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Field-Amplified Sample Injection and Sweeping Micellar Electrokinetic Chromatography in Analysis of Glyphosate and Aminomethylphosphonic Acid in Wheat

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Highlights

- Derivatization of glyphosate and its metabolite AMPA using FMOC-Cl, was optimized
- Field Amplified Sample Injection Sweeping MEKC, enhanced UV sensitivity
- Double step SPE (C18/SAX) was developed for wheat flour aqueous extracts cleanup
- The validated method was applied to commercial and experimental wheat flour samples
- LOQs of glyphosate and AMPA were 0.1 and 0.05 mg/kg, respectively

Abstract

Glyphosate, a widely used herbicide, has been classified as probably carcinogenic to humans by the International Agency for Research on Cancer (IARC). In the present study a method based on Field-Amplified Sample Injection and Sweeping Micellar Electrokinetic Chromatography (FASI sweep-MEKC) has been developed and validated for determination of glyphosate and its microbial metabolite aminomethylphosphonic acid (AMPA) in wheat flour. The method involved a preliminary solid phase extraction for cleanup of the aqueous extracts from wheat flour, based sequentially on C18 and strong anion exchange cartridges, followed by derivatization using 9-fluorenylmethylchloroformate. Optimization of sample cleanup and derivatization procedure was carried out by a HPLC-UV method, whereas FASI sweep-MEKC was applied for achieving the sensitivity necessary for analysis of real samples. To this regard, optimum conditions involved the use of an extended path fused-silica capillary (80 cm total length, 50 μm, i.d.) filled with a high concentration buffer (sodium phosphate 100 mM, pH 2.2). Electrokinetic sampling was carried out at −10 kV with injection time of 700 s and the separation of the loaded analytes was performed under MEKC conditions using sodium phosphate buffer 50 mM at pH 2.2, supplemented with sodium dodecyl sulfate, 100 mM. The method was validated for linearity, precision, accuracy and sensitivity, showing that using conventional UV detection (210 nm) the achieved limit of quantitation (LOQ) values for both the analytes were widely lower than those set by Authorities. In particular, LOQ for glyphosate and AMPA were found to be 5 and 2.5 ng/mL, respectively, corresponding to 0.1 and 0.05 mg/kg, in wheat flour. The method, applied to commercially available real samples (wheat flour from different manufacturers) and to an experimental sample obtained by cv. Svevo wheat, can be considered as a convenient alternative to the existing approaches in analysis of complex matrices.

Keywords: Glyphosate; Aminomethylphosphonic acid; Field-Amplified Sample Injection (FASI); Sweeping MEKC; Derivatization; Solid Phase Extraction (SPE).

1. Introduction

Glyphosate, the common name for *N*-(phosphonomethyl)glycine, is a component of a number of products used as herbicides in agriculture to combat undesired plants competing with cultivated crops. It is widely applied as a pre-sowing or pre-emergence treatments to control weeds, but also before crop harvest to facilitate better harvesting by regulating plant growth and ripening. The use of glyphosate has increased sharply with the development of genetically modified glyphosate-resistant crop varieties and nowadays it is commonly detected in air, water and food [1]. In 2015 the compound has been classified as "probably carcinogenic to humans" (Group 2A of the International Agency for Research on Cancer, IARC) [2], however in 2016 the European Union commission (EU) established that based on the available information, there is no evidence to link glyphosate to cancer and it should not be classified as a substance that causes genetic damage (mutagen) or disrupts reproduction: as a consequence, on December 2017, EU renewed the approval of glyphosate for 5 years [3]. This divergence has contributed to an intensive scientific work on the effect of glyphosate exposure to the humans and environment [1, 4] and in 2018 the European Food Safety Authority (EFSA) released a review of the existing maximum residue levels (MRLs) for glyphosate, reporting values within 0.5 – 50 mg/kg depending on the considered sample (*e.g.*, plants derived food, meat etc..). Currently the MRL in wheat grain is set by EU at 10 mg/kg; when setting MRLs, EFSA referred to the acceptable daily intake (ADI) for everyday exposure to glyphosate at 0.5 mg glyphosate/kg of bodyweight/day [5]. Independent studies suggested to set ADI at a lower level with respect to those considered by EFSA (*i.e.*, 0.025 mg/kg) , thus accordingly, the current MRL in different food should be lower than what it has been defined by the authorities [6]. As it can be argued, in this dynamic framework there are still open questions that can be closed and answered with new and elevated analytical strategies for quantitation of glyphosate and its main (microbial) metabolite aminomethylphosphonic acid (AMPA) [7]. Comprehensive reviews show that liquid chromatography coupled to mass spectrometry

(LC-MS) is the most suited technique for analysis of glyphosate and related compounds in food, environmental and biological samples [8, 9]. Their determination however, is challenging as due to the amphoteric character, low masses, high water solubility, low volatility, tendency to interact with stainless steel surfaces and lack of chemical groups that could facilitate detection [10]. Most of the proposed chromatographic methods are based on pre-column derivatization and as highlighted by Arkan and Molnár-Perl, 9-fluorenylmethylchloroformate (FMOC-Cl) is often selected as the labeling reagent for quantitation in different matrices (biological, environmental, food samples), using MS as well as fluorescence (FL) detection [11]. Updating the literature to the last years, FMOC-Cl is confirmed as the most convenient reagent for derivatization of glyphosate and subsequent analysis by LC-FL and LC-MS in food and environmental samples; the reported LOQ values ranged in a quite wide window depending on the sample type [12 - 21]. Electromigration techniques represent a good alternative to LC, as recently reviewed by Gauglitz *et al*., who pointed out the high matrix tolerance of both capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), as one of the main advantages in analysis of glyphosate and related compounds. However the required low limits of detection represents a challenging issue because of the poor sensitivity of CE due to the small injected sample volumes (typically 1 – 10 nL) [22].

