%)
6 (n = 5)		84 (10%)
5 (n = 5)	91 (8%)	
0.5 (n = 7)	87 (10%)	
0.4 (n = 7)		78 (16%)

4. Conclusion

The proposed sample preparation procedure for the determination of glyphosate and AMPA in tomato fruit only requires a small amount of sample (0.5 g) and exclusively uses aqueous solutions for the obtainment of two purified extracts, one containing the pesticide and the other containing the metabolite. A one-step cleanup on ion-exchange phase was only necessary to perform an appropriate purification of the two crude extracts issued from an MSPD column. By comparison with previously reported sample preparation methods for the same analytes in plant material, the developed procedure has the advantage of eliminating the use of toxic solvents (chloroform, hexane, ethylacetate, commonly used in extraction/purification of analytes) and the requirement of exhaustive cleanup (including partitioning, charcoal, elimination of pigments and/or several ion-exchange columns). Yet, good recoveries and reproducibility were obtained in

spite of matrix complexity. Besides, the estimated LODs were comparable to those reported in literature for the determination of glyphosate and AMPA in similar matrixes. Therefore, this sample pretreatment is well adapted for measuring residues of these pesticides in tomato fruit and can be an interesting alternative to the more classical extraction methods.

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