# A Comparison of Pre- and PostColumn Sample Treatment for the Analysis of Glyphosate

Application Note

Environmental Analysis

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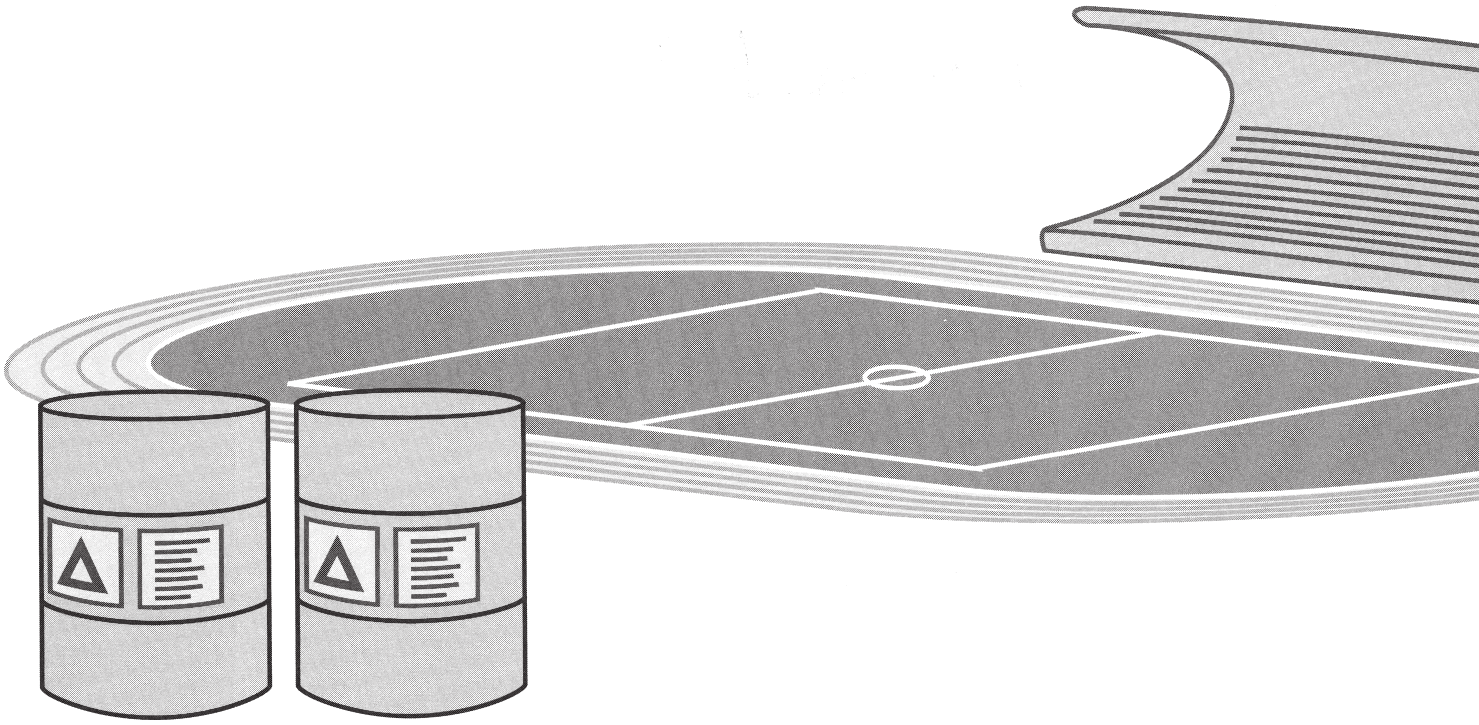

The active ingredient in several sports field weedkillers, glyphosate, and its main metabolite aminomethyl phosphonic acid (AMPA) can be analyzed to low (1–5) ppb levels by HPLC, although the absence of chromophores in both compounds makes labeling with fluorogenic reagents necessary. Precolumn derivatization with fluorenylmethyl chloroformate (FMOC) gives low detection limits and has the advantage of simplicity, since it uses the automated injector program facility of the HP 1050 Series autosampler. Post-column oxidation of the glyphosate with hypochlorite, followed by derivatization with *o*-phthalaldehyde (OPA) requires additional equipment, but has the advantage of superior selectivity. It is thus better suited to samples in complex matrices.

