Quantitative analysis of glyphosate, glufosinate and AMPA in irrigation water by *in situ* derivatization - dispersive liquid-liquid microextraction combined with UPLC-MS/MS

Edgar Pinto[1,2],
António Gomes Soares[3],
Isabel M.P.L.V.O. Ferreira[1]

[1] LAQV/REQUIMTE, Department of Chemical Sciences, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Porto, Portugal

[2] Department of Environmental Health, Polytechnic Institute of Porto, Escola Superior de Saúde, CISA/Research Center in Environment and Health, R. Dr. António Bernardino de Almeida 400, 4200-072, Porto, Portugal

[3] Embrapa Food Technology, Rio de Janeiro-RJ, Guaratiba, Brazil

Abstract

A novel method was developed for the sensitive, cheap and fast quantitation of glyphosate, glufosinate and aminomethylphosphonic acid (AMPA) in irrigation water by in-situ derivatization and dispersive liquid-liquid extraction (DLLME) combined with ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Water samples were filtered with a 0.22 μm nylon filter, pH adjusted to 9 with ammonium bicarbonate and derivatized with fluorenylmethyloxycarbonyl chloride (FMOC-Cl). Afterwards, DLLME was applied to concentrate the compounds of interest, which were then analyzed by UPLC-MS/MS. The best results were obtained when acetone and dichloromethane were used as dispersive and extraction solvents, respectively. Two-level full factorial designs and a central composite design were applied to select the most appropriate derivatization and DLLME conditions. The method performance was evaluated according to the SANTE/11945/2015 guidelines and was linear in the 1.0 to 200 μg/L range for glyphosate, glufosinate and AMPA, with $r^2 \geq 0.997$ and individual residuals <13%. Repeatability (RSD_r) and within-laboratory reproducibility (RSD_{wr}) ranged from 2.7 to 9.1% and from 3.4 to 14.3%, respectively, and the trueness between 94.9 and 118.1%. The limits of detection were of 0.35, 0.05 and 0.10 μg/L for glyphosate, glufosinate and AMPA, respectively and the limits of quantitation were of 1.0 μg/L for all three compounds. The developed method was successfully applied to the analysis of irrigation water (surface and groundwater). No sign of the three compounds was detect in the groundwater samples but glyphosate was quantified in surface waters.

Keywords – Glyphosate; AMPA; glufosinate; DLLME; UPLC-MS/MS; derivatization

1. Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum, post-emergence, non-selective and systemic herbicide, which is widely used for weed control due to its effectiveness against broadleaf plants. In 2014, the global agricultural and non-agricultural use of glyphosate was of more than 800.000 tonnes, placing it as the world's best-selling pesticide [1]. Glyphosate-based formulations are known to have a broad-spectrum herbicidal activity, which greatly simplifies weed control management. Glyphosate acts as an enzyme inhibitor, affecting the synthesis of aromatic amino acids by blocking the shikimic acid pathway [2]. Once applied to cultures, glyphosate can be taken up by plants or adsorbed to soil particles [3]. If taken by plants, glyphosate is very little metabolized to its main metabolite, aminomethylphosphonic acid (AMPA). Yet, in soil or water, glyphosate undergoes rapid conversion to AMPA [4]. Glufosinate is an organophosphorus herbicide that is also widely used in weed control. It affects the nitrogen metabolism of plants by inhibiting glutamine synthetase, which catalyzes the condensation of glutamate with ammonia to yield glutamine [5]. Glyphosate is highly soluble in water and its ability to bind to mineral components makes it persistent in the environment. Due to its low mobility in soil, it is not likely to found glyphosate in groundwater, but it can contaminate surface waters by soil erosion and runoffs or even by its direct use on fields near aquatic environments [4, 6]. Moreover, glyphosate is chemically stable in water and is not subject to photochemical degradation [7]. Glufosinate is also hydrolytically stable in typical environmental conditions and it is not degraded by photolysis in water. Although few studies exist on the potential of glufosinate to leach, it seems that glufosinate is highly mobile in soil and has the potential to contaminate groundwater [5].

Glyphosate was recently classified as "probably carcinogenic to humans" (Group 2A) by the International Agency for Research on Cancer. The volume 112 of the IARC Monographs

states that “there is a strong evidence that exposure to glyphosate or glyphosate-based formulations is genotoxic based on studies in humans *in vitro* and studies in experimental animals” and that “there is a strong evidence that glyphosate, glyphosate-based formulations and aminomethylphosphonic acid can act to induce oxidative stress” [8]. Glufosinate has been classified as a “safer” herbicide. The European Food Safety Authority (EFSA) evaluated the information available in the literature about glufosinate and concluded that “there was no evidence of genotoxicity or carcinogenicity” and that “there was no indication of delayed neurotoxicity”. However, glufosinate was shown to induce pre- and post-implantation losses, vaginal bleedings, abortions and dead fetuses in rats and thus it was classified as “Possible risk of impaired fertility”. Because of that, an acute reference dose (ARfD) of 0.045 mg/kg bw/day was set for the general population [4].

The determination of glyphosate, AMPA and glufosinate at a low ppb level is considered challenging because of their unique physicochemical properties (high polar nature, amphoteric, low volatility, small molecule size, absence of chromophores or fluorophores, etc.). Usually, two analytical approaches can be applied for the determination of polar pesticides: direct analysis and derivatization. Direct analysis is usually more straightforward as they do not require a time-consuming derivatization step. However, direct analysis is only achievable by ion chromatography (IC)[9], hydrophilic interaction chromatography (HILIC)[10] or mixed-mode hydrophilic interaction/weak anion-exchange columns[11] coupled to mass spectrometry. These methods rely on specific chromatographic columns or on complex and expensive instrumentation, which makes the analysis of these compounds very difficult to perform. Derivatization offers the possibility of improving the chromatographic behavior of these polar pesticides under a conventional reverse-phase separation and can also enables the detection of these compounds by UV or fluorescence, thus offering cost-effective solutions. A careful literature review shows that 9-fluorenylmethyl chloroformate (FMOC) is the most

widely used derivatizing reagent for glyphosate analysis [12-14]. In fact, FMOC has been successfully used in the derivatization of primary and secondary amines in many fields of analytical chemistry [15]. However, FMOC derivatization has a major drawback when the analysis is to be performed on MS systems, which is the use of non-volatile buffers when adjusting the sample pH to carry out the derivatization reaction. In fact, most works devoted to glyphosate analysis by FMOC derivatization coupled to LC-MS analysis have used borate buffer in the derivatization step [12, 13, 16], yet none have addressed the limitations of using this non-volatile buffer in this type of analysis. It is well-known that the use of non-volatile buffers in LC-MS systems is related to poor signal stability and salt deposits in specific parts of the LC-MS instrument such as the sample cone, making impossible the analysis of series of several samples and undermining the outstanding detection and quantitation capabilities of MS systems. In addition to this limitation, most works published until now rely on the use of solid-phase extraction (SPE) or large volume injection after FMOC derivatization in order to improve the overall method sensitivity, which allows the detection of glyphosate and other polar pesticides at the level of μg or ng/L [14, 16]. However, these strategies make the analysis more expensive and time-consuming. Dispersive liquid-liquid extraction (DLLME) has now been extensively used in the determination of different types of pesticides in both environmental and food matrices. Compared to other extraction techniques, DLLME offers several advantages such as low cost, easy-to-use and quickness [17, 18].