Regardless the separation approach (LC or CE), the detection mode (FL, UV, MS) and matrix type, a sample cleanup and a pre-concentration step is applied in determination of glyphosate and related compounds in real samples. Direct analysis (no derivatization) of glyphosate in breast milk by LC-MS/MS was carried out upon removal of fat by centrifugation, and proteins by ultrafiltration in one step through centrifugal filtration using a molecular weight cut-off filter [23]. Solid Phase Extraction (SPE) has been widely applied in several types of samples; for analysis of relatively simple matrix as river waters, SPE using anion exchanger resin in micro-pipette tip format was adopted for quantitation by MEKC upon glyphosate derivatization using naphthalene-2,3-dicarboxaldehyde as a fluorogenic reagent, obtaining LOQ values in the low nM range [24]. More complex matrices as soybean and corn, required the use of Oasis HLB SPE cartridge to retain suspended particulates and non-polar interferences for direct LC-MS analysis [25]. Double step SPE, combining a C18 cartridge to remove protein and weak-polar interferences, followed by a strong anion exchange (SAX) cartridge, to remove neutral and basic substances, was applied for analysis of glyphosate residue in plant-derived food. The subsequent quantitation was carried out directly by LC-MS performing separation on hydrophilic interaction/weak anion-exchange (HILIC/WAX) stationary phase [26]. A further example of double step SPE was given by Nagatomi *et al*., reporting the application of Oasis MCX as a strong cation-exchanger able to remove from the complex matrices (malt and corn samples), interfering components such as amines or pigments; a subsequent enrichment by SAX cartridge, was

found to be necessary for sensitive quantitation, achieving LOQ of 10 μg/kg [27]. A double SPE procedure for sample cleanup was also proposed for analysis of glyphosate and AMPA in leaves of *Coffea arabica.* The first SPE step involved the purification of the sample (acidified aqueous extract of the powder of plant material) by elution through a Strata X cartridge, then the non-retained analytes were derivatized with FMOC-Cl and a second SPE step on Strata X was carried out to retain the derivatives that were successively eluted using methanol [21]. Derivatization of the analytes by FMOC-Cl and subsequent SPE on Strata X cartridge was approached for analysis in natural waters [28]. In a study by Ghanem *et al.*, SAX resin was selected for trapping glyphosate and AMPA allowing for a subsequent derivatization on the solid support by FMOC-Cl. Interestingly, it was shown that the retention on the commercially chloride SAX resin was not sufficient because of the poor competition of the analytes with the counter-ion chloride. By converting the latter to HCO_3^- using a preliminary washing of the resin with bicarbonate solution, effective retention of the phosphonic analytes was achieved [29]. Dispersive SPE using magnetic Fe_3O_4@Al_2O_3 nanoparticles was applied for extraction/pre-concentration of glyphosate and AMPA based on the strong affinity of alumina for analytes containing phosphate groups within a large pH range. The quantitation was performed by CE and electrochemiluminescence detection [30]. Similarly, Fe_3O_4 nanoparticles were used as a support to immobilize Ti^{4+} using polydopamine as bridging molecules; the obtained material was applied for magnetic solid phase extraction of glyphosate and AMPA in water samples by FMOC-Cl derivatization for CE analysis using UV detection at 203 nm [31]. Both homemade and commercially available molecularly imprinted polymers (MIPs) were used for cleanup and pre-concentration from natural waters followed by derivatization with FMOC-Cl and UHPLC-MS/MS determination; it was found that the commercially available MIP provided recovery of glyphosate and AMPA of 68% and 82% respectively, achieving a concentration factor up to 100-fold [32, 33].

The aim of the present study was to provide an alternative method for analysis of glyphosate and AMPA in wheat flour (commercially available and experimental samples) based on the use of conventional CE equipment with UV detection. In order to fulfill the limit established by Authorities on the maximum residue level for both the analytes, a SPE procedure for sample cleanup and concentration was combined with Field-Amplified Sample Injection and sweeping MEKC (FASI sweep-MEKC) [34]. While commercially available samples showed to do not contain glyphosate and AMPA at the detection limit of the method, an experimental sample obtained by wheat cv. Svevo revealed the presence of 243 mg/kg of glyphosate.

2. Material and methods

2.1. Materials

The reference compounds glyphosate and taurine were from Sigma-Aldrich (Milano, Italy) and aminomethylphosphonic acid (AMPA) was from Acros Organics (Thermo Fisher, Karlsruhe, Germany); the derivatization reagent 9-fluorenylmethylchloroformate (FMOC-Cl) was from Sigma-Aldrich (Milan, Italy). The components of electrophoretic and HPLC buffers as well as solutions and eluents for SPE procedures *i.e.*, sodium tetraborate, phosphoric acid (85%, w/w in water), hydrochloric acid (37%), ammonium hydroxide solution (ACS grade, 28.0-30.0%), ammonium acetate, sodium acetate, sodium hydroxide, sodium dodecyl sulfate (SDS), methanol (HPLC grade) and acetonitrile (HPLC grade), were purchased from Sigma-Aldrich. Common wheat flour for method optimization and used as a blank sample, was obtained from a local store. In addition, 10 durum wheat plants (cv. Svevo) were placed in a growth chamber and sprayed with glyphosate in pre-harvest. Grains were collected and used for the subsequent analyses as an experimental sample. Ultrapure water, for the preparation of the running buffers, samples and standard solutions was obtained by Elix Systems (Millipore, Billerica, MA, USA).

2.2. Solutions and derivatization reaction

Standard stock solutions of glyphosate and AMPA were prepared individually in water (about 2 mg/mL) and stored at 4-8°C for one week. Working standard solutions were prepared daily by appropriate dilution with water in order to obtain the desired final concentrations for optimization and method validation experiments. Taurine (aqueous solution 0.2 μg/mL) was used as the internal standard in CE analysis. Derivatization reagent FMOC-Cl, was prepared daily in acetonitrile at the concentration of 6 mM. Phosphate buffer at 100 mM concentration for CE analysis was prepared by diluting the proper amount of concentrated phosphoric acid solution in water and adjusting the pH to 2.2 using sodium hydroxide 1.0 M. MEKC analysis was performed using a background electrolyte composed of a 50 mM sodium phosphate solution pH 2.2 (prepared as above described) supplemented with SDS at 100 mM concentration; the obtained solution was ultrasonicated and filtered (syringe filter 0.45 μm, regenerated cellulose). Ammonium acetate buffer used as a component of the mobile phase in HPLC analysis, was prepared at 10 mM concentration by dissolving the proper amount of the salt in water; the pH was adjusted to 8.5 using ammonium hydroxide (prepared at about 10%, v/v, by dilution of the commercially available concentrated solution). The buffer was filtered through a membrane filter 0.20 μm (nylon membrane, Merck Millipore Ltd., Tullagreen, Irl.).

