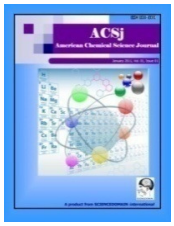

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A Sequential Injection Analysis Method for the Determination of Glyphosate and Aminomethylphosphonic Acid in Water Samples

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

A conventional laboratory method for the determination of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) is high performance liquid chromatography (HPLC) followed by post column derivatization with o-phthaldialdehyde (OPA). However, AMPA is partly decomposed in the process causing a deviation in the AMPA detection or even making a simultaneous detection of glyphosate and AMPA with the described postcolumn procedure impossible. We used a compact sequential injection analysis system and optimized the process conditions for both analytes independently from each other. The process conditions were adjusted to the different chemical characteristics of AMPA (primary amine) and glyphosate (secondary amine), which needs to be oxidized to a primary amine prior to the derivatization. An ion exchange column was included in the system to eliminate amines interfering with the method. Limits of detection of 16 and 9 µg L^{-1} for glyphosate and AMPA, respectively are similar to those achieved with HPLC methods reported in literature, but compared to conventional HPLC methods only small amounts of reagents are consumed.

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Keywords: Glyphosate; roundup; AMPA; sequential injection analysis; SIA; phosphororganic analysis.

1. INTRODUCTION

Glyphosate (N-(phosphonomethyl)glycine) is the active ingredient of one of the most widely applied herbicides, introduced by Monsanto (St. Louis, MO, USA) in the 70s under the name of Roundup. It is applied to control weeds in both agricultural and non-agricultural areas all over the world. Its use frequently involves cultivation of genetically modified crops ("roundup ready") that tolerate this herbicide. Due to the large quantities of glyphosate applied, there are increasing concerns about its impact on the environment. Studies showed that the commercial formulation of glyphosate poses a risk to fish populations [1,2] and acts as a potent endocrine disruptor in juvenile rats [3]. Stachowski-Haberkorn et al. [4] found that exposure to Roundup at a concentration of 1 µg L^{-1} affects natural coastal microbial communities. A recent study sponsored by the BUND (Friends of the Earth Germany) found glyphosate residues in human urine samples from 18 European countries thus showing to which extent the general public is exposed to glyphosate. Residues of this herbicide were found in 70% of the German urine samples [5].

Glyphosate is reported to leach into the water phase [6,7], where its degradation by microorganisms is significantly reduced, compared to the conditions in soil [8]. Its halflife in water ranges from 35 to 63 days [9]. Glyphosate is metabolized to aminomethyl-phosphonic acid (AMPA) through microbial and photolytic degradation. In order to evaluate the fate of glyphosate in the environment, both substances must be analyzed simultaneously. In Denmark, Norway, and the Netherlands, glyphosate and AMPA are frequently detected in streams and groundwater. Maximum values of 15 µg L^{-1} of glyphosate in Norwegian streams were reported in 2002 [10].

Several laboratory methods for the detection of glyphosate and AMPA have been developed. Mostly HPLC with fluorescence detection after pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) [11], or post column derivatization with o-phthaldialdehyde (OPA) [12], and HPLC-MS methods [13,14] have been proposed. GC-MS has been used for the analysis of both substances [15]. Electrochemical methods, e.g. capacitively coupled contactless conductivity detection [16] or coulometric detection at copper microelectrodes [17] have also been investigated. UV/Vis detection after derivatization with p-toluenesulphonyl chloride [18] or 4-methoxybenzenesulfonyl fluoride [19] has also been reported.

A small number of flow methods for the detection of glyphosate have been described. Chemiluminescence detection of glyphosate and its mono-isopropylamine salt, using flow injection analysis, has been proposed [20]. Masini and Colombo developed a sequential injection analysis (SIA) method, based on the OPA derivatization of glyphosate, to study its adsorption on soil and sediments [21]. Subsequently the same authors introduced a sequential-injection reversed-phase chromato-graphy method to detect glyphosate and AMPA without mutual interferences [22]. A multi-pumping flow system detecting the reaction product of glyphosate and p-dimethyl-aminocinnamaldehyde (p-DAC) using UV/Vis spectrometry has also been described [23].