The aim of this study was to develop and optimize a novel, inexpensive, fast and easy-to-use method based on *in situ* derivatization and dispersive liquid-liquid microextraction combined with UPLC-MS/MS for the sensitive determination of glyphosate, glufosinate and AMPA in irrigation water.

2. Materials and Methods

2.1. Chemicals and standard solutions

Glyphosate (Pestanal®), Glyphosate-2-^{13}C, aminomethylphosphonic acid (AMPA, 99%), glufosinate-ammonium (Pestanal®), fluorenylmethyloxycarbonyl chloride (FMOC-Cl, ≥99%), ammonium bicarbonate and ammonium hydroxide solution (28-30%) were purchased from Sigma (St. Louis, MO). Acetonitrile and methanol (both LC-MS grade) were purchased from J.T. Baker (Phillipsburg, NJ). Acetone, formic acid (98-100%), hydrochloric acid (HCl, 37%, v/v), dichloromethane, chloroform, tetrachloroethylene and trichloroethylene were obtained from Tedia (Rio de Janeiro, Brazil). Ultrapure water was obtained from a Milli-Q® Gradient A10 system (Merck Millipore, USA).

Only plastic ware was used in all analytical procedure. Standard stock solutions of glyphosate, glufosinate and AMPA were prepared by dissolving each compound in ultrapure water at a final concentration of 1000 mg/L. Working standard solutions were prepared daily from the stock solutions by adequate dilution with water. A stock solution of glyphosate (2-^{13}C) was prepared at ca. 1000 µg/mL in ultrapure water. A mixed intermediate internal standard (IS) stock solution containing 1 mg/L of glyphosate (2-^{13}C) was prepared in ultrapure water. Mixed calibration standard solutions of glyphosate, glufosinate and AMPA were prepared daily in the range 1.0−200 µg/L in ultrapure water. The ammonium bicarbonate buffer (0.1M, pH 9) was prepared by dissolving 3.95±0.01 g of ammonium bicarbonate in 500 mL of ultrapure water and adjusting the pH to 9.0±0.02 with an ammonium hydroxide solution (0.1M, v/v). After that, the volume was made up with ultrapure water to 1000 mL. A 6M (v/v) HCl solution was prepared by transferring 49.3 mL of HCl (37%, w/w) to a volumetric flask and the volume made up with ultrapure water to 100 mL. The derivatizing reagent FMOC-Cl was prepared in acetone at a concentration of 5.5 mg/mL.

2.2. Instrumentation and chromatographic conditions

Analysis was performed on an ACQUITY ultra-performance liquid chromatography system (UPLC^{TM}) coupled to a Xevo TQD triple quadrupole mass spectrometer (Waters, Milford, MA). Chromatographic separation was achieved using an ACQUITY UPLC BEH C_{18} (1.7 μm, 2.1 × 50 mm) column (Waters, Milford, MA), operated at 40 °C at a flow rate of 0.4 mL/min with a mobile phase system consisting of solvent (A) water:acetonitrile (95:5, v/v) containing formic acid (1%, v/v), and solvent (B) water:acetonitrile (5:95, v/v) containing formic acid (1%, v/v). The solvent gradient program was as follow: (1) 0−0.5 min, 0% B; (2) 0.5−5 min, 100% B; (3) 5−6 min, 100% B; (4) 6−6.5 min, 0% B. After reaching the initial conditions, the column was re-equilibrated for 4 min before the next injection. The total run time was 10 min and the injection volume was 10 μL.

The mass spectrometer was equipped with orthogonal Z-spray-electrospray interface operating in positive ion mode. The optimized MS parameters were as follows: capillary voltage, 3.00 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; desolvation gas flow, 800 L/h; and cone gas, 50 L/h. High purity nitrogen (>99.999%) and argon (>99.999%) were used as the cone and collision gases, respectively. Two MRM transitions (quantitation and confirmation) were selected and optimized for glyphosate, glufosinate, AMPA and glyphosate (2-^{13}C). MRM transition, cone voltages and collision energies for each compound are listed in supplementary material (Table S1). Data acquisition was performed by the MassLynx V4.1 software.

2.3. Derivatization and dispersive liquid-liquid microextraction (DLLME)

A 2.5 mL volume of standard solution or sample was mixed with 2.5 mL of bicarbonate buffer (0.1M, pH 9) and 50 μL of IS (1 mg/L) in a 15 mL centrifuge tube. After that, 650 μL

of FMOC-Cl (5.5 mg/mL in acetone) was added to the previous solution, which was subjected to vortexing for 30 s and allowed to react for 10 min at room temperature. Afterwards, 100 µL of 6M HCl was added to stop the derivatization reaction. The derivatized pesticides were then extracted by DLLME by adding 700 mg of NaCl and 230 µL of dichloromethane (extraction solvent) to form a cloudy solution, which was hand-shaken for 20s and vortexed for 30s. The previous solution was then centrifuged for 5 min at 5000 rpm and the sedimented phase was aspirated using a 250 uL Hamilton syringe (Hamilton Bonaduz AG, Switzerland) and transferred into a 1.5 mL Eppendorf tube. The sedimented phase was then evaporated in a gentle stream of N_2 and reconstituted in 200 µL of water:acetonitrile (90:10, v/v). Finally, 10 µL was injected into the UPLC-MS/MS system for analysis.

2.4. Experimental design

Several trials were conducted to optimize the derivatization reaction and the DLLME conditions for the quantitative analysis of glyphosate, glufosinate and AMPA in water. Two types of experimental designs were used in this work: (i) a two-level (2^k) factorial design that was aimed to screen the main variables affecting both the derivatization reaction and the DLLME procedure and (ii) a central composite design (CCD) that was used to optimized the significant variables selected from the previous experimental design. The response was the sum of all pesticides peak areas. Two different full factorial designs were created at low (−1) and high (+1) levels with five central points. The first full factorial design was created to evaluate the significance of five variables involved in the derivatization, i.e., FMOC-Cl concentration (0.5-10 mg/mL), FMOC-Cl volume (400-800 µL), derivatization time (5-55 min), derivatization temperature (25-75 ºC) and pH (9-10). After fixing the most suitable derivatization conditions, the second factorial design was carried out, which was aimed to evaluate the significance of three variables involved in the DLLME, i.e. dispersive solvent

volume (400-800 µL), extraction solvent volume (100-250 µL) and amount of NaCl (600-800 mg).