Derivatization and calibration graphs were performed as follow: aliquots of 100 μL of standard solutions containing glyphosate and AMPA in the concentration range of 0.5 – 15.0 μg/mL (HPLC) and 0.01 – 1.5 μg/mL (CE), were mixed with 20 μL of sodium tetraborate buffer (0.1 M), 20 μL of ultrapure water (in CE analysis, instead of water, the internal standard solution was added) and 20 μL of derivatization reagent solution. After mixing, the solution was kept at room temperature for 10 min

before dilution with water, by adding volume of 160 μL or 500 μL for HPLC or CE analysis, respectively. The final obtained clear solution was filtered through a syringe filter (0.45 μm, regenerated cellulose), and injected into the HPLC or CE apparatus for analysis. All standard stocks and working solutions were stored in polypropylene containers.

2.3. Sample preparation and SPE procedure

Extraction of glyphosate and AMPA from wheat flour was performed according to Granby *et al.*, with slight modifications [35]. Aliquots of wheat flour (1.0 g) were extracted with 5 mL of ultrapure water by sonication at room temperature for 10 min; after centrifugation at 2000 rcf for 10 min, the supernatant was removed and extraction was repeated. The combined supernatants were mixed and subjected to SPE method.

The optimized SPE procedure consisted in two steps; the first involved the use of a C18 cartridge (Strata C18-E 500 mg/3 mL, 55 μm, Phenomenex, Castel Maggiore, Bologna, Italy) that was preconditioned sequentially with 3 mL of methanol and 3 mL of water. Then the supernatant from extraction of wheat flour was loaded and the cartridge was washed with 1 mL of water; the effluent containing not retained compounds, was collected and subjected to the second SPE step using a SAX cartridge (500 mg/3 mL, Scharlau, Barcelona, Spain) preconditioned sequentially with 3 mL of methanol and 3 mL of water. In order to convert the counter-ion chloride of the commercially available SAX sorbent in the acetate form, a 4 mL volume of sodium acetate 2 M solution was loaded to the cartridge; then a rinsing with 4 mL of ultrapure water (till pH <9) was carried out before loading the effluent from the C18 cartridge. After sample loading, the cartridge was washed using methanol (3 mL) and vacuum was applied. Interferences were selectively removed by washing the cartridge with 1 mL of 0.05 M hydrochloric acid (the eluate was discharged), then elution of glyphosate and AMPA was achieved using 2 mL of 0.1 M hydrochloric acid. The obtained eluate was diluted 1 to 10 with ultrapure water, then aliquots of 100 μL of the solution were subjected to derivatization procedure for HPLC or CE analysis as described at *Section 2.2*.

2.4. CE instrumentation and analysis

The instrument for CE analysis was a G1600 HP^{3D}CE from Agilent Technologies (Waldbronn, Germany) using the software Rev. A. 09. 01. Agilent Chemstation. Fused-silica extended path capillaries (G1600-62232, Agilent Technologies) were 80 cm total length and 71.5 cm effective length, with the inner diameter (i.d.) of 50 μm (outer diameter 360 μm). New capillaries were conditioned by flushing in the following order, sodium hydroxide 1 M, sodium hydroxide 0.1 M and water (10 min each). The CE analysis was performed by applying a FASI sweep-MEKC procedure consisting in loading the capillary with a solution of sodium phosphate buffer 100 mM (pH 2.2), then a plug of water was loaded by hydrodynamic injection at 50 mbar x 10 s and the sample was

introduced by electrokinetic injection at −10 kV for 700 s. The separation voltage was −25 kV introducing both the capillary ends in the separation buffer composed of a 50 mM sodium phosphate solution (pH 2.2) supplemented with SDS at 100 mM concentration. Between injections, the capillary was flushed sequentially with sodium hydroxide 1 M, water and sodium phosphate buffer 100 mM at pH 2.2 (3 min each). The separation was performed at a constant temperature of 25°C and the detection wavelength was 210 nm.

2.5. HPLC instrumentation and analysis

HPLC separations were carried out using a Liquid Chromatograph 1050 Ti series (Agilent Technologies) equipped with a DAD detector (detection was set at 250 nm). The separation column was a narrow bore C18 Zorbax Eclipse plus 150 x 2.1 mm (3.5 μm) by Agilent Technologies. Analyses were performed using a mobile phase composed of (A) 10 mM solution of ammonium acetate (pH 8.5) and (B) acetonitrile, under gradient elution from A/B 85/15 (v/v) to A/B 45/55 (v/v) in 15 min at the flow rate of 0.3 mL/min. Sample injections were manually done by a Rehodyne Model 7125 injector (volume 20 μL).

3. Results and discussion

3.1. Optimization of derivatization conditions

Derivatization by FMOC-Cl is a well-known approach for determination of glyphosate and AMPA using LC with either MS and optical detection. FMOC-Cl reacts with primary and secondary amino groups under alkaline conditions obtained by buffering the sample with addition of tetraborate solution; the volume ratio of sample *vs.* tetraborate buffer, depends on its concentration (generally 0.025 – 1 M) and the matrix type/pH [11].