DIN 38407-22 [24] (DIN: German Institute for Standardization) and EPA method 547 [25] describe a continuous post column procedure for the detection of both substances. Here the analysis of glyphosate and AMPA is based on the o-phthaldialdehyde (OPA) derivatization of primary amines in presence of a thiol as a nucleophile to form a fluorescent product (Fig. 1). AMPA as a primary amine is directly derivatized. Glyphosate is a secondary amine and needs to be oxidized with calciumhypochlorite ($Ca(OCl)_2$) to glycine before the derivatization reaction.

However, AMPA is partly decomposed by hypochlorite causing an error in the AMPA detection or even making a simultaneous detection of glyphosate and AMPA with the described post column procedure impossible [26]. To solve this problem we applied a SIA system to optimize the process parameters individually for glyphosate and AMPA.

The OPA derivatization of glyphosate and AMPA is very sensitive and the detection is realized using an optical detector which allows its implementation in a SIA system. The SIA itself was chosen due to its flexibility and high reliability [27] as well as due to its potential implementation in a compact and automated system, allowing for unattended monitoring of contaminants.

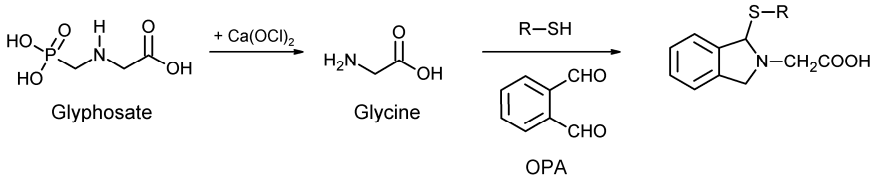

Fig. 1. Derivatization reaction: Glyphosate is oxidized by calciumhypochlorite to glycine which reacts with OPA in the presence of a thiol to form a fluorescent isoindole derivative [24]

2. EXPERIMENTAL

2.1 Chemicals and Reagents

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Seelze, Germany) or Merck (Darmstadt, Germany) unless stated otherwise. All reagents were prepared with purified water obtained from a Milli-Q system (Millipore, Schwalbach, Germany) and were filtered (0.2 µm) and degassed. The carrier solution was 0.005 M KH_2PO_4. The pH of the mobile phase utilized for chromatographic separation was adjusted to 2.0 with H_3PO_4. A stock solution of $Ca(OCl)_2$ (for synthesis) with a concentration of 2.3 g L^{-1} was prepared. The stock solution was stored at -20°C. The working solution of $Ca(OCl)_2$ was prepared according to the following procedure: 1 M phosphate buffer stock: 1.5 g of NaCl and 13.6 g of KH_2PO_4 were dissolved in 100 mL purified water and Na_2HPO_4 solution (17.8 g L^{-1}) was added until a pH of 7.0 was reached. 65.6 µL of the $Ca(OCl)_2$ stock solution was added to 10 mL of the phosphate buffer in order to obtain a final $Ca(OCl)_2$ concentration of 15 mg L^{-1}. For the OPA reagent a borate buffer solution was prepared by dissolving 4.1 g of Na_2CO_3, 1.3 g of H_3BO_3 and 1.9 g of K_2SO_4 in about 95 mL purified water. The pH was adjusted to 10.0 with NaOH (30 wt%). 160 mg of o-phthaldialdehyde and 200 mg of N-acetyl-L-cysteine was dissolved in 2 mL ethanol and added to the borate buffer. 0.4 mL Brij 35 solution (10%) was added and the final volume was adjusted with water to 100 mL. This solution was stored at room temperature in a dark glass bottle. Glyphosate and AMPA stock solutions of 1 g L^{-1} were prepared weekly in 0.005 M KH_2PO_4 at pH 2.0. An amino acid kit was purchased from Ultra Scientific (N. Kingstown, RI, USA) containing L-alanine, L-arginine, creatine, L-glutamic acid, glycine, L-histidine, hydroxyl-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, sarcosine, L-serine, L-threonine, L-tryptophan, L-valine, and L-norleucine. Water samples were collected from the Elbe river near Wedel (Germany). The samples were filtered through 0.45 µm mixed cellulose ester membranes.