The significant variables FMOC-Cl concentration (X), dispersive solvent (Y) and extraction solvent (Z) were selected from the previous experimental designs and were further optimized by applying a CCD consisting of a complete 20-factorial design with six center points and two axial points on the axis of each design variable at a distance of α =1.682 from the design center. Experiments were carried out as follows: FMOC-Cl concentration (3−8 mg/mL), dispersive solvent volume (400−800 µL) and extraction solvent volume (100−250 µL). The complete experimental design (see the Supporting Information) was performed using the Design Expert Trial Version 10 (Stat-Ease, Inc., Minneapolis, MN) software. The appropriate fitting model for the response was selected based on the comparison of various statistical parameters such as R^2, Q^2, lack of fit and adequate precision. After the fitting of the mathematical model, the desirability function was studied for the optimization of independent variables for desirable responses.

2.7 Figures of merit

Validation of the proposed method was carried out according to European SANTE guideline 11945/2015 [19]. The analytical parameters evaluated during the validation process on surface and groundwater matrices were: specificity, linearity, LOD, LOQ, precision, trueness and matrix effect. To evaluate specificity, reagent blanks were analyzed to check for false positive results and for interfering compounds. To assess the method linearity, eight mixed standard solutions of glyphosate, glufosinate and AMPA at the concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 200 µg/L were subjected to the derivatization and DLLME procedures described above. Calibration curves were constructed by the least-squares linear regression model, using the ratio between the peak area of each pesticide and the peak area of IS. The limits of

detection (LODs) were calculated based on the three times the standard deviation of five consecutive blank injections divided by the slope of the calibration curve ($LOD = \frac{3 \times SD_{blank}}{slope\ of\ the\ calibration\ curve}$) and the limits of quantitation (LOQs) were calculated based on the lowest spike level for which the criteria for trueness (i.e. 70-120%) and precision (≤20%) was met. Precision was calculated using 15 determinations (i.e., three concentration levels in quintuplicate). The repeatability (RSD_r) was calculated from the results of five replicate analyses in a single day of standards at 1, 10 and 100 µg/L and the within-laboratory reproducibility (RSD_{wr}) was calculated from results obtained in four consecutive days. Trueness was calculated based on the analysis of spiked surface and groundwater at three different levels (1, 10, and 100 µg/L). For that, a 1 mL volume of mixed standard solutions (at 10, 100 and 1000 µg/L of each pesticide) was added to 10 mL volumetric flask and the volume was made-up with surface or groundwater. Trueness, on the basis of the recovery percentage R, % = [(concentration of the spiked sample − concentration of the unspiked sample)/added concentration] × 100, was estimated from quintuplicate experiments performed with two different ground and surface waters. Matrix effect was evaluated by comparing the response of solvent standards and matrix-matched standards at three concentration levels, i.e., 1, 10 and 100 µg/L.

3. Results and Discussion

3.1 Selection of dispersive and extraction solvents

A working standard solution containing glyphosate, glufosinate and AMPA each at 200 µg/L was used to study the most suitable dispersive and extraction solvents. Three dispersive solvents (methanol, acetonitrile and acetone) and four extraction solvents (chloroform, dichloromethane, trichloroethylene and tetrachloroethylene) were evaluated. The working standard solution was derivatized and subjected to the DLLME procedure. The analysis of

each solvent was carried out in triplicate. The use of acetone as a dispersive solvent and dichloromethane as the extraction solvent led to higher peak areas for all of the tested pesticides (Fig. 1), indicating that higher sensitivity is obtained with these solvents. Thus, they were selected for the following experiments.

3.2 Two-Level Full Factorial Design

The same 200 μg/L standard solution that was used to perform the selection of the dispersive and extraction solvents was used in the two-step experimental design (two-level full factorial design for screening and CCD for optimization). The main effects of the eight studied variables (FMOC-Cl concentration, FMOC-Cl volume, derivatization time, derivatization temperature, pH, dispersive solvent volume, extraction solvent volume and amount of NaCl) are presented in Fig. 2 in the form of a Pareto chart. The magnitude of the effects is highlighted in an ordered bar chart, where the bar length of the vertical axis is proportional to the significance of the variables. For the derivatization procedure, the significance of the variables FMOC-Cl concentration, FMOC-Cl volume, derivatization time, derivatization temperature and pH was evaluated. From those, the variables FMOC-Cl concentration and FMOC-Cl volume as well as their interaction were statistically significant ($p < 0.05$), with a positive effect on the response (Fig. 2A). Thus, these variables were selected for further assessment in the CCD. With regard to the extraction procedure, the significance of the variables dispersive solvent volume, extraction solvent volume and amount of NaCl was assessed. The variables dispersive solvent volume and extraction solvent volume as well as the interaction between them were statistically significant ($p < 0.05$) and, thus, were considered for optimization (Fig. 2B). Because the variables “FMOC-Cl volume” and “dispersive solvent volume” tested in the first and second full factorial designs, respectively, rely on the use of acetone, the two variables were designated throughout the optimization

procedure as just “dispersive solvent volume”. The variables derivatization time, derivatization temperature, pH and amount of NaCl showed no significant effect on the peak area of the analyzed pesticides and, thus, were fixed for further experiments at 10 min, room temperature, pH 9 and 700 mg, respectively. Faster reaction times without the need of heating were achieved without compromising the method sensitivity.

3.3 Central composite design (CCD)

For CCD, 20 experiments were carried out with the significant variables FMOC-Cl concentration (X), dispersive solvent volume (Y) and extraction solvent volume (Z) selected from the screening experiments. The sum of all pesticides peak areas as a function of X, Y and Z was used as a response for the CCD. The generated models were validated by two diagnostic residuals, the R^2 and Q^2. Typical values indicating good models are $R^2 > 0.75$ and $Q^2 > 0.60$ [20]. In this study, the values of R^2 predicted and R^2 adjusted were 0.97 and 0.99, respectively, and the Q^2 value was 0.98, which are indicative of the goodness of the model. Adequate precision, measured as a signal-to-noise ratio, was 44.9 (greater than 4 as desirable), indicating the adequacy of the present model. As shown in Table 1, the F value was 302.5, which indicates that the regression model is statistically significant ($p < 0.01$) at the 99% confidence level. The terms X, Y, Z, XY, YZ, X^2, Y^2, and Z^2 showed a p-value lower than 0.05, indicating the significance of the model terms. The lack-of-fit was 0.22, non-significant as desired, and the quadratic model was valid.