With the aim to develop a FASI sweep-MEKC method for sensitive determination of glyphosate and AMPA, it was considered of importance maintaining the concentration of borate in the sample mixture as low as possible to have a significant conductivity mismatch between sample and background electrolyte (BGE) [34]. Thus, while the selection of the general derivatization conditions (*e.g.*, molar ratio analytes/reagent, reaction time and temperature) was done according to literature data [11, 13], the effect of tetraborate concentration on derivatization efficiency was evaluated in detail. The optimization experiments were carried out by HPLC-UV whose analytical response can be considered not significantly affected by sample matrix composition. A narrow bore C18 Zorbax Eclipse plus column was used under gradient elution in acetonitrile/ammonium acetate (10 mM, pH 8.5) mobile phase; in Table SM1 of Supplementary Material, the validation parameters of the applied HPLC method, are reported. Standard mixtures of about 2 μg/mL of the analytes and taurine (the latter intended to be used as an internal standard) were derivatized by addition of FMOC-Cl solution

in acetonitrile (6 mM, providing a reagent/analyte molar ratio of about 100) at room temperature for 10 min in the presence of tetraborate solution (pH 9.2) at different concentration (0.075 – 0.2 M); according to the volume added (20 μL), tetraborate final concentration in the sample was within 9 – 25 mM. The evolution of the peak area of the compounds is reported in the graph of Fig. 1 (solid line). As it can be seen, no significant differences in the response is observed at different tetraborate concentration, thus apparently the most convenient condition for derivatization should be that using tetraborate solution at the lower concentration since it would allow for best performance of FASI sweep-MEKC. However, it has to be taken into account that glyphosate and AMPA in real samples, are eluted by SAX SPE using hydrochloric acid 0.1 M solution (see *Experimental Section 2.3*), thus derivatization experiments were also carried out on analytes solutions prepared in the same solvent; as expected, the derivatization efficiency of all the three solutes was very low (data not shown) as the final pH of the reaction mixture was not enough alkaline to allow the suitable reactivity of amine group. As it can be seen in graph of Fig. 1 (dotted lines), only when the concentration of hydrochloric acid was 0.01 M, the derivatization efficiency was comparable to that obtained for analytes in aqueous solutions at tetraborate concentration at least 0.1 M. It can be concluded that in order to carry out efficient FMOC-Cl derivatization, the eluate from SPE SAX cartridge must be diluted at least 10-fold.

3.2. Sample extraction and optimization of the SPE method

Extraction of the analytes was carried out according to a general method using an ultrasonic bath to treat 1.0 g of wheat flour with 5 mL of water (10 min, twice) [35]. The aqueous extract from spiked sample was passed through a C18 cartridge, then the not retained solution was loaded into the SAX cartridge in order to trap the analytes by means of the electrostatic interaction of the anionic phosphate groups with quaternary amine of the sorbent. According to the pKa values of the most acidic phosphate function of the two solutes (pKa_1 of glyphosate and AMPA are reported to be 0.7 and 0.9, respectively [29]), it was considered to do not necessary the pH adjustment of the aqueous extract before its application to SAX cartridge. However, since AMPA was detected in the flow-through of the loading, the counter-ion chloride of the standard SAX sorbent was exchanged with one having lower selectivity and allowing for better retention of the analyte, according to the following order: OH^- < acetate < formate < HCO_3^- < Cl^- [29]. By a systematic evaluation (Supplementary Material, Table SM2) it was found that acetate counter-ion allowed the best retention of AMPA both during loading and washing steps of SPE. Washing steps based on methanol (3 mL) to remove hydrophobic interferences, and 0.05 M hydrochloric acid (1 mL), were carried out before elution of the analytes using 0.1 M hydrochloric acid (2 mL). As above discussed, FMOC-Cl derivatization requires the dilution with water (1:10, v/v) of the obtained eluate and subsequent alkalinization using 0.1 M

tetraborate buffer. In Fig. 2 are reported the chromatograms of: A) a standard solution of glyphosate and AMPA each at 2.5 μg/mL; B) blank (wheat flour subjected to the extraction and SPE procedure) and C) spiked sample containing the same level of the analytes as in the standard solution. In order to assess the matrix effect, it is also reported a chromatogram of a blank sample fortified, post-SPE procedure, with the two compounds at the same concentration level (Fig. 2D). No significant differences were found between the analytical response of standard solution and that of the blank sample spiked with the analytes post-SPE procedure. Moreover, the recovery estimated by comparing the analytical response (HPLC-UV peak area) of the considered derivatives in standard aqueous solutions with those obtained in spiked samples subjected to the described procedure (extraction and double step SPE), were 105.2% (4.16%, n = 3) and 85.5% (3.80%, n = 3) for glyphosate and AMPA, respectively. It has to be underlined that the optimization of SPE procedure was carried out by HPLC-UV whose performance, although not suitable for a definitive analytical method because of the limited sensitivity (Table SM1), can be considered not affected by sample composition. Thus, the obtained results suggested that the proposed SPE method was worth to be applied in combination with the sensitive FASI sweep-MEKC in analysis of real samples whose content in glyphosate and AMPA is expected to be much lower with respect to the spiked samples considered at the present stage of the investigation.

3.3. Development of FASI sweep-MEKC

Online sample pre-concentration techniques are applied to improve the sensitivity in CE according to different approaches as discussed in many reviews [36 - 39]. The anionic properties of the considered FMOC-derivatives, prompted us to apply a FASI method for their electrokinetic sampling [34, 40]; in the scheme depicted in Supplementary Material (Scheme SM1) are reported the main steps of the applied method, described as following. The fused-silica capillary was conditioned and filled with a high concentration buffer (HCB) at low-pH in order to strongly suppress the electroosmotic flow (EOF). The sample for optimization experiments was prepared using aqueous solution of FMOC-derivatives by glyphosate and AMPA; in addition FMOC-derivative of taurine was included as an internal standard. Before injection, the derivatization mixture was diluted with water (see details in *Section 2.2.*) to reduce the concentration of borate ions from derivatization mixture, in order to have high conductivity mismatch between sample and HCB thus providing best conditions for sample stacking [34, 36 - 40]. For injection, the capillary end was inserted into the vial containing at least 0.5 mL sample solution whose pH was weakly alkaline. The application of a negative voltage (the anode at the outlet end) allowed for the electrokinetic loading of the anionic analytes. The latter, entering the low-pH HCB underwent to a decrease of negative charge because of the reduced dissociation of the anionic groups of both glyphosate and AMPA conjugated with the less acidic phosphonic

functions (reported pKa of 5.6 and 5.9, respectively) and carboxylic group of glyphosate (pKa 2.2) [29]. The described conditions, in combination with the low electric field experienced because of the high ionic strength across the capillary, led the compounds to be stacked creating a sample zone of higher concentration with respect to that in sample vial. The duration of electrokinetic injection was set to be quite long (*e.g.*, > 10 min) to increase sensitivity; afterward both the capillary ends were transferred to the vials containing the BGE with a conductivity similar to that of HCB at the same pH, and supplemented with anionic surfactant to perform MEKC separation. A negative voltage (–25 kV) was applied allowing the anionic micelles entering the sample zone to give sweeping and separation by MEKC mechanism [41]. The optimization of the most important parameters involved in FASI sweep-MEKC is discussed below.