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## Introduction

Glyphosate, the N-(phosphonomethyl) derivative of the amino acid glycine, is a widely-used broad-spectrum, non-selective and post-emergence herbicide. Its application to plants inhibits the production of specific enzymes so that the synthesis of aromatic amino acids is interrupted. Compared with triazines, glyphosate is strongly absorbed on soil particles and subsequently undergoes microbial degradation to the main metabolite, aminomethyl phosphonic acid (AMPA) (figure 1) and finally to ammonia and carbon dioxide.[1] It is the active ingredient of several commercial weedkillers, and is used against weeds, for example couch grass, in the farming of cereals, potatoes, vines and mushrooms; it is very often used to maintain sports fields and lawns.

The wide use of glyphosate in agriculture can result in its presence in ground water, vegetable matter and milk. Because of the suspected toxicity of glyphosate and its metabolite, tolerance levels have been set for food and drinking water. German regulations, for example, limit the maximum residue limits (MRL) to 80 mg/kg for mushrooms, 10 mg/kg for cereals, and 0.1 mg/kg for plant food, and the limit set by the European Community for drinking water is 0.1 µg/l.[2] The United States Environmental Protection Agency (EPA) has set a detection limit for glyphosate in drinking water of 6 µg/l.[3]

Because of the low MRLs and the wide variety of matrices, selectivity and sensitivity play an important role in the analysis of glyphosate. High selectivity is required for complex matrices such as food samples and the highest sensitivity is needed to monitor glyphosate and its metabolite in water samples.

Many different methods for the analysis of glyphosate and its metabolite AMPA have been described, including gas chromatography,[4] thin-layer chromatography[5] and high performance liquid chromatography (HPLC). All these methods, however, require special manual derivatization steps or tedious cleanup procedures making them time-consuming. For the HPLC analyses, the absence of chromophores makes it necessary for the analytes to be labeled with reagents either before the column[6] or after the column.[7] We have compared pre- and postcolumn derivatization methods in the light of the different requirements for the analyses of drinking water and agricultural produce.

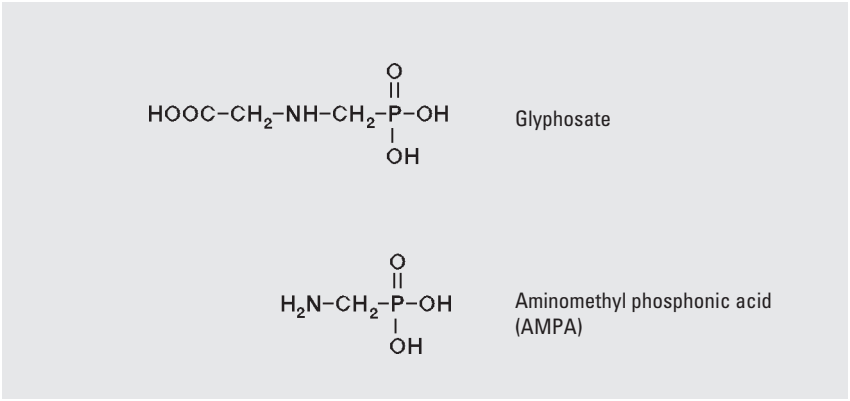


**Figure 1**
**Glyphosate and its main metabolite aminomethyl phosphonic acid (AMPA)**

## Experimental

For the chromatographic analyses, we used the HP 1050 Series autosampler with variable-volume auto-injector, the HP 1050 Series quaternary pump with temperature-controlled column compartment and the HP 1046A programmable fluorescence detector. We used the programmable injection facility of the auto-injector for automated precolumn derivatization. For postcolumn derivatization, we used a Postcolumn Reaction System (PRS) from Pickering Laboratories. Figure 2 shows a schematic diagram of the complete analysis setup.