Three-dimensional surface and contour plots were drawn to investigate the interactive effect of two factors on the sum of pesticides peak areas (Fig. 3). Fig. 3A shows the combined effect of the variables dispersive solvent volume and FMOC-Cl concentration. Fig. 3B shows the combined effect of the variables extraction solvent volume and FMOC-Cl concentration,

while Fig. 3C shows the combined effect of the extraction solvent and dispersive solvent volumes.

To conclude the CCD optimization, the desirability indices were defined to maximize the response (i.e., the peak areas of the analyzed pesticides). The following optimum conditions were selected: 5.5 mg/mL FMOC-Cl, 650 μL of dispersive solvent, and 230 μL of dichloromethane. Confirmatory experiments were conducted with the selected parameters and the obtained response (7.41×10^4) was within the 95% prediction response interval (from 7.22×10^4 to 7.64×10^4), which indicates the adequacy of the obtained model.

3.4. Method performance

In order to evaluate the performance of the proposed method, its figures of merit were studied and are presented in Tables 2, 3 and 4. The chromatograms displayed in Fig. 4A and 4B show no indication of interfering compounds eluting near the retention times of glyphosate, glufosinate and AMPA, which proves the good selectivity of the proposed method. In fact, the use of tandem mass spectrometry provides a high degree of selectivity and specificity. With respect to the linearity of the method, the calibration curves for glyphosate, glufosinate and AMPA constructed between 1 and 200 μg/L with a weighting factor of $1/x^2$ showed a correlation coefficient (r^2) above 0.997, a non-significant lack of fit and individual residuals deviations <13%, which are all prove of the good linearity of the method. The LODs for glyphosate, glufosinate and AMPA were 0.35, 0.05 and 0.10 μg/L and the LOQs for all three compounds was of 1 μg/L (Table 2). A chromatogram of all three compounds at the LOQ level (i.e., 1 μg/L) is present in supplementary material (Fig. S2). The retention times (mean±SD) of glyphosate, AMPA and glufosinate were 3.21±0.02 min, 3.37±0.01 min and 3.47±0.01 min, respectively. The maximum deviation was of ±0.02 min, which is well below the maximum tolerance deviation stated in SANTE guidelines (±0.1 min). The repeatability

(RSD_r) and within-laboratory reproducibility (RSD_{wr}), expressed as percent relative standard deviation (% RSD), ranged from 2.7 to 9.1% and from 3.4 to 14.3%, respectively (Table 3). The RSD values obtained were below 15%, meeting the SANTE guideline of RSD ≤20%. The trueness of the method was evaluated using recovery studies with spiked surface and groundwater matrices at three levels. The mean recoveries calculated for glyphosate, glufosinate and AMPA are shown in Table 4 and are within the range required by the SANTE guideline (between 70% and 120%). In order to evaluate the matrix effect, the response of a spiked surface water at 1, 10 and 100 µg/L was compared with the corresponding solvent standards. Calibration curves were constructed with both solvent standards and matrix-matched standards and the slopes were compared using a Student's t-test. No statistical difference ($p > 0.05$) was observed between the slopes of the solvent standard curve and the matrix-matched standard curve for all the three compounds thus, no matrix effect was considered to be present.

Overall, this method has three important characteristics that stand it out from the rest: (**1**) it relies on the use of a volatile buffer (ammonium bicarbonate) in contrast with the other published methods that use non-volatile buffers such as borate [12, 13, 16]. The use of non-volatile buffers has a detrimental effect on the instrument response due to deposit buildup in several parts of the mass spectrometer, resulting in poor signal stability. Thus, the use of the volatile buffer ammonium bicarbonate in the present method greatly improved the analysis of these compounds by LC-MS at both short and long-term analysis; (**2**) quickness – the use of DLLME makes the method faster compared to the use of SPE that needs column conditioning and equilibration, sample loading, washing and, finally, the elution of the analytes of interest; all these steps together makes SPE more time-consuming than DLLME [11, 16]; and (**3**) similar LODs and LOQs with lower cost of analysis – the LODs and LOQs obtained by this method are similar to those obtained in other published works that rely on expensive SPE columns,

specialized instrumentation or large injections volumes. For example, Mallet (2014) developed an automated derivatization protocol for the analysis of glyphosate, glufosinate and AMPA in tap and surface waters that relied on FMOC derivatization and on-line SPE coupled to LC-MS/MS and obtained a LOQ of 1 µg/L for glyphosate, glufosinate and AMPA [21]. A similar work was performed by Ibanez et al. (2005), which used FMOC derivatization and on-line SPE with an OASIS HLB cartridge column prior to LC–MS/MS analysis, and a LOQ of 0.05 µg/L was obtained for the same pesticides [22]. This lower LOQ was obtained by Ibanez el al. because they have used a volume of 4.3 mL in the SPE procedure and Mallet only used 0.5 mL. With regard to other analytical techniques, Guo et al. (2007) report LODs of 1.2 and 1.3 µg/L for glyphosate and glufosinate, respectively, using large volume injection (500 µL) and IC-ICP-MS [9].

3.5. Analysis of real samples

To assess the applicability of the proposed method, glyphosate, glufosinate and AMPA were determined in irrigation waters (surface and groundwater). Eight samples (four surface and four groundwaters) were collected in different locations in plastic containers, stored at 4º C and analyzed in the next day. Irrigation water samples were pre-filtered with a 0.22 µm nylon filter with no further treatment and the derivatization and DLLME procedures were performed. No sign of glyphosate, glufosinate or AMPA was detected in the analyzed groundwaters. With regard to surface waters, glyphosate was quantified at 4.2, 2.6, 10.1 and 7.7 µg/L (Figure 4C) while glufosinate and AMPA were below the LOD.

4. Conclusion

The present method offers a cheap and fast quantitative analysis of glyphosate, glufosinate and AMPA in irrigation water. The method validation, performed according to the EU SANTE guidelines, demonstrates that the proposed method is accurate, precise and robust. The optimized conditions using *in situ* derivatization and DLLME followed by UPLC-MS/MS enabled the analysis of glyphosate, glufosinate and AMPA with LODs of 0.35, 0.05 and 0.10 µg/L, respectively, and LOQs of 1.0 µg/L for all three compounds. The use of the volatile buffer ammonium bicarbonate avoids the common problems of poor signal stability and salt deposits LC-MS instruments observed with the use of non-volatile buffers such as borate buffer. The developed method was successfully applied in the analysis of glyphosate, glufosinate and AMPA in surface and groundwater. No sign of glufosinate and AMPA was detected in the analyzed waters. Glyphosate was only detected in surface waters.