2.2 Instrumentation

The experimental setup is depicted in (Fig. 2). An HPLC-pump (Knauer Smartline 100, Berlin, Germany) is connected to an ion exchange column (Hamilton PRP-X400, Reno, NV, USA) via a 6-ports/3-channel injection valve (Knauer A1370). This setup is coupled via a T-piece to a 7-port/1-channel valve (Knauer A1374) which is connected via a holding coil (PTFE tubing, 0.8 mm i.d.) to a 2.5 mL syringe pump (Hamilton PSD/4). An Agilent fluorescence detector (1200 Series, Boeblingen, Germany) with an 8 µL flow cell is employed in this setup (ex. 230 nm, em. 445 nm). A heated reactor (45°C) containing 1 m woven PTFE tubing (0.8 mm i.d.) in a heating block is used as reaction coil (Global FIA, Fox Island, WA, USA). All other connections are made of PTFE tubing (0.5 mm i.d.). The syringe pump and the valve are connected via USB to a personal computer and are controlled by scripts written in the python programming language according to Frank [27,28].

The HPLC pump continuously delivers carrier solution (pH 2.0) through the ion exchange column. As long as no sample is drawn into the holding coil, the carrier solution goes through an auxiliary tubing to the waste. A glyphosate measurement cycle begins with the manual introduction of the sample into the injection valve which is switched to inject the sample. After the retention time of glyphosate in the ion exchange column (258 s) sample (145 µL) and $Ca(OCl)_2$ (12.5 µL) are drawn from the auxiliary tubing into the holding coil by the syringe pump. To improve the mixing, the sample-reagent segment is divided into 5 alternating sections starting and ending with $Ca(OCl)_2$. The sample-reagent segment is mixed by flow reversal (one time) through the valve and injected into the heated

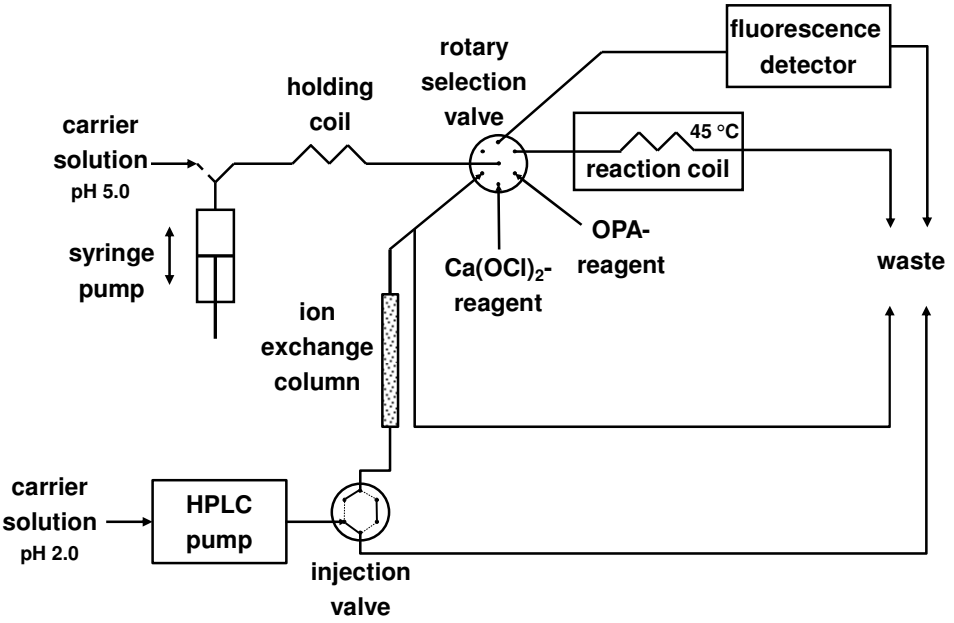

Fig. 2. Scheme of the SIA system utilized for the detection of glyphosate and AMPA