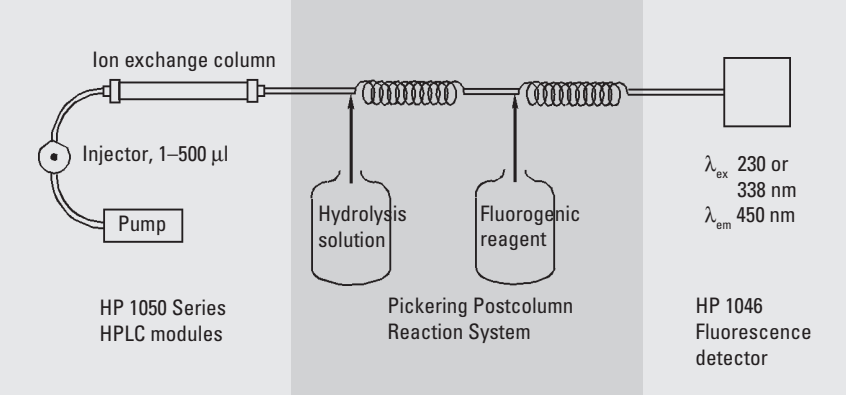


**Figure 2**
**Analysis set-up for postcolumn derivatization**

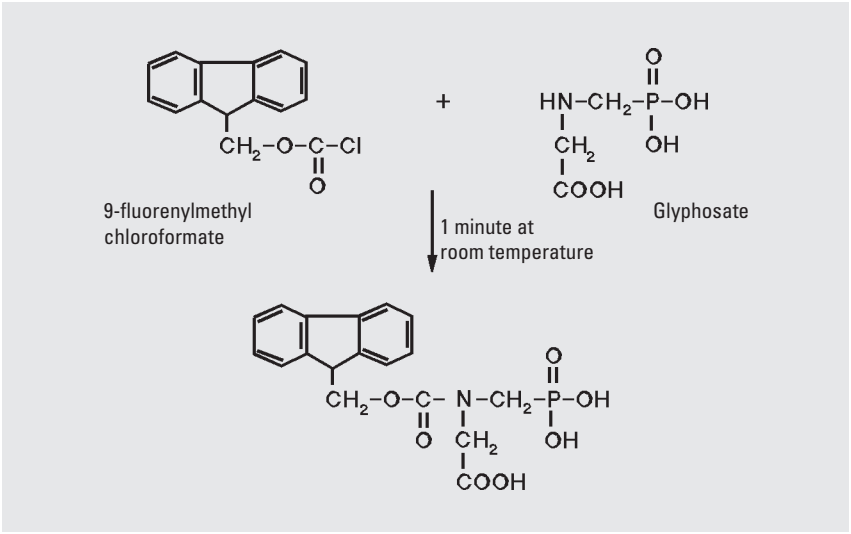


**Figure 3**
**Precolumn reaction for glyphosate with FMOC**

## Automated precolumn derivatization

The reaction of glyphosate with fluorenylmethyl chloroformate FMOC,[8] used for the automated precolumn derivatization, is shown in figure 3. The reaction was performed automatically in the auto-injector, using an injector program. Table 1 lists the various program steps of the automated precolumn derivatization.

In step 1 of the program, a borate buffer (necessary to maintain the correct reaction pH of 10.4), was drawn into a capillary, then FMOC (step 3), sample (step 5) and again FMOC (step 7) were drawn into the capillary. Between each step, the surface of the needle was cleaned by washing with acetonitrile (steps 2, 4, 6 and 8). The sample and reagent volumes were mixed by moving them back and forth inside the capillary (step 9). The resulting FMOC derivatives of glyphosate and AMPA were injected after a 1 minute wait. Derivatization was performed at ambient temperature. The derivatives were separated on a reversed phase column and were detected with a fluorescence detector using an excitation wavelength of 266 nm and an emission wavelength of 305 nm, with a 280 nm cut-off filter.

| | | | |
|---|---|---|---|
| **1** | **draw :** | **2 µl from vial 2** | draws borate buffer (0.4N, pH 10.4) |
| **2** | **draw :** | **0 µl from vial 3** | rinses tip by dipping in vial containing acetonitrile |
| **3** | **draw :** | **1 µl from vial 1** | draws FMOC (1mg/ml in acetonitrile) |
| **4** | **draw :** | **0 µl from vial 3** | rinses tip by dipping in vial containing acetonitrile |
| **5** | **draw :** | **1 µl from sample** | |
| **6** | **draw :** | **0 µl from vial 3** | rinses tip by dipping in vial containing acetonitrile |
| **7** | **draw :** | **1 µl from vial 1** | draws FMOC |
| **8** | **draw :** | **0 µl from vial 3** | rinses tip by dipping in vial containing acetonitrile |
| **9** | **mix :** | **7 µl cycles: 10** | mixes reagent and sample for derivatization reaction |
| | | **mixing speed: 300 µl/min** | |
| **10** | **wait :** | **1 min** | |
| **11** | **inject** | | |