References

1. C. M. Benbrook, *Environmental Sciences Europe*, 2016, **28**, 3.
2. L. Pollegioni, E. Schonbrunn and D. Siehl, *Febs J*, 2011, **278**, 2753-2766.
3. M. Arias-Estévez, E. López-Periago, E. Martínez-Carballo, J. Simal-Gándara, J.-C. Mejuto and L. García-Río, *Agriculture, Ecosystems & Environment*, 2008, **123**, 247-260.
4. EFSA, *EFSA Journal*, 2015, **13**, 107 pp.
5. EFSA, *EFSA Scientific Report*, 2005, **27**, 1-81.
6. V. C. Aparicio, E. De Gerónimo, D. Marino, J. Primost, P. Carriquiriborde and J. L. Costa, *Chemosphere*, 2013, **93**, 1866-1873.
7. P. Mercurio, F. Flores, J. F. Mueller, S. Carter and A. P. Negri, *Mar Pollut Bull*, 2014, **85**, 385-390.
8. IARC, *IARC Monographs*, 2017, **112**, 464 pp.
9. Z.-X. Guo, Q. Cai and Z. Yang, *Rapid Communications in Mass Spectrometry*, 2007, **21**, 1606-1612.
10. P. K. Jensen, C. E. Wujcik, M. K. McGuire and M. A. McGuire, *J Environ Sci Heal B*, 2016, **51**, 254-259.
11. M. X. Chen, Z. Y. Cao, Y. Jiang and Z. W. Zhu, *J Chromatogr A*, 2013, **1272**, 90-99.
12. S. Ehling and T. M. Reddy, *J Agr Food Chem*, 2015, **63**, 10562-10568.
13. S. Goscinny, H. Unterluggauer, J. Aldrian, V. Hanot and S. Masselter, *Food Anal Method*, 2012, **5**, 1177-1185.
14. T. Arkan and I. Molnar-Perl, *Microchem J*, 2015, **121**, 99-106.
15. A. Jambor and I. Molnar-Perl, *J Chromatogr A*, 2009, **1216**, 3064-3077.
16. T. Poiger, I. J. Buerge, A. Bachli, M. D. Muller and M. E. Balmer, *Environ Sci Pollut R*, 2017, **24**, 1588-1596.
17. A. Melo, S. C. Cunha, C. Mansilha, A. Aguiar, O. Pinho and I. M. P. L. V. O. Ferreira, *Food Chemistry*, 2012, **135**, 1071-1077.
18. W. Ahmad, A. A. Al-Sibaai, A. S. Bashammakh, H. Alwael and M. S. El-Shahawi, *Trac-Trend Anal Chem*, 2015, **72**, 181-192.
19. SANTE, *EUROPEAN COMMISSION*, 2015, 34.
20. E. Pinto, A. Melo and I. M. P. L. V. O. Ferreira, *J Agr Food Chem*, 2014, **62**, 4276-4284.
21. Mallet, *Waters app note*, 2014, 6.
22. M. Ibáñez, Ó. J. Pozo, J. V. Sancho, F. J. López and F. Hernández, *J Chromatogr A*, 2005, **1081**, 145-155.

421 **Table 1**. Analysis of variance (ANOVA) for the response surface model for the sum of pesticides peak areas

Source	Sum of squares	Degrees of freedom	Mean square	F value	Probability > F	Remarks
Model	1.45×10^{10}	9	1.62×10^{9}	302.5	< 0.0001	significant
X – FMOC concentration	7.40×10^{8}	1	7.40×10^{8}	138.5	< 0.0001	significant
Y – Dispersive solvent	8.22×10^{8}	1	8.22×10^{8}	154.0	< 0.0001	significant
Z – Extraction solvent	5.82×10^{9}	1	5.82×10^{9}	1090.6	< 0.0001	significant
XY	1.04×10^{8}	1	1.04×10^{8}	19.5	0.0013	significant
XZ	2.14×10^{7}	1	2.14×10^{7}	4.00	0.0733	not significant
YZ	1.69×10^{8}	1	1.69×10^{8}	31.6	0.0002	significant
X^2	3.42×10^{9}	1	3.42×10^{9}	639.5	< 0.0001	significant
Y^2	2.68×10^{9}	1	2.68×10^{9}	501.9	< 0.0001	significant
Z^2	2.10×10^{9}	1	2.10×10^{9}	393.1	< 0.0001	significant
Residual	5.34×10^{7}	10	5.34×10^{6}			
Lack of Fit	9.51×10^{6}	5	1.90×10^{6}	0.22	0.9407	not significant
Pure Error	4.39×10^{7}	5	8.78×10^{6}			
Total	1.46×10^{10}	19				

422

423

424 **Table 2**. Linear dynamic range, determination coefficients (r^2), residuals, retention times, limits of detection (LOD) and limits of quantitation

425 (LOQ)

Compound	Linear dynamic range (µg/L)	r^2	Maximum individual residual (%)	Retention times (min)	LOD (µg/L)	LOQ (µg/L)
Glyphosate	1 – 200	0.998	11.3	3.21±0.02	0.35	1.0
Glufosinate	1 – 200	0.997	10.5	3.47±0.01	0.05	1.0
AMPA	1 – 200	0.997	12.8	3.37±0.01	0.10	1.0

426

427 **Table 3**. Repeatability (RSD_r) and within-laboratory reproducibility (RSD_{wr}) for peak areas

428 evaluated at three concentration levels[a]

Compound	RSDr (n = 5)			RSD_{wr} (n = 5 × 4 days)		
	1 µg/L	10 µg/L	100 µg/L	1 µg/L	10 µg/L	100 µg/L
Glyphosate	9.1	4.1	3.3	14.3	7.0	5.1
Glufosinate	6.7	4.7	2.7	8.6	6.7	3.4
AMPA	8.5	3.3	2.8	12.2	4.1	3.8

429 [a] data are presented as % RSD

430 **Table 4**. Trueness results for glyphosate, glufosinate and AMPA in irrigation water matrices.