3.3.1. MEKC and separation conditions

Sodium dodecyl sulfate (SDS) was chosen as the surfactant for development of the MEKC system; preliminary experiments conducted on a BGE composed of 50 mM sodium phosphate buffer at pH 2.2, containing SDS at 100 mM concentration, showed that glyphosate and AMPA derivatives can be easily separated each other and from reagent excess and its degradation products.

3.3.2. Selection of electrolyte (high concentration buffer, HCB) pH and concentration

The electrophoretic behavior of FMOC-derivatives was evaluated by CZE experiments in a fused-silica capillary (71.5 cm effective length, 50 μm i.d.) at 25°C and –25 kV in a 100 mM sodium phosphate buffer. Under strongly acidic conditions (pH 2.5), in the presence of weak EOF, the apparent mobility expressed as the mean of three determinations was found to be $-13.6 \times 10^{-9} m^2 V^{-1} s^{-1}$ and $-14.0 \times 10^{-9} m^2 V^{-1} s^{-1}$, for glyphosate and AMPA derivatives, respectively. By lowering the pH to 2.2, the apparent mobility was significantly diminished to $-9.75 \times 10^{-9} m^2 V^{-1} s^{-1}$ (glyphosate) and $-10.2 \times 10^{-9} m^2 V^{-1} s^{-1}$ (AMPA). Hence, operating in FASI fashion with a pH 2.2 HCB, allows for a significant decrease of migration velocity of the analytes across the capillary, thus resulting in efficient stacking. HCB solutions with pH values lower than 2.2 did not provide any significant stacking improvements. HCB solutions of concentrations > 100 mM, were not used because produced high electric current, irregular baseline and system peaks (spikes).

3.3.3. Electrokinetic injection length

Injection time affects response in FASI by influencing the length of the sample zone and accordingly, the amount of analytes loaded into the capillary. The optimization was carried out by applying a constant voltage of −10 kV since at higher values, electric current instability and breakdown was often observed. Using as HCB, a 100 mM sodium phosphate buffer at pH 2.2, the electric current generated during injection was stable at about −12 μA. In Fig. 3 are shown the electrokinetic chromatograms obtained in analysis of glyphosate and AMPA (about 0.015-0.02 μg/mL) in the

presence of taurine as the internal standard. The compounds were sampled at different injection time *i.e.*, 300 s, 500 s and 700 s and separation was then performed by MEKC. As it can be seen, the responses (peak height) increased with injection time and no peaks distortions were observed up to 700 s. Performing longer injections was not convenient since the response was not significantly improved.

3.4. Validation of FASI sweep-MEKC

The optimized method was validated assessing linearity, sensitivity, precision and accuracy in order to be applied in analysis of glyphosate and AMPA in wheat flour samples. Since electrokinetic injection is susceptible to the variation in the composition of the sample matrix, taurine was added as an internal standard, before derivatization. Being the mobility of taurine FMOC-derivative, similar to those of the analytes, the variation in matrix composition among samples (and standard solutions) that could result in different sampling rate, will reflect at the same extent on the internal standard and analytes. Thus the peak area ratio (analyte/internal standard) can be used for normalization of the responses in quantitation experiments [42]. Linearity was established by regression analysis of peak area ratio *vs.* the analytes concentration (μg/mL), within a range extended at two orders of magnitude. The data are reported in Supplementary Material Table SM3.Coefficients of determination r^2 were 0.999 and no trends for residual distribution were observed. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated by the signal-to-noise ratio defined as 3 (LOD) and 10 times (LOQ) the noise, assumed as the distribution of the response at zero analyte concentration. The LOQ values related to the solutions subjected to the derivatization procedure were found to be 2.5 and 5 ng/mL for AMPA and glyphosate, respectively. According to the sample preparation method, the LOQ values corresponded to 0.05 and 0.1 mg/kg for AMPA and glyphosate, respectively, thus the method widely fulfils the limit established by EU in wheat grain (10 mg/kg). With respect to the data obtained by the HPLC-UV method of the present study, the sensitivity of FASI sweep-MEKC was found to be about 100-fold higher. Among the sensitivity values reported in literature for glyphosate and AMPA by electromigration techniques, the results by See *et al.,* deserve to be mentioned since using the combination of field enhanced sample injection and contactless conductivity detection, LOD of 0.5 nM was achieved [43]. However the method was applied on relatively simple samples represented by drinking water. Simultaneous electrophoretic concentration and separation (SECS) of some herbicides in beer, prior to stacking and UV detection, allowed to achieve LOQ values of 10 and 20 ng/mL for glyphosate and AMPA, respectively [44]. Very high sensitivity (LOD 0.05 μg/L) in analysis of more complex samples such as extracts from food, was achieved by derivatization with fluorescein isothiocyanate (FITC) followed by separation on microchip device and LIF (Laser Induced Fluorescence) detection [45]. Applications on samples similar to those of the present study

(wheat and soybean extracts), reported LOD values of 2.5 μM (about 0.4 μg/mL) and 0.06 μg/mL by CE-MS and CE with electrochemiluminescence detection, respectively [46, 47]. In Table 1 are reported the main analytical characteristics of the present method compared to those of published methods applied to the determination of glyphosate and AMPA in food and related samples, by different techniques. Among the considered methods, and with exception of that proposed by Wuethrich *et al.*, [44], it is noteworthy that the present approach is the sole using simple and affordable UV detection while allowing sensitivity in the order of that reported for most of the published methods using LC-MS. System precision was assessed by repeated injections (n = 6) of a standard mixture of the analytes at the LOQ concentration levels. The obtained RSD% values of the peak area ratio (analyte to internal standard) were 2.03% and 4.32% for AMPA and glyphosate, respectively; the RSD% values of migration time were 1.57% and 1.68% for AMPA and glyphosate, respectively (Supplementary Material Table SM3). These data suggest that the instrumental variability at low concentration of the analytes is adequate and consistent with those obtained by CE methods applied to similar samples.