**Table 1**
**Injector program for derivatizing glyphosate and its metabolite AMPA**

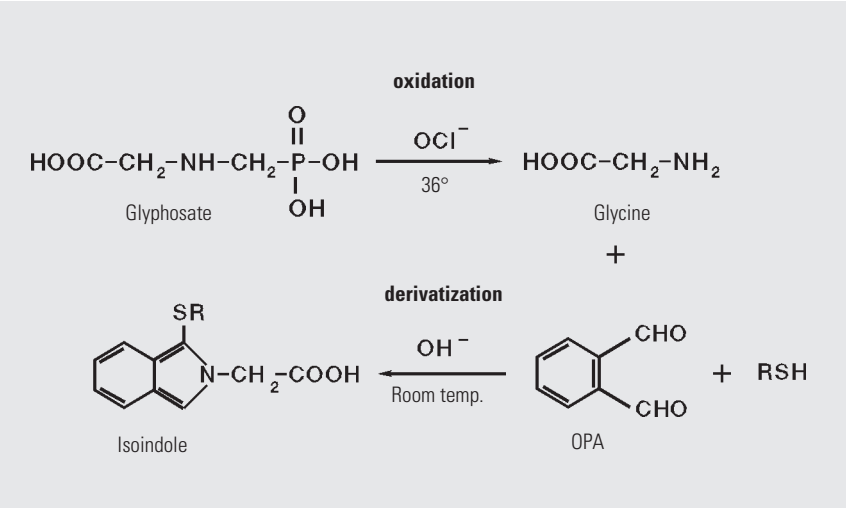


**Figure 4**
**Two step postcolumn reaction for glyphosate**

## Postcolumn derivatization

For postcolumn derivatization experiments a Pickering glyphosate Postcolumn Reaction System EG5000XX (Pickering laboratories, Inc. 1951 Colony Street, Mountain View, CA 94043) was connected between the HPLC column and the fluorescence detector. After separation on the anion exchange column, the glyphosate underwent a two-stage derivatization which is shown in figure 4. The glyphosate was first hydrolyzed to glycine by an hypochlorite solution and the glycine then derivatized with *o*-phthalaldehyde (OPA) reagent to form a fluorescent isoindole. The amino group of AMPA (see figure 1) reacted directly with the OPA. The isoindole derivatives were detected by fluorescence with excitation wavelength of 230 nm or 338 nm and an emission wavelength of 450 nm with a

370 nm cut-off filter. Chemicals and solvents were obtained from Baker (FRG).

The hydrolysis solution was made up by dissolving 8 g dipotassium hydrogen phosphate, 5 g potassium chloride and 150 ml sodium hypochlorite solution (5% active chloride, supplied by Aldrich-Chemie D7924 Steinheim) in 1 liter of water. pH was measured as 9.1. If necessary, the sodium hypochlorite solution can be replaced by 15 mg calcium hypochlorite. The fluorogenic reagent can be either a premixed OPA solution (Fluoraldehyde®, Pierce), or a mix of 1 g *o*-phthaldaldehyde and 1 ml 2-mercaptoethanol dissolved in 10 ml methanol and added to 1 l of 0.05 N sodium borate solution (19.1 g sodium borate in water). pH was measured as 9.2.

The analysis was performed according to a method published by the EPA[3] for the analysis of glyphosate in drinking water. This method requires that the two compounds, glyphosate and AMPA, are monitored at maximum residue limits of 6 ppb (6 µg/l).

Because of the amphoteric character of glyphosate (–NH– and –COOH, $–PO_3H_2$), the separation of glyphosate and AMPA can be performed on either an anion exchange (SAX) with 0.0025 M potassium dihydrogen phosphate, adjusted to pH 2.1 with phosphoric acid, as mobile phase (elution order AMPA–glyphosate) or on a cation exchange ($K^+$ form) with 0.005 M potassium dihydrogen phosphate, adjusted to pH 2.0 with phosphoric acid, (elution order glyphosate–AMPA). We chose the anion-exchange column (SAX-300 100 × 4.6 mm id, part number 79919QA-754) for our experiments. At pH 2.1, any amino acids in the matrix (for example from food samples) behave like neutral molecules and are not retained. Additionally, if sample enrichment is necessary, glyphosate can be retained on an anion exchange column at neutral pH.[9]