Matrix	Spiking level (μg/L)	Glyphosate		Glufosinate		AMPA	
		Mean recoveries (%)	RSD (%)	Mean recoveries (%)	RSD (%)	Mean recoveries (%)	RSD (%)
Groundwater 1	1	109.2	7.3	94.9	4.3	96.1	5.4
	10	97.2	3.8	98.3	1.0	103.0	1.7
	100	102.2	4.3	101.5	1.9	109.2	2.8
Groundwater 2	1	107.1	5.7	102.3	5.2	97.7	6.0
	10	99.6	3.6	103.3	3.8	105.5	3.7
	100	105.6	3.0	104.1	2.4	109.3	5.7
Surface water 1	1	114.7	10.1	103.1	5.6	98.2	7.4
	10	110.5	8.6	97.5	1.6	102.4	2.3
	100	117.6	6.9	102.8	2.6	108.9	8.8
Surface water 2	1	118.1	12.3	103.2	7.5	103.6	9.1
	10	98.7	7.5	96.6	6.3	105.4	8.2
	100	112.3	9.1	103.2	3.6	113.6	4.4

Figures Caption

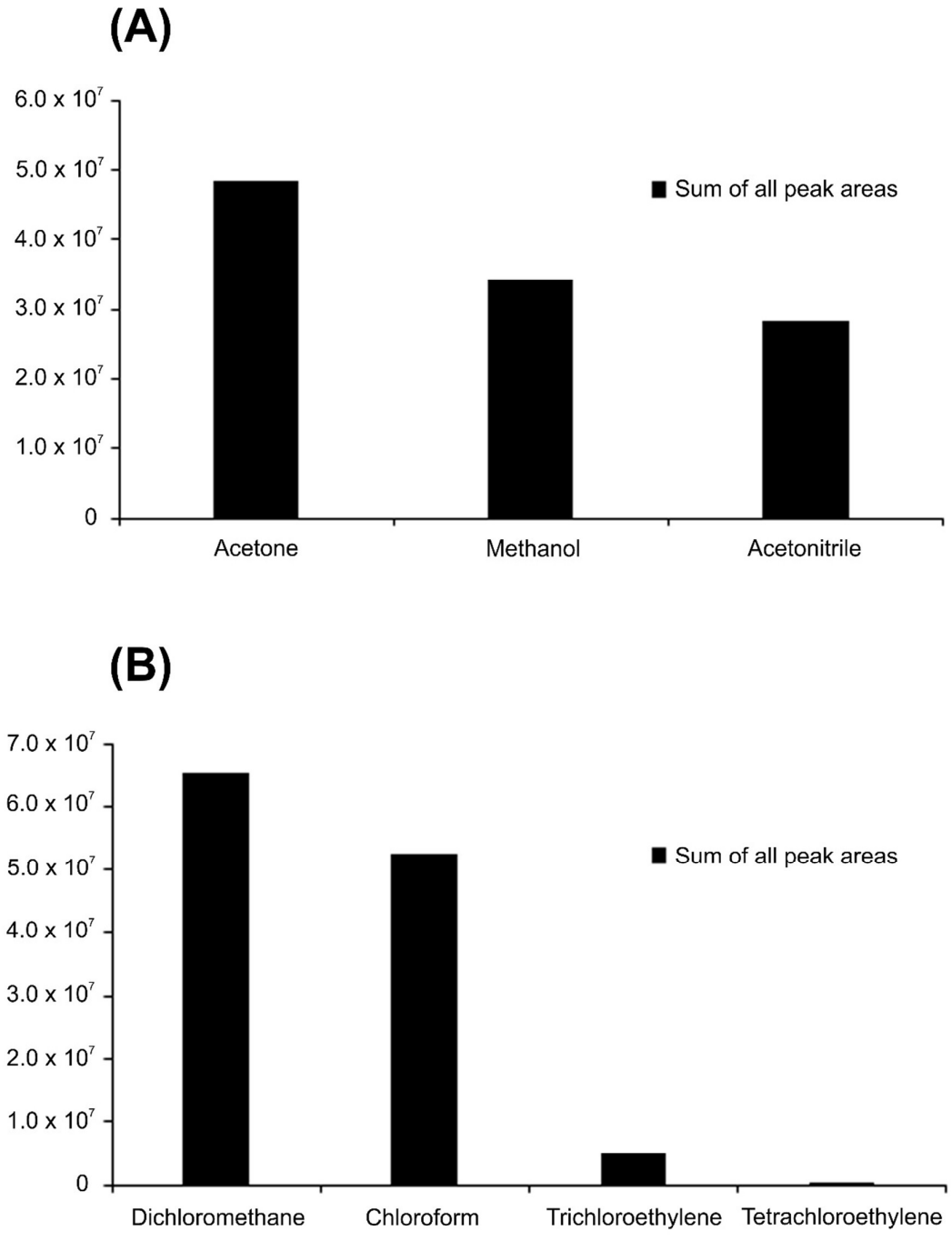
Fig. 1 Selection of (A) dispersive and (B) extraction solvents.

Fig. 2 Main effect Pareto chart for the two-level factorial design of the screening experiments of (A) derivatization and (B) extraction.

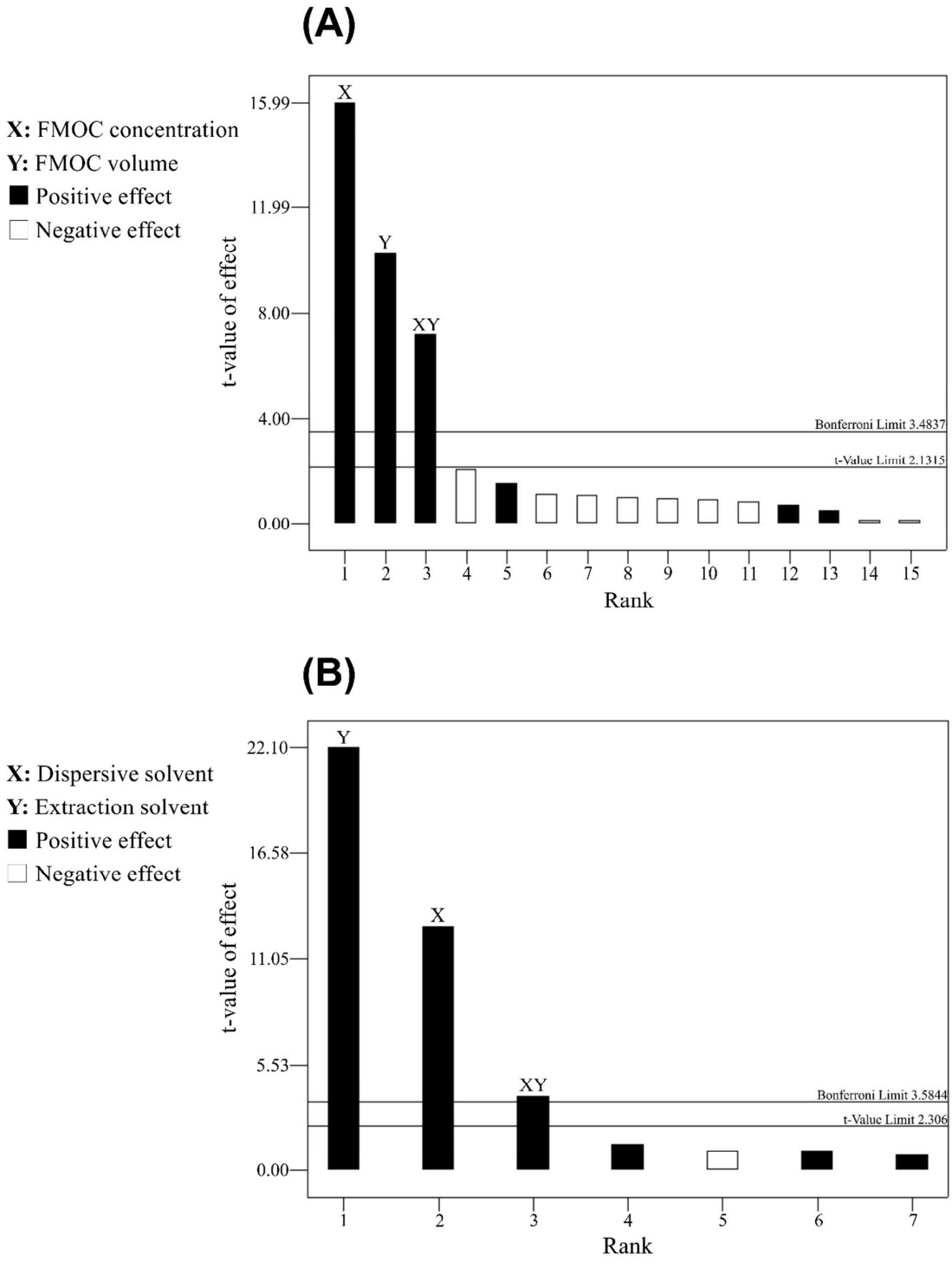
Fig. 3 Response surface plots for the CCD: (A) FMOC concentration *vs*. dispersive solvent volume, (B) FMOC concentration *vs*. extraction solvent volume, and (C) dispersive solvent volume *vs*. extraction solvent volume.

