The method illustrates the usefulness of semiautomation to improve the speed and reproducibility of analytical methods.

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# Determination of Glyphosate Herbicide and (Aminomethyl)phosphonic Acid in Natural Waters by Liquid Chromatography Using Pre-Column Fluorogenic Labeling with 9-Fluorenylmethyl Chloroformate

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**An analytical method has been developed for determination of glyphosate herbicide and its major metabolite, (aminomethyl)phosphonic acid (AMPA), in natural waters. Sample pretreatment consisted of filtration, addition of phosphate buffer, concentration by rotary evaporation, and a final filtration before derivatization with 9-fluorenylmethyl chloroformate. The derivatives were separated by anion exchange liquid chromatography and measured with a fluorescence detector. Standard curves were linear over 3 orders of magnitude and minimal detectable quantities were 10 ng/mL for glyphosate and 5 ng/mL for AMPA. The 20-fold concentration factor realized in sample preparation corresponds to ppb method detection limits for glyphosate and AMPA in natural waters. Recovery and storage studies were performed and are discussed.**

Glyphosate [*N*-(phosphonomethyl) glycine; Roundup®] is a broad spectrum, nonselective, post-emergence herbicide that has found widespread agricultural and domestic use. Recently, it has been introduced for the control of aquatic weeds (Rodeo®). Analytical methods development for the determination of glyphosate in environmental samples has not been avidly pursued, largely because of its low mammalian toxicity ($LD_{50}$ = 1568 mg/kg) and subsequent low risk of environmental pollution. Nevertheless, the effect of glyphosate on nontarget organisms and its overall environmental fate cannot be fully evaluated unless techniques possessing suitable sensitivity and selectivity are available.

Several chromatographic methods have been developed for the analysis of glyphosate and its major metabolite, (aminomethyl)phosphonic acid (AMPA), including gas chromatography (GC) after chemical derivatization (1–3), thin layer chromatography (4, 5), and liquid chromatography (LC; 6, 7). Recently, these methods and several others have been reviewed (8). The ionic, water-soluble character of glyphosate and AMPA make analysis by LC advantageous over GC. Although glyphosate and AMPA cannot be sensitively measured by conventional photometric LC detectors, highly fluorescent derivatives can be formed pre-column, using 9-fluorenylmethyl chloroformate (FMOCC1) (6), or post-column with orthophthalaldehyde-mercaptoethanol (OPA-MERC) (7). The post-column procedure forms derivatives on-line but it requires more instrumentation and careful maintenance. Conversely, the pre-column method is rapid and simple and requires minimal equipment and analyst experience.

Analysis of glyphosate and AMPA by LC as FMOC derivatives has been applied to vegetation (9, 10) and water and soil (11). Glass (11) reported good recoveries and detection limits for glyphosate in water but the procedure required ion-exchange cleanup and AMPA was not determined. We have applied this pre-column LC procedure to the analysis of glyphosate residues in natural waters. We report a shortened sample preparation and include the determination of AMPA. Method limitations and recoveries from fortified water samples are discussed.

## Experimental

### *Sample Preparation*

Natural waters investigated included rainwater, lake water, and river water from a forest watershed; samples were collected and frozen in polyethylene bottles until analyzed. Periodically, frozen samples were thawed and thoroughly shaken to mix, and about 150 mL was filtered through Whatman No. 1 paper. In recovery experiments, samples were fortified with herbicide and metabolite before this filtration. A 100 mL aliquot of this water was placed into 250 mL round-bottom flask and 1 mL 0.1M $K_2HPO_4$ was added. Samples were concentrated to near-dryness by rotary evaporation (Buchi Model R; Brinkmann) at 30–50°C and diluted to 5.0 mL by carefully rinsing the flask twice with 2 mL washes of deion-

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**Table 1. Capacity factors for AMPA and glyphosate derivatives on selected columns[a]**

| Column[b] | Derivative | |
|---|---|---|
| | AMPA | Glyphosate |
| $\mu NH_2$ (Alltech) | 1.14 | 2.45 |
| $N(CH_3)_2$ (Macherey-Nagel) | 1.38 | 1.77 |
| μCarbohydrate (Waters)[c] | 1.10 | 3.10 |
| SAX (Altex) | 0.70 | >5.7 |

[a]75% v/v mixture of 0.05M $KH_2PO_4$ (pH 6.0) in acetonitrile.
[b]SAX column is a strong anion exchanger; all others are weak anion exchangers.
[c]75% v/v mixture of 0.1M $KH_2PO_4$ (pH 6.0) in acetonitrile.

ized water. They were stored at 4°C if not analyzed immediately.

### *Standard Preparation*

Fifty mg glyphosate (Monsanto) or AMPA (Sigma) was dissolved in 500 mL deionized water to yield 100 μg/mL stock solutions. Mixed standards (glyphosate and AMPA) covering the range of 0.005–10 μg/mL were prepared by appropriate dilutions of the stock solutions in deionized water. Standard solutions were refrigerated and no degradation was observed over 6 months.