| | | |
|---|---|---|
| Column | | ODS Hypersil, 100 x 4.6 mm, 5 µm |
| Eluant | Channel A | 0.002M $KH_2PO_4$ 7% acetonitrile adjusted to pH 7.0 |
| | Channel B | acetonitrile |
| Column temperature | | 40°C |
| Fluorescence detection | | $\lambda_{ex}$ 266 nm $\lambda_{em}$ 305 nm with a cut-off filter at 280 nm. |
| Injection volumes | | 1 µl and 10 µl |

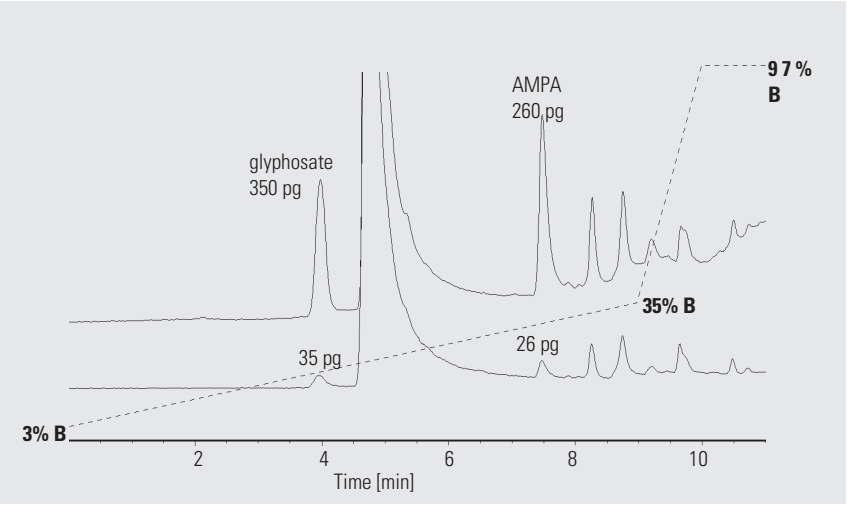


**Figure 5**
**Analysis of two samples after precolumn derivatization (upper trace 1 µl injection of 350 ppb glyphosate and 260 ppb AMPA, lower trace 10 µl of a 100-fold dilution)**

## Results and discussion

### Automated precolumn derivatization

Figure 5 (previous page) shows the separation of glyphosate and AMPA. The upper trace shows the results of a 1 µl injection of water containing 350 ppb glyphosate and 260 ppb AMPA. The lower trace shows the results of a 10 µl injection of water containing 3.5 ppb glyphosate and 2.6 ppb AMPA. The additional peaks in the chromatogram derive from either FMOC reagents or from by-products of the derivatization.

We found that the derivatization yield was constant for volumes of up to 10 µl of sample, resulting in a detection limit of approximately 1 ppb of each compound. Over eight runs, the method's repeatability was better than 0.5% RSD for retention times and better than 2.5% for peak areas when injecting 10 µl and better than 5% when injecting 1 µl sample volume.

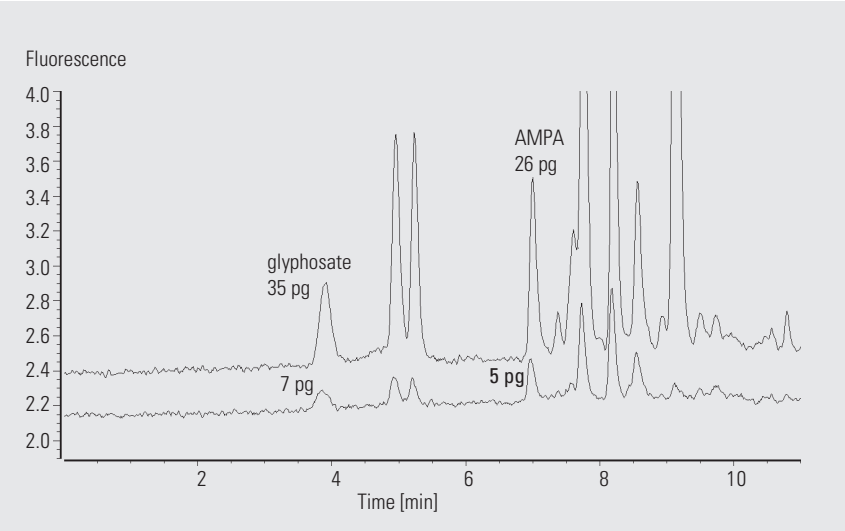


**Figure 6**
**Analysis of 100 µl injection after manual derivatization of glyphosate and AMPA at different concentrations (upper trace 350 ng/l glyphosate, 260ng/l AMPA, lower trace 70 ng/l glyphosate, 52 ng/l AMPA)**