In Fig. 4 are reported the electrokinetic chromatograms of: A) a standard solution of glyphosate and AMPA each at about 0.015 μg/mL; B) blank (wheat flour subjected to the extraction and SPE procedure) and C) spiked sample containing the same level of the analytes as in the standard solution (level of about 0.3 mg/kg); it is also reported the electrokinetic chromatogram of the blank sample fortified, post-SPE procedure, with the analytes at the same concentration level (Fig. 4D). By following the above described experimental setup, the recovery assumed as the method accuracy was assessed; as it can be seen, in Supplementary Material Table SM4, recovery estimated at three spiking levels (0.3, 0.7 and 1.0 mg/kg) ranged within 77.9 – 114 %, with RSD% (n = 3) lower than 6.5%. The obtained values are comparable to those reported in similar studies (Table 1). As an example, by means of LC-MS/MS for analysis of extracts from soybean upon SPE, the recovery at the level of 0.1 – 2.0 mg/kg was found to be 89-107% for glyphosate and 57-111% for AMPA [25]. Nagatomi *et al.*, in analysis of malt using a double SPE treatment and direct LC-MS/MS analysis, reported recovery values of 96.3% and 72.2% for glyphosate and AMPA, respectively [27]. In conclusion the proposed method, involving standard derivatization and conventional UV detection showed to be adequate for the intended analytical purposes.

3.5. Analysis of real samples

The validated analytical method was applied to real samples represented by commercially available wheat flour (four samples from different manufacturers) and a sample constituted of grains collected from plants treated with glyphosate in pre-harvest. The results of determination on the commercially available samples revealed that both glyphosate and AMPA were not contained at the detection limit

of the method. The content of glyphosate in the experimental sample was found to be higher than the upper limit established in linearity assessment. Upon proper dilution of the eluted solution from SPE, the application of the method allowed to quantify glyphosate at the level of 243 mg/kg (RSD%, n = 3, 5.6%), whereas AMPA was not detected.

Conclusions

Quantitation of glyphosate and AMPA in wheat flour samples was approached using capillary electrophoresis by a method involving a strategy based on: (i) application of an optimized sample cleanup consisting in a double SPE (C18 and SAX) of the aqueous extracts from wheat flour; (ii) derivatization using FMOC-Cl and (iii) FASI sweep-MEKC. The method was validated showing to be suitable for the analysis of residual levels of the analytes achieving limit of quantitation widely lower than the MRLs established by the Authorities. Owing to the fluorescence properties of FMOC tag, the method could be applied by using CE with LIF detection for a further sensitivity improvement addressed to assess the distribution of glyphosate in different tissues of wheat plants.

Acknowledgments

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Legend of Figures

Glyphosate FASI sweep-MEKC, Gotti et al.

Figure 1

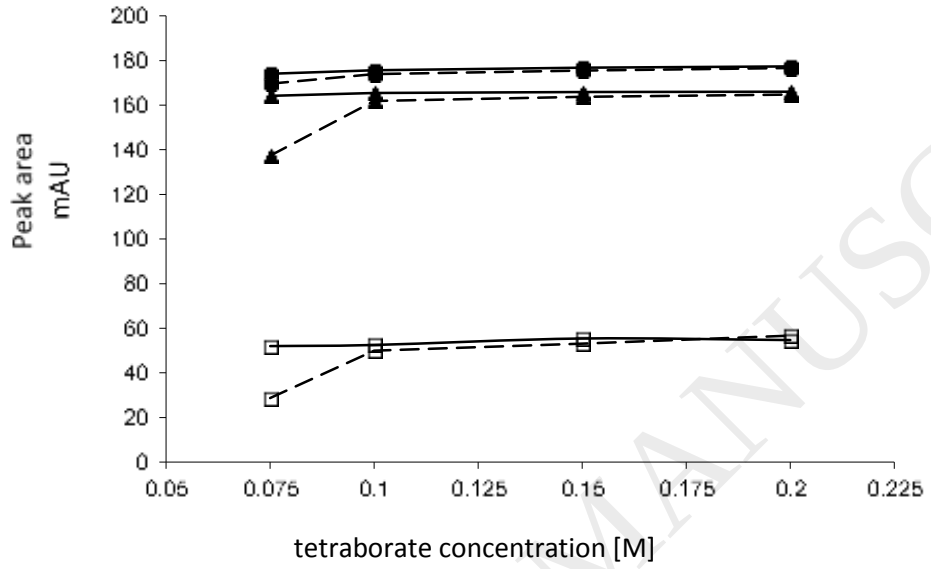

Figure 1. Evolution of the HPLC-UV peak area for FMOC-derivatives of glyphosate (□), AMPA (▲) and taurine (●). The solid lines are related to aqueous solution of the analytes; the dotted lines are related to hydrochloric acid solution 0.01 M of the analytes. Concentration of each of the analytes is about 2.5 μg/mL. The reported values are the mean of three determination (RSD% < 2.2%).