reaction coil (45°C). The flow is stopped to ensure a reaction time of 40 seconds. Then the central reaction zone (125 µL) and OPA solution (187.5 µL) are drawn into the holding coil. Again the sample-reagent segment is divided into 5 alternating sections starting and ending with OPA solution. This segment is pumped through the detector. For the detection of AMPA sample (125 µL) and OPA (187.5 µL) are aspirated in 5 alternating sections into the holding coil after the retention time of AMPA in the ion exchange column (377 s). The sample-reagent segment is directly pumped through the detector. The retention times of both analytes in the ion exchange column were determined in a classical post-column derivatization set-up and subsequently optimized for the SIA set-up.

3. RESULTS AND DISCUSSION

3.1 Parameter Optimization

The influence of process parameters for both oxidation and derivatization reactions were studied in order to maximize the sensitivity of the method. Here sensitivity is defined as the change in detector signal (in LU s) divided by the change in analyte concentration (in µg L^{-1}) (LU: luminescence units) [29]. For each parameter several analyte concentrations were measured and the slope of the resulting calibration curve was evaluated. The following optimization steps were performed without the utilization of the ion exchange column.

The DIN standard 38407-22 [24] proposes to set the temperature of the reaction coil used for the oxidation reaction to 40°C. In literature, temperatures between 30 and 62°C have been suggested for the oxidation step of glyphosate to glycine. Consequently, the effect of the temperature on the oxidation reaction in the described SIA system was investigated (Fig. 3). An increase in sensitivity is observed up to a temperature of 45°C. Higher temperatures do not lead to a further increase in sensitivity. In accordance with these results, a temperature of 45°C was chosen for subsequent analyses. No air bubble formation could be observed at this temperature.

The oxidation rate of glyphosate was also optimized by adjusting the retention time of the sample-$Ca(OCl)_2$ segment in the heated reaction coil in the range of 0 to 60 seconds (Fig. 4). Maximum sensitivity is observed at retention times higher than 30 s. Accordingly a retention time of 40 s was employed in the further method development.

In order to achieve good sensitivity of the SIA method the interpenetration of sample and reagent zones plays an important role. The influence of flow reversal and the number of segments was examined for both reactions. Flow

reversal means that sample and reagent are mixed after the aspiration into the holding coil by being pumped into the reaction coil and directly aspirated back into the holding coil before being delivered to the reaction coil (oxidation) or the detector (derivatization). Mixing sample and $Ca(OCl)_2$ reagent through flow reversal showed an improvement of sensitivity of 6% after one mixing step, whereas more than one mixing step caused a decrease in sensitivity probably caused by the increased dispersion. Using flow reversal to mix sample and OPA reagent resulted in a slight decrease in sensitivity. On the basis of these results, one mixing step for the oxidation was added to the method. Increasing the number of segments from 3 (reagent-sample-reagent) to 5 (at constant reaction volumes) led to an increase in sensitivity of 28% when applied to the OPA derivatization. A higher number of segments did not result in a further improvement of the method. For the oxidation reaction an increase in number of segments showed only a minor increase in sensitivity. A number of 5 segments was chosen for both reactions in this method.

The optimum amount of reagent was determined for both reactions. (Fig. 5) shows the influence of the $Ca(OCl)_2$-sample volume ratio on the sensitivity. Only small amounts of the $Ca(OCl)_2$ reagent are required for the oxidation of glyphosate. A $Ca(OCl)_2$ proportion of more than 8.6% (relating to the sample volume) leads to a significant decrease in sensitivity, supposedly due to an oxidation of the derivatizing reagent.

The optimum amount of OPA reagent lies within the same range for glyphosate and AMPA (Fig. 6). For the derivatization of both analytes an OPA reagent amount of 1.5 times the sample volume is favourable.

(Fig. 7) shows calibration curves of both glyphosate and AMPA in the mobile phase at pH 2.0. Both curves prove linearity in the concentration range of interest with an R^2 of 0.991 for glyphosate, and 0.996 for AMPA, respectively. Each measurement was repeated three times. The high intercept values of the calibration curves are due to the blank value of the OPA-solution.