For lower detection limits, larger sample volumes have to be used. There are three possible solutions :

a) 25 ml sample is derivatized manually with FMOC then extracted with two 30 ml aliquots of methylene chloride, as described by Gauch, Leuenberger and Mueller.[10] The methylene chloride extracts are concentrated and 20 µl are injected. Detection limits are 20 ng/l;

b) The sample is derivatized manually with FMOC, and a large volume (up to 500 µl) is injected without extraction or concentration. Figure 6 shows chromatograms of standards at different amounts, and figure 7 shows chromatograms of standards overlaid on a water sample taken from a well. The detection limits were below 50 ng/l;

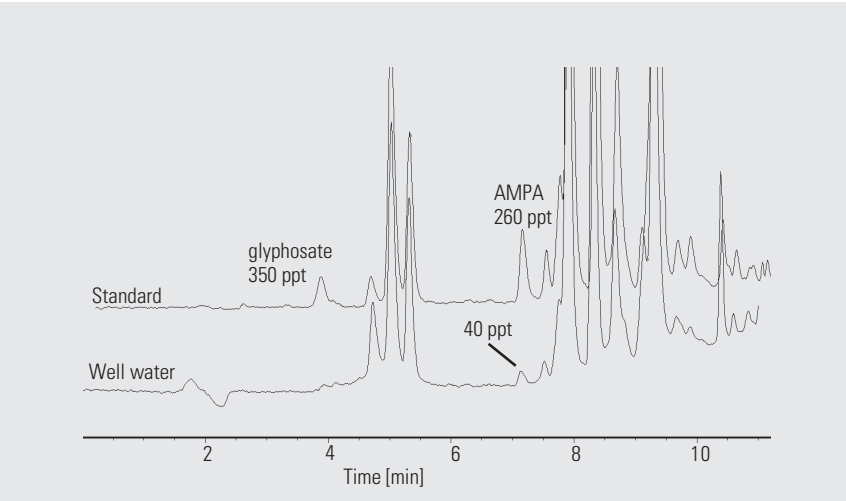


**Figure 7**
**Analysis of 100 ml of well water overlaid with a standard**

c) The sample is derivatized automatically with FMOC in the larger volume of a sample vial (rather than the capillary) using the HP 1050 Series autosampler with the extended 100 vial tray and rotary mixer accessory. Reagents and borate buffer (20 µl each) are added to a 500 µl sample vial and mixed in the vial using the rotary mixer; all steps are performed automatically using the injector program. The derivatization is completed within one minute, and volumes as large as 100 µl can be injected onto the column. An example of a large volume injection is shown in figure 8. Injection of volumes larger than 100 µl should be avoided because compounds from reagents may interfere with the analytes' derivatives. With the injection volume of 100 µl, the detection limit is about 50 ng/l for AMPA and 100 ng/l for glyphosate. Further investigations to improve the reproducibility of this method of derivatization are in progress.