Figure 2

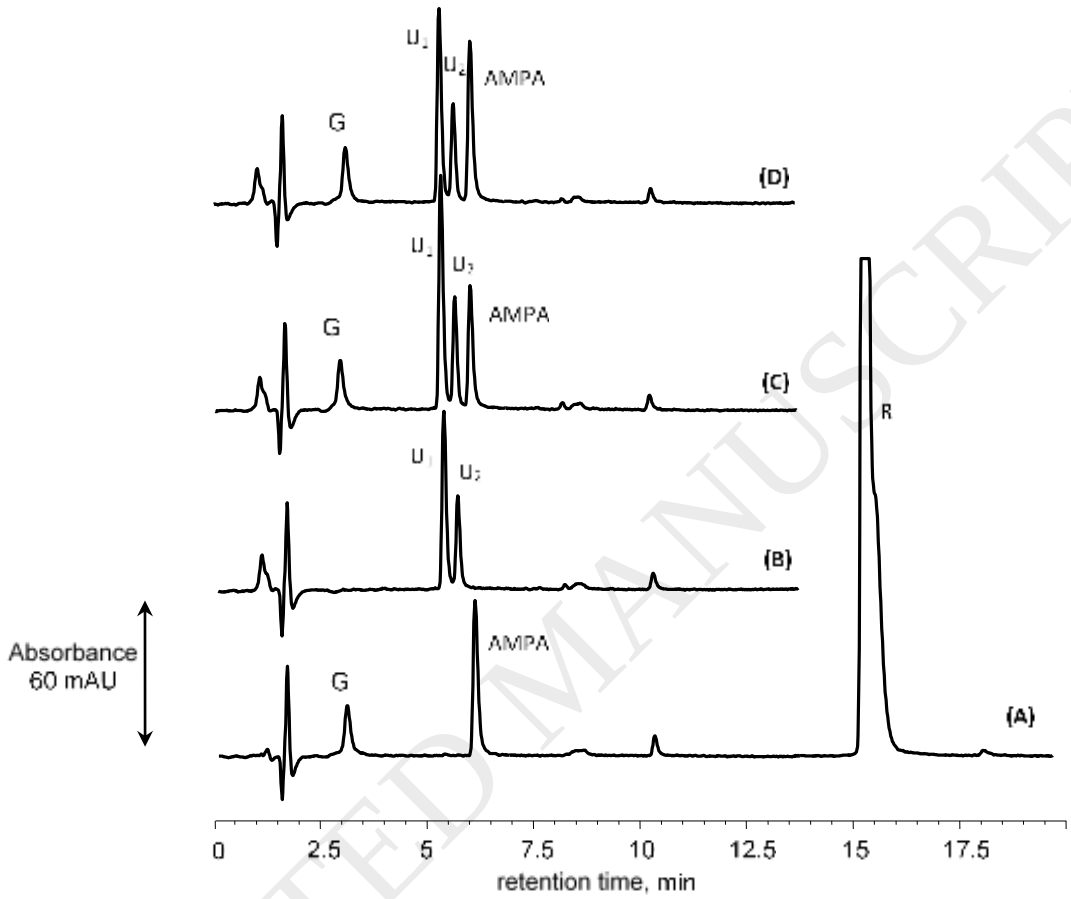

Figure 2. HPLC-UV chromatograms of: A) standard solution of glyphosate (G) and AMPA (2.5 μg/mL); B) blank sample (wheat flour subjected to the extraction and SPE procedure); C) spiked sample containing the same level of the analytes as in the standard solution; D) blank sample fortified post-SPE procedure, with glyphosate and AMPA at the same concentration level of standard solution. Symbols: U_1 and U_2 are for unknown compounds; the peak of reagent excess (R) is out of scale and it has been reported only in chromatogram (A). Chromatographic conditions are described in *Experimental Section 2.5*.

Glyphosate FASI sweep-MEKC, Gotti et al.

Figure 3.

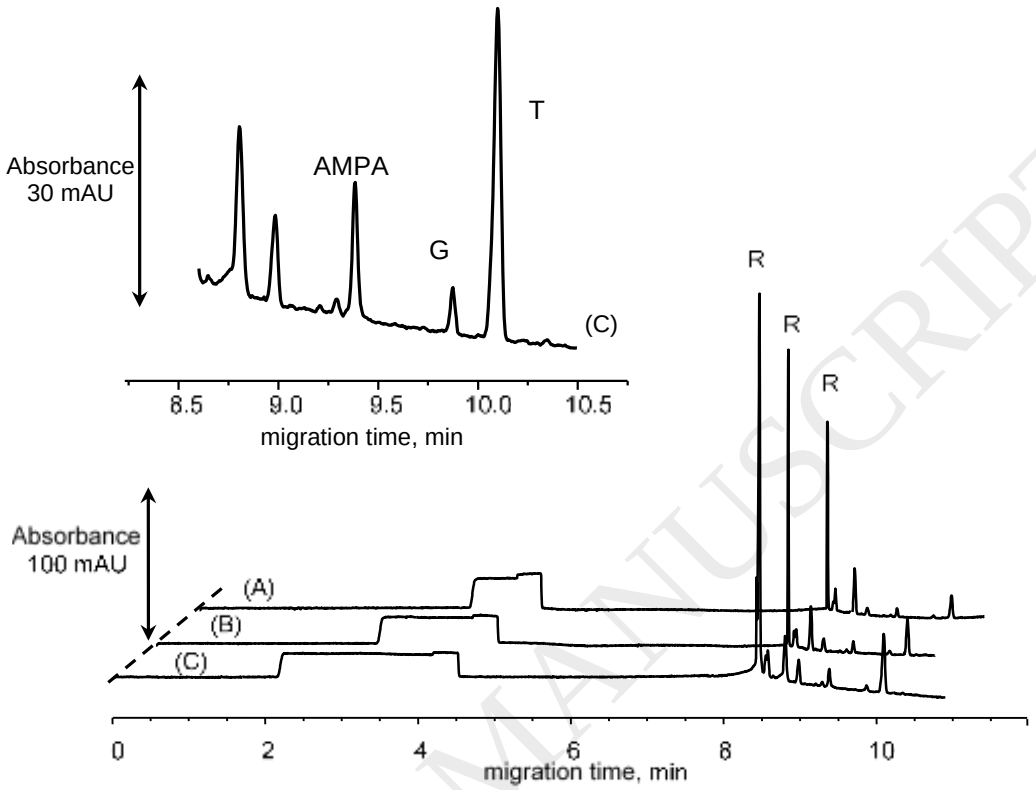

Figure 3. Electrokinetic chromatograms of standard solutions of glyphosate (G), AMPA and taurine (T, the internal standard) derivatized with FMOC-Cl. Conditions: HCB sodium phosphate buffer 100 mM, pH 2.2; BGE sodium phosphate buffer 50 mM, pH 2.2 supplemented with SDS, 100 mM. Electrokinetic injection at −10 kV for (A) 300 s, (B) 500 s and (C) 700 s. Other conditions: fused-

silica capillary 80 cm total length, (50 μm i.d., extended path); voltage –25 kV; temperature 25°C; detection at 210 nm. Sample concentration about 0.015-0.02 μg/mL. The inset reports the magnification of the electrokinetic chromatogram C (optimum conditions) in the timeframe related to the migration of the analytes. Symbols as in Figure 2; T is taurine, the internal standard.