Limits of detection (LOD) are 16 µg L^{-1} (glyphosate), and 9 µg L^{-1} (AMPA), respectively. They are calculated according to a signal-to-noise ratio of 3 using the equation $LOD = 3 \cdot \sigma / S$ (σ: standard deviation of 10 blank measurements; S: slope of calibration curve) [30].

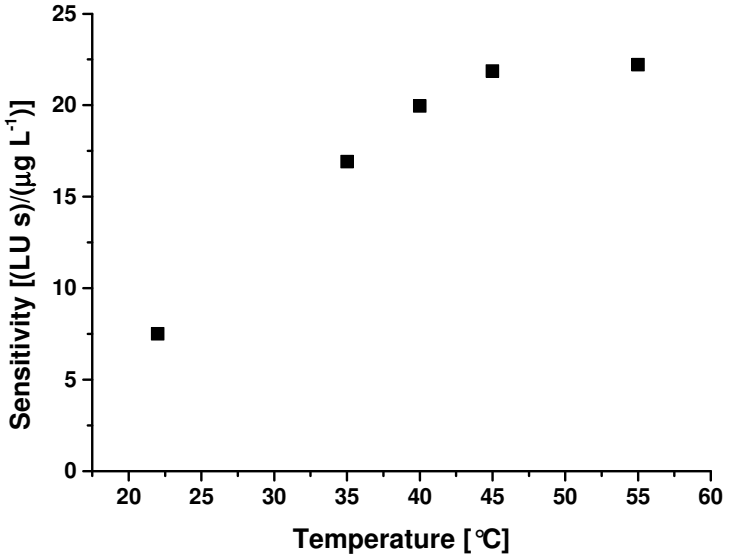

Fig. 3. Influence of the temperature of the heated reaction coil on the oxidation of glyphosate with $Ca(OCl)_2$ at a reaction time of 40 s

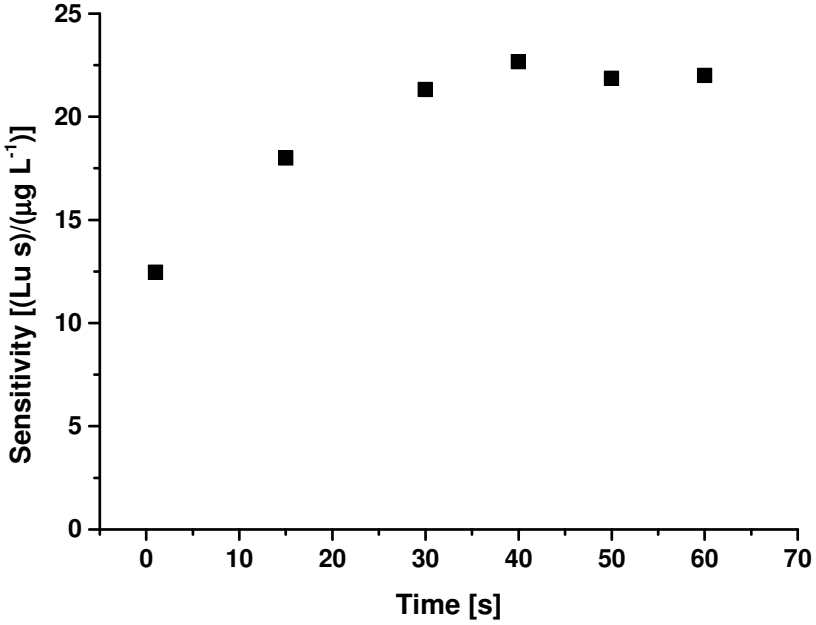

Fig. 4. Influence of the retention time of the sample-$Ca(OCl)_2$ segment in the heated reaction coil