### *Derivatization*

Approximately 0.5–1 mL sample concentrate was passed through a Gelman GA-8 (0.2 μm) filter and into a 2 mL glass vial. A 0.10 mL aliquot of filtered sample was placed in a small glass culture tube, followed by 0.90 mL 0.025M borate buffer, 0.90 mL LC grade acetone, and 0.10 mL 0.01M 9-fluorenylmethyl chloroformate (FMOCC1; Aldrich) in acetone. Tubes were shaken and allowed to react 20 min at room temperature. Excess reagent was removed by three 1 mL washes of ethyl ether (top layer). Samples were analyzed within 8 h.

### *Liquid Chromatography*

The instrument used consisted of an Altex Model 110A pump, Rheodyne Model 7125 injector (200 μL loop), 0.4 × 25 cm $\mu NH_2$ column (Alltech Assoc.), Aminco spectrophotofluorometer (excitation 270 nm; emission 315 nm) equipped with 50 μL flow cell, and Soltec strip chart recorder (50 mV). Mobile phase consisted of 75% (v/v) mixture of 0.05M $KH_2PO_4$ (pH 6.0 with KOH) in acetonitrile (Fisher LC grade) delivered at 1.0 mL/min. Glyphosate and AMPA were measured by comparing peak heights of samples to external standard curve of at least 3 points.

During the development of this method, the following LC columns (all 0.4 × 25 cm) were also evaluated: $N(CH_3)_2$ (Macherey-Nagel), μCarbohydrate (Waters), and SAX (Altex). Also, a Gilson Specta/Glo filter fluorometer (excitation 280 nm; emission 300–400 nm) equipped with 8 μL flow cell was compared with the spectrophotofluorometric instrument.

### *Storage Study*

Two separate storage studies were performed. Control water samples with added propionic acid (3 mL/300 mL sample) were fortified with glyphosate and AMPA at 0.05 and 0.50 ppm and stored in a refrigerator (4°C) for 3 months. Control water samples (no propionic acid) were fortified with glyphosate at 0.05 and 0.50 ppm and frozen (0°C) for 3 months. Subsequently, these samples were prepared and analyzed as described above.

## Results and Discussion

Since glyphosate and AMPA are zwitterionic molecules, formation of their respective FMOC derivatives by reaction of the amine (analyte) and the acid chloride (FMOCC1) yields anionic compounds which can easily be separated by anion exchange liquid chromatography (6). Several anion exchange stationary phases were examined, and in all cases, AMPA-FMOC, the weaker acid, eluted before glyphosate-FMOC (Table 1). Efficiency on all of these columns ranged from 2000 to 3000 theoretical plates. For the 4 different stationary phases examined, maximum retention of AMPA-FMOC was observed with the dimethylamine moiety ($N(CH_3)_2$), while glyphosate-FMOC was retained longest on the SAX column under the conditions tested. These conditions (0.1–0.05M phosphate buffer with 25% acetonitrile) offered the best compromise between good sensitivity and reasonable retention time. Phosphate buffer was the only salt evaluated and other buffers could significantly affect separation behavior. Substitution of methanol for acetonitrile resulted in significant deterioration of efficiency.

Practical application of chromatographic methods to environmental samples requires that analyte retention be controlled such that interference peaks can be circumvented. In anion exchange, retention usually can be increased by a decrease in ionic strength of buffer and/or an increase in pH (12). On silica-based stationary phases, a decrease in the percentage of organic modifier will also increase retention. Our experience with the columns examined has been that a decrease in ionic strength or percentage of acetonitrile increases retention at the expense of significantly degraded peak shape and sensitivity (see Figure 1).

Glyphosate-FMOC retention could easily be controlled by changing the buffer pH, especially on the SAX column. However, for AMPA-FMOC, varying the pH did not significantly increase retention on any of the stationary phases evaluated. Retention increased slightly from pH 4 to 6, but decreased as pH was increased to 8. Roseboom and Berkhoff (10) reported that the mobile phase pH (5–8) had no effect on the retention