Glyphosate FASI sweep-MEKC, Gotti et al.

Figure 4

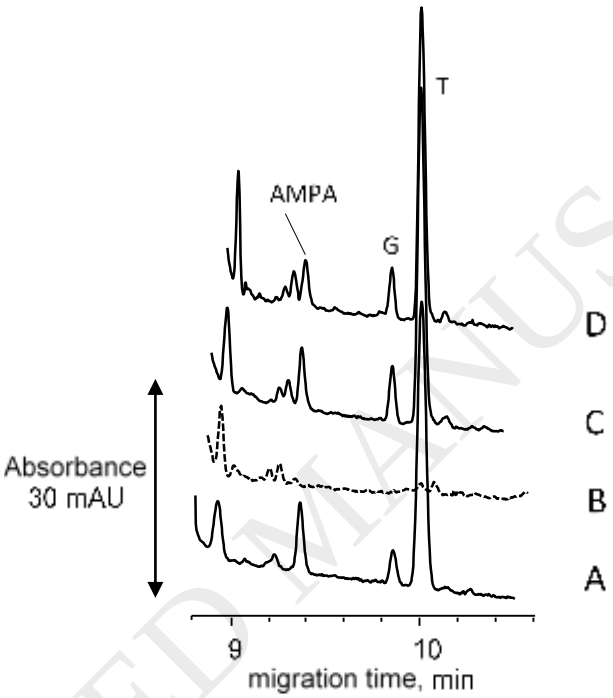

Figure 4. Electrokinetic chromatograms by FASI sweep-MEKC of: A) standard solution of AMPA and glyphosate (0.015 μg/mL); B) blank sample (wheat flour subjected to the extraction and SPE procedure); C) spiked sample containing the same level of the analytes as in the standard solution; D) blank sample fortified post-SPE procedure, with AMPA and glyphosate at the same concentration level of standard solution. Symbols as in Figure 2; T is taurine, the internal standard.

Table 1. Main characteristics of the present FASI sweep-MEKC and HPLC-UV methods compared to published methods applied in analysis of glyphosate in food and similar samples.

Technique	Sample	AMPA	Sample prep	Derivatization	LOQ (or LOD)[1]	Recovery %	ref
LC-MS	Nutritional ingredients	yes	SLE[2]	FMOC-Cl[3]	0.05 μg/g	94.3% (G)[4] 89.7% (A)[5]	14
LC-MS	Food products	no	SLE	FMOC-Cl	5 μg/kg	91-114%	15
LC-MS	Plant matrices	yes	SLE, SPE	FMOC-Cl	0.5 ng/g	80-120%	17
LC-MS	*Coffea arabica* leaves	yes	SLE, SPE	FMOC-Cl	41 μg/kg (G) 111 μg/kg (A)	80-107%(G) 93-97% (A)	21
LC-MS GC-MS	Breast milk	no	UF[6] LLE[7], SPE	 TFAA HFB[8]	1 ng/mL (both methods)	97-110% 54-70%	23
LC-MS	Soybean; corn	yes	SLE, SPE	no	14 ng/g (G) (LOD) 18 ng/g (A)	100-107 (G) 96-108 (A)	25
LC-MS	Plant-derived food	no	SLE, SPE	no	0.016-0.026 mg/kg	83.1-100.8%	26
LC-MS	Beer; barley tea; malt; corn	yes	SLE, SPE	no	10 μg/kg	89.2-97.5% (G) 72.2-103.8% (A)	27
CE-ECL[9]	Guava fruit	yes	SLE, SPEnp[10]	no	0.01μg/g (G) (LOD)	46% (G) 6.4% (A)	30
LC-MS	Cereals	no	SLE	no	0.02 mg/kg (LOD)	77-93%	35
CE-UV (sweep-MEKC)	Beer	yes	SECS[11]	no	20.0 ng/mL (A) 10.0 ng/mL (G)	100.5-115.1 (A) 91.0-109.3 (G)	44
Microchip CE-LIF[12]	Soybean, broccoli	no	SLE	FITC[13]	0.17 μg/L[14]	84-101%	45
CE-MS	Wheat	yes	SLE	no	2.5μM (G) (LOD)[15]	nr	46
CE-ECL	Soybean	yes	SLE	no	0.6 μg/g (G) (LOD)	92.7% (G)	47
CE-UV (sweep-MEKC) HPLC-UV	Wheat	yes	SLE, SPE	FMOC-Cl	5 ng/mL; 0.1 mg/kg (G)[16] 2.5 ng/mL; 0.05 mg/kg (A) 300 ng/mL; 12 mg/kg (G) 100 ng/mL; 4 mg/kg (A)	108.2-114.0% (G) 77.9-86.8% (A) 105.2% (G) 85.5% (A)	present study

1. LOQ if not indicated
2. SLE: Solid-Liquid extraction (using water or other solvents)
3. FMOC-Cl: 9-fluorenylmethylchloroformate
4. G: Glyphosate

5. A: AMPA
6. UF: Ultrafiltration (molecular weight cut-off filter 30 kDa)
7. LLE: Liquid-Liquid extraction
8. Derivatization for GC-MS analysis with trifluoroacetic acid anhydride and heptafluorobutanol
9. ECL: Electrochemiluminescence
10. SPEnp: SPE using magnetic nanoparticles based on $Fe_3O_4@Al_2O_3$
11. SECS: simultaneous electrophoretic concentration and separation
12. LIF: Laser induced fluorescence
13. FITC: fluorescein isothiocyanate
14. LOQ calculated on standard solution (LOQ values on matrix was not reported)
15. According to the reported sample preparation, LOD is 0.8 mg/kg
16. LOQ data are referred to both standard solution (ng/mL) and content in real samples (mg/kg)
nr: not reported