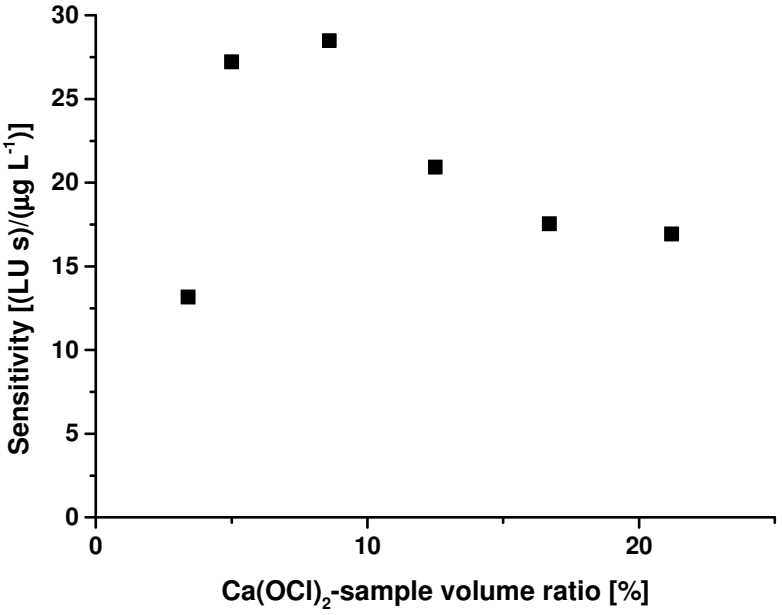

Fig. 5. Influence of the $Ca(OCl)_2$-sample volume ratio

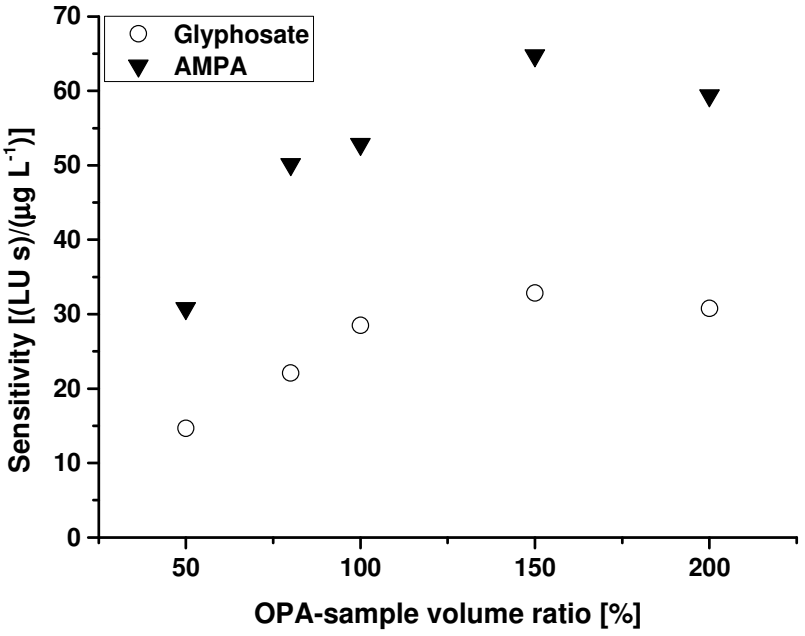

Fig. 6. Influence of the OPA-sample volume ratio

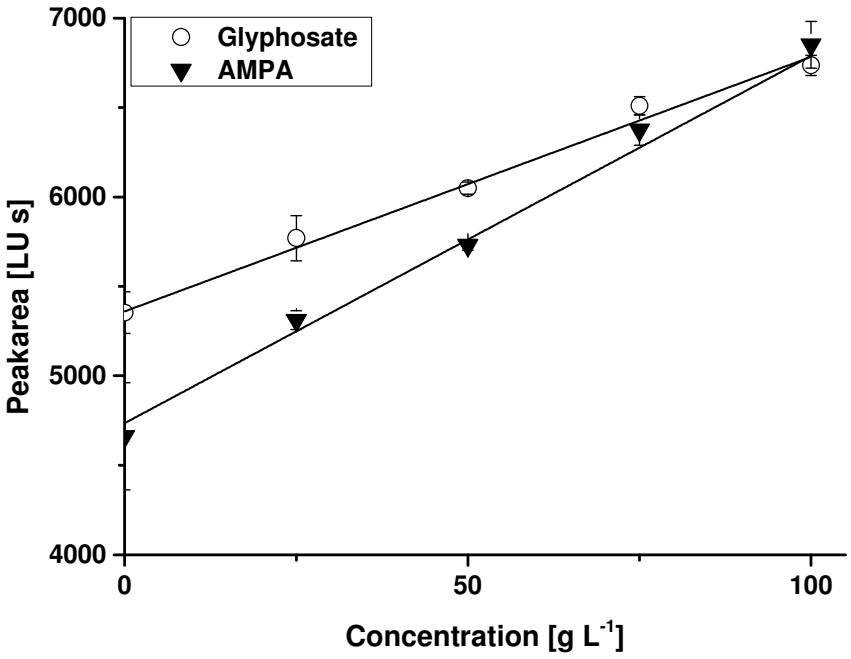

Fig. 7. Calibration curves of glyphosate and AMPA

3.2 Interference Study

The proposed method is bound to be disturbed by other amines which are omnipresent in natural waters. In order to enhance the selectivity of the method, an ion exchange column was integrated in the process. The poly(styrene-divinylbenzene) sulfonate cation exchange support column separates the analytes according to charge [31].

Ammonia and 19 frequent amino acids were tested as model amines for this interference study. Ammonia (50 µg L^{-1}) or a mixture of 19 amino acids (10 µg L^{-1} each) at environmentally relevant concentrations were added to glyphosate and AMPA standards (100 µg L^{-1}).

The addition of ammonia or 19 amino acids to glyphosate and AMPA standards show no increase in the signal (Fig. 8), demonstrating a complete elimination of these interferences by the ion exchange column.

Natural organic matter, present in natural waters, is prone to interfere with fluorimetric methods [22]. We applied this method to spiked real water samples from the Elbe river in order to evaluate possible interferences caused by the real water matrix. Samples were spiked with glyphosate and AMPA at concentration levels of 100 µg L^{-1} and the fluorescence signal was compared to that of the analyte standards in mobile phase at 100 µg/L (Fig. 9). No influence of the complex real water matrix on the fluorescence signal could be observed, indicating a high accuracy of the method applied to real water samples.

The detection of the analytes in spiked real water samples (tenfold) showed standard deviations of ±3% (glyphosate) and ±1.9% (AMPA). These results demonstrate a good precision of the proposed SIA method with regard to determining glyphosate and AMPA in real water samples.