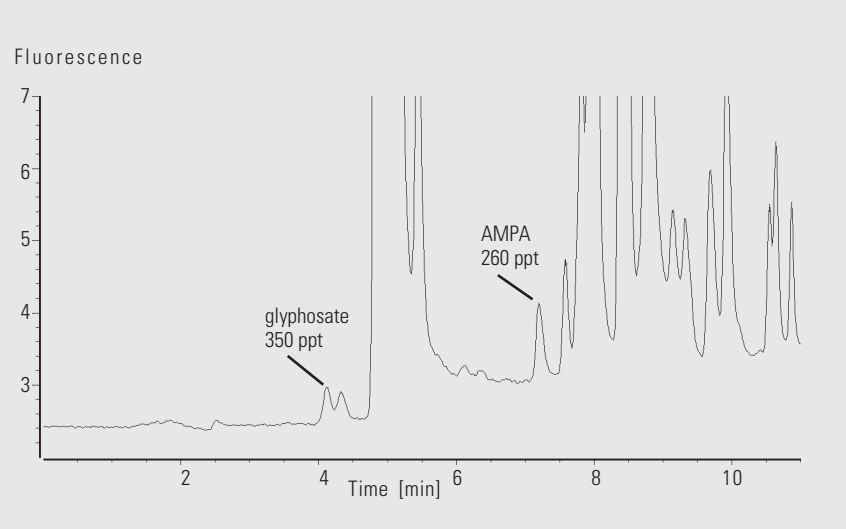


**Figure 8**
**Analysis of 10 ml of sample after automated precolumn derivatization in the vial using a rotary mixer (35 pg glyphosate and 26 pg AMPA)**

## Postcolumn derivatization

Chromatograms of the separation of glyphosate and AMPA at different concentrations are shown in figure 9. In the lower chromatogram, 2.6 ng AMPA and 3.5 ng glyphosate in 500 µl are injected representing 5.2 µg/l AMPA and 7 µg/l glyphosate. Compared with the chromatograms obtained after precolumn derivatization, the selectivity is

| | |
|---|---|
| Column | SAX 300, 100 x 4.6 mm |
| Eluant | 0.34g/l $KH_2PO_4$, 0.1% $H_3PO_4$, adjusted to pH 2.1 |
| Flow rate | 0.8 ml/min |
| Column temperature | 40°C |
| Fluorescence detection | $\lambda_{ex}$ 230 nm<br>$\lambda_{em}$ 450 nm with a cut-off filter at 370 nm |
| Injection volume | 500 µl |

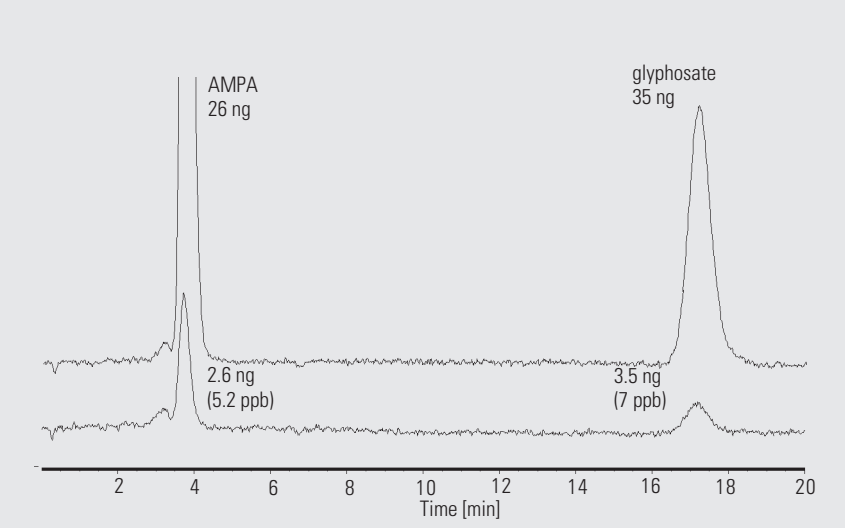


**Figure 9**
**Chromatogram of glyphosate and AMPA with post-column derivatization**

impressively high. No peaks from reagents or side reactions are visible. Detection limits are in the low ppb range. If lower detection limits are required, the sample can be enriched by passing it through an anion-exchange column at pH 7.2. A procedure for this has been described.[11]

Reproducibility of the method was measured over 10 runs. The standard deviation of the retention times was 0.8% RSD and the repeatability of the peak areas was 2.1% RSD for a 35 pg sample and 100 µl injection volume.

## Conclusion

Glyphosate and its metabolite AMPA can be determined by either pre- or postcolumn automated derivatization techniques. Detection limits for precolumn derivatization are slightly better and the instrumentation is simpler, requiring no more than the HP 1050 Series autosampler with injector program. The advantage of the postcolumn reaction method is higher selectivity. This is important for the analysis of food samples, where the matrix contains amino acids that are derivatized during the precolumn derivatization and interfere with glyphosate and AMPA in the chromatography. Thus the determination of glyphosate and its metabolite in water samples is dealt with adequately by precolumn derivatization, while more complex matrices are better handled by the postcolumn derivatization technique.

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