Fig. 4 Ion chromatogram of glyphosate, glufosinate and AMPA: (A) derivatized blank solution, (B) 10 µg/L standard solution; (C) surface waters

441 **Fig. 1**

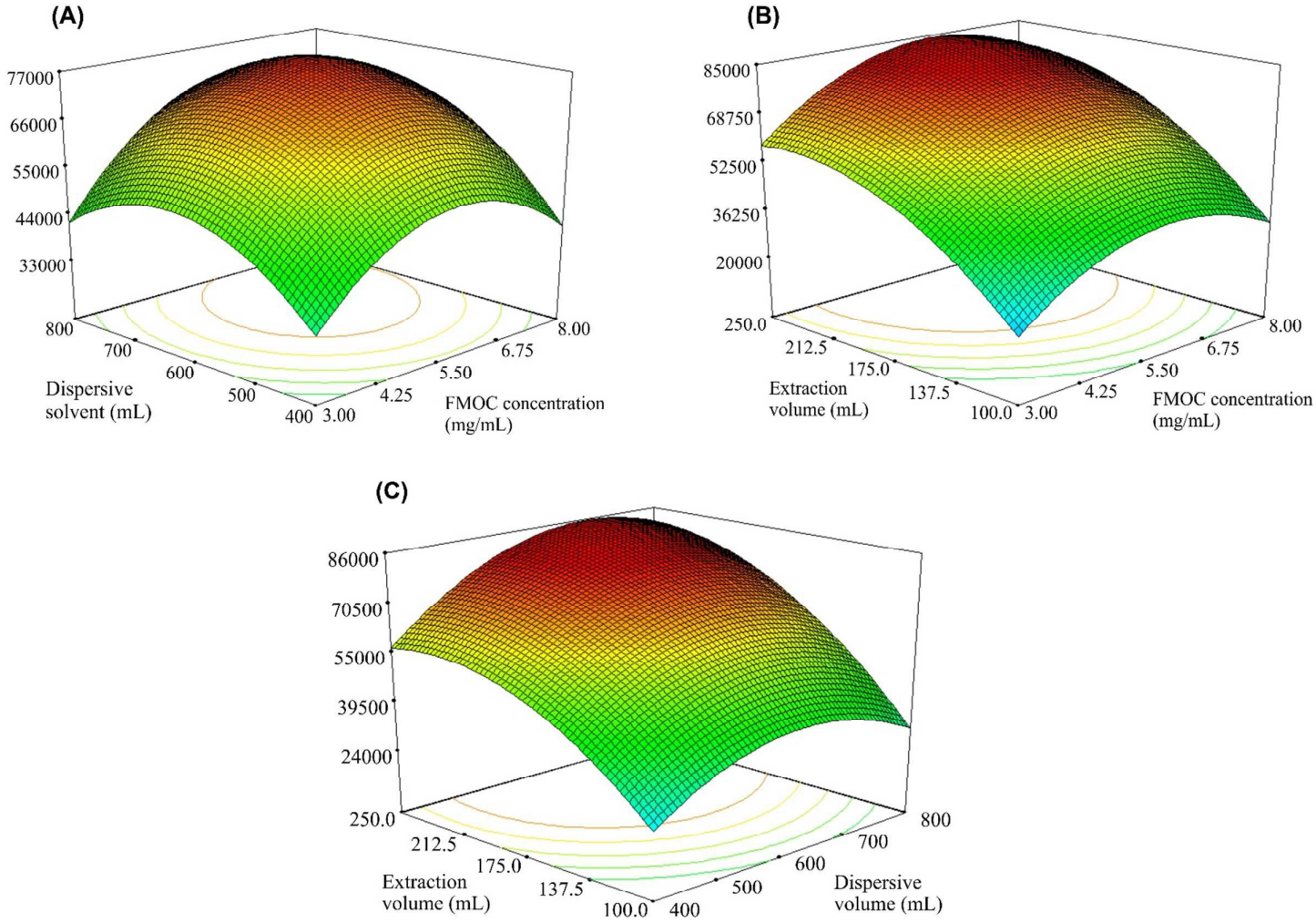

(A)
6.0 x 10^7
5.0 x 10^7
4.0 x 10^7
3.0 x 10^7
2.0 x 10^7
1.0 x 10^7
0
Sum of all peak areas
Acetone
Methanol
Acetonitrile
(B)
7.0 x 10^7
6.0 x 10^7
5.0 x 10^7
4.0 x 10^7
3.0 x 10^7
2.0 x 10^7
1.0 x 10^7
0
Sum of all peak areas
Dichloromethane
Chloroform
Trichloroethylene
Tetrachloroethylene