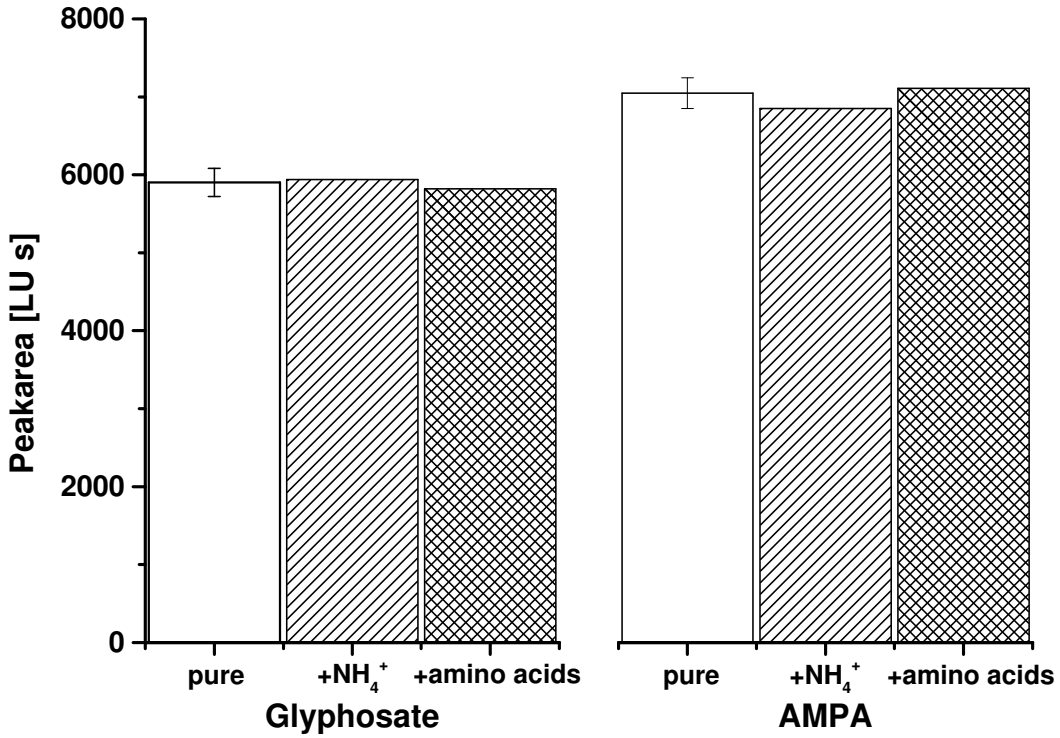

Fig. 8. Influence of ammonia and amino acids on the detection of glyphosate and AMPA

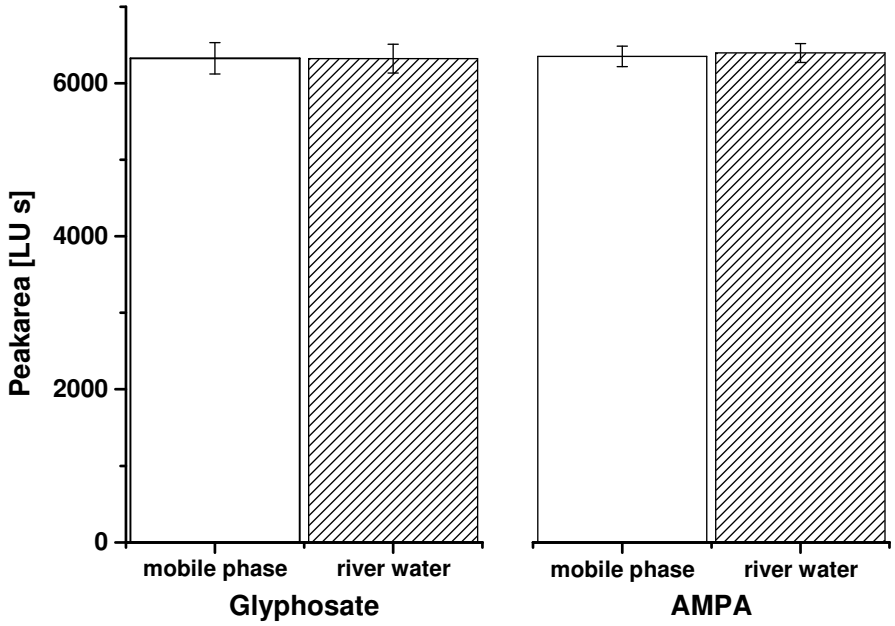

Fig. 9. Fluorescence signal of different water matrices spiked with 100 µg L^{-1} glyphosate and AMPA

4. CONCLUSION

A sequential injection analysis method was developed to detect glyphosate and its degradation product AMPA in water samples. Good sensitivity could be achieved with limits of detection of 16, and 9 µg L^{-1} (96, and 81 nmol L^{-1}) for glyphosate, and AMPA, respectively.

With the proposed SIA method we optimized the detection of glyphosate and AMPA independently from each other according to their different chemical characteristics as primary and secondary amines using the same system. Compared to conventional HPLC methods only small amounts of reagents are required. During a combined analysis of glyphosate and AMPA 12.5 µL of the $Ca(OCl)_2$ reagent and 375 µL of the OPA reagent are consumed. Other amines (ammonia, amino acids) interfering with the OPA derivatization could be eliminated by integrating an ion exchange column into the system. The method was applied to spiked real water samples (Elbe river) showing high accuracy and precision.

The SIA system was successfully configured and optimized. In order to use this system for the evaluation of water samples from natural origin a preceding sample preparation will be necessary. A selective preconcentration to lower the detection limits of this method is required in places where the concentration of both contaminants is below the stated detection limits.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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