444 **Fig. 2**


(A)
X: FMOC concentration
Y: FMOC volume
Positive effect
Negative effect
t-value of effect
15.99
11.99
8.00
4.00
0.00
X
Y
XY
Bonferroni Limit 3.4837
t-Value Limit 2.1315
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Rank
(B)
X: Dispersive solvent
Y: Extraction solvent
Positive effect
Negative effect
t-value of effect
22.10
16.58
11.05
5.53
0.00
Y
X
XY
Bonferroni Limit 3.5844
t-Value Limit 2.306
1 2 3 4 5 6 7
Rank

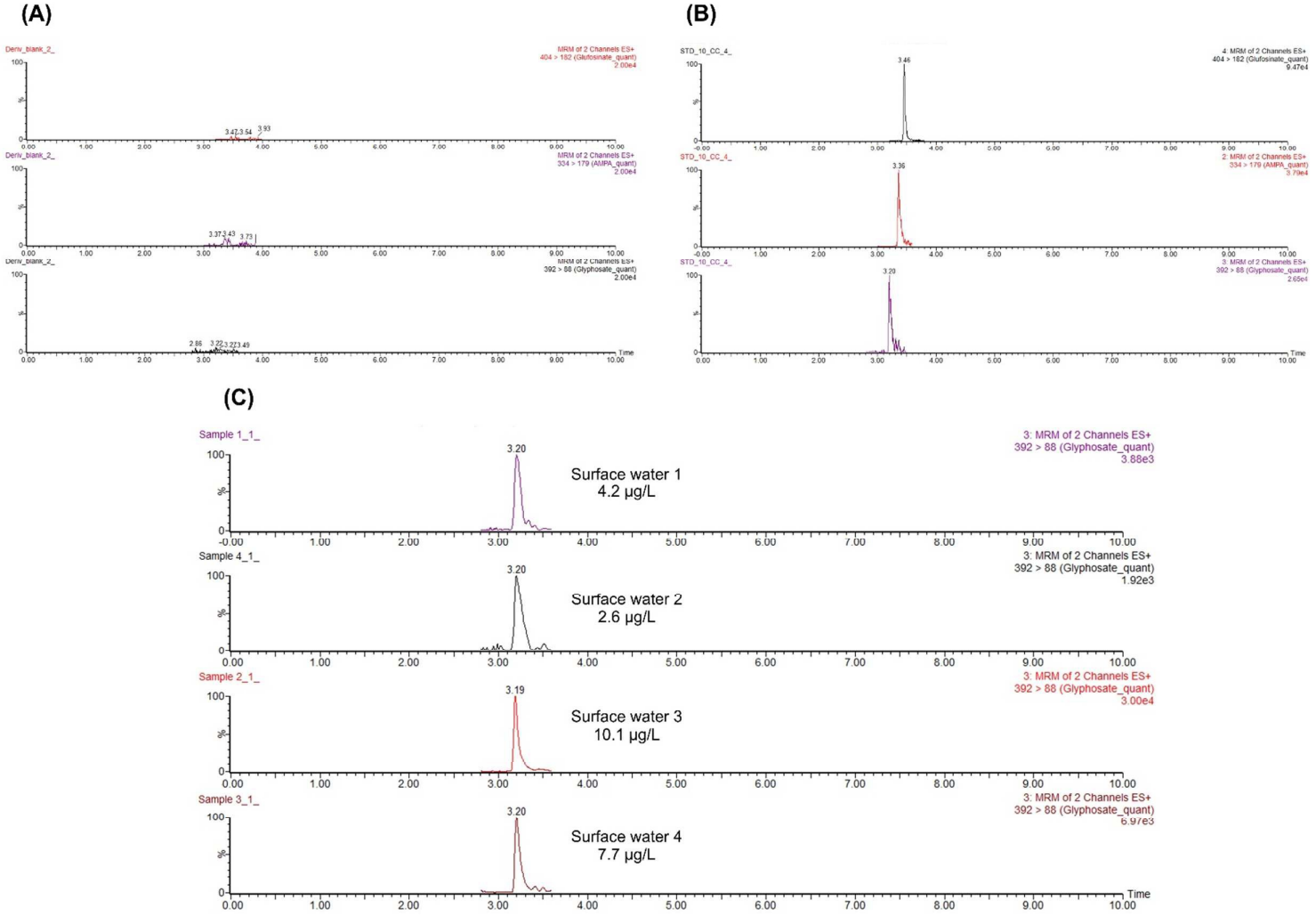
445

446 **Fig. 3**

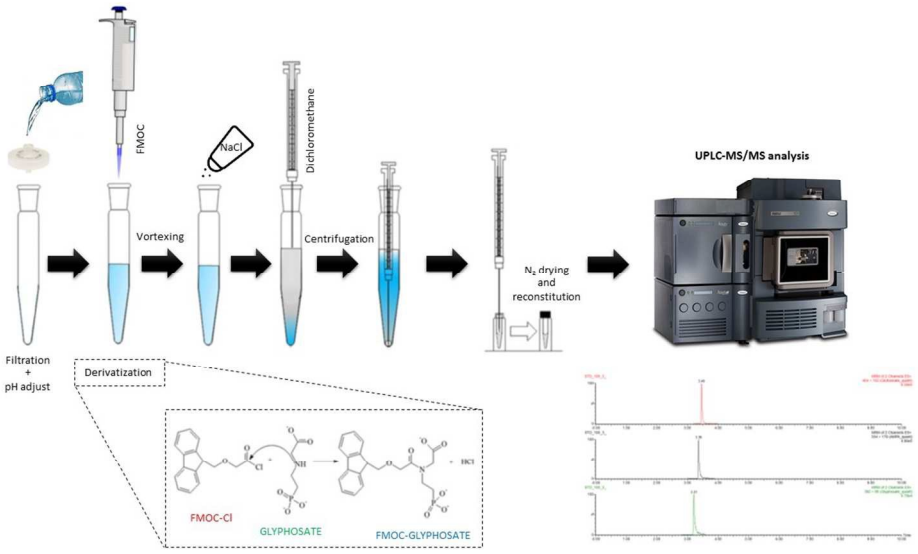

(A)
77000
66000
55000
44000
33000
800
700
600
500
400
Dispersive solvent (mL)
3.00
4.25
5.50
6.75
8.00
FMOC concentration (mg/mL)
(B)
85000
68750
52500
36250
20000
250.0
212.5
175.0
137.5
100.0
Extraction volume (mL)
3.00
4.25
5.50
6.75
8.00
FMOC concentration (mg/mL)
(C)
86000
70500
55000
39500
24000
250.0
212.5
175.0
137.5
100.0
Extraction volume (mL)
400
500
600
700
800
Dispersive volume (mL)

447

448 **Fig. 4**


(A)
(B)
(C)
Surface water 1
4.2 µg/L
Surface water 2
2.6 µg/L
Surface water 3
10.1 µg/L
Surface water 4
7.7 µg/L
Time

449



338x190mm (96 x 96 DPI)