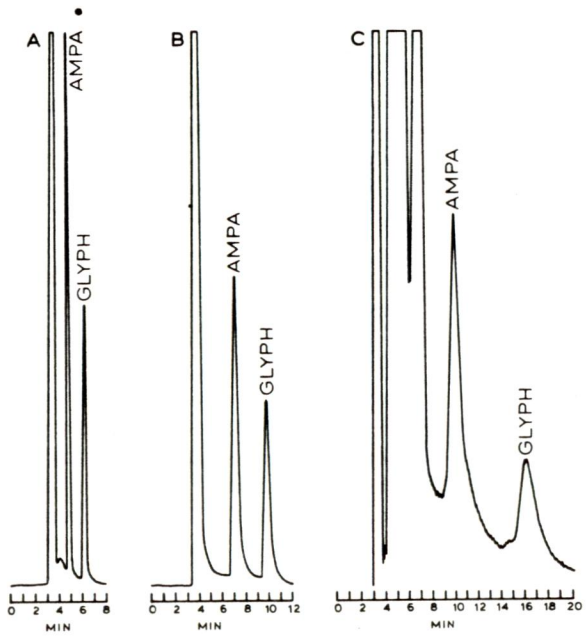


**Figure 1. Chromatograms of AMPA-FMOC and glyphosate-FMOC standards on $\mu NH_2$ column with different mobile phases, demonstrating decrease in efficiency with decreased percent organic modifier or buffer ionic strength: (A) 75% 0.10M $KH_2PO_4$, pH 6/25% $CH_3CN$; (B) 90% 0.10M $KH_2PO_4$, pH 6/10% $CH_3CN$; (C) 90% 0.05M $KH_2PO_4$ pH 6/10% $CH_3CN$.**

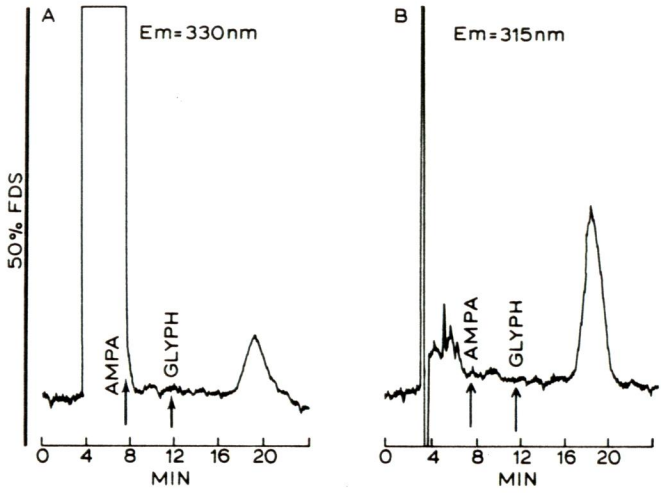


**Figure 2. Chromatograms of derivatized control forest water showing effect of emission wavelength on interference peaks (excitation wavelength 295 nm; mobile phase 75% 0.05M $KH_2PO_4$, pH 6/25% $CH_3CN$; $\mu NH_2$ column).**

of AMPA-FMOC on a Hypersil APS column. On the weak anion exchange columns, the cationic character of the stationary phase decreases as pH approaches the pK value (about 9); thus retention should decrease as pH is increased above 6. On the strong anion exchange column (SAX), the cationic character should not significantly decrease until the pH is greater than about 9, but increasing the mobile phase pH did not increase retention of AMPA-FMOC on this column. This was unfortunate because AMPA-FMOC eluted very early in the chromatogram which increased the possibility for potential interferences. This is further confounded by the fact that AMPA resembles many amino acids which are certain to be found in most agricultural samples. For better control of AMPA-FMOC retention by anion exchange, a change in the buffer salt offers a good possibility. Since multi-charged ions are generally held on ion exchangers more strongly, phosphate buffers at a high pH will compete strongly for ion exchange sites on the stationary phase. It is also possible to separate the AMPA-FMOC and glyphosate-FMOC derivatives by ion-pair or micellar liquid chromatography.

Two fluorescence detectors were evaluated; a filter fluorometer and a spectrophotofluorometer (SPF). Typically, filter fluorometers offer better sensitivity because of a higher optical transmissivity while the SPF has better selectivity because of the narrow bandpass. Nevertheless, for the 2 systems evaluated here, the spectrophotofluorometric instrument was 20–50 times more sensitive. One reason for this difference was its larger diameter light path (50 μL cell) compared to the filter fluorometer (8 μL). A smaller cell decreases band spreading in an LC detector, but the relatively wide peaks that are typical of ion-exchange LC are usually not significantly affected by larger cells. Another important difference in these 2 detectors was the higher intensity of the spectrophotofluorometric light source (200 W xenon arc) compared to the filter fluorometer (5 W mercury lamp). In addition, it was found that the selectivity of the spectrophotofluorometer allowed spectral resolution of some sample interferences. A shift in emission wavelength from 330 to 315 nm virtually eliminated forest water sample interferences that eluted early from the column (Figure 2). It should also be noted that these derivatives are good chromophores and can be detected by UV absorbance at 263 nm, however, with a significant sacrifice in sensitivity.

For most of our applications, we chose the $\mu NH_2$ (Alltech) column because it gave good separation and the cost was about ⅓ that of the other columns tested. With this column, a mobile phase of 0.05M phosphate (pH 6.0), and the spectrophotofluorometric detector, standard curves for glyphosate and AMPA were linear from about 0.01 to 10 μg/mL, or 3 orders of magnitude. Minimum detectable quantities (S/N = 3) were about 0.01 μg/mL (0.1 ng) for glyphosate = FMOC and 0.005 μg/mL (0.05 ng) for AMPA-FMOC (example calculation: 0.1 mL of a 0.01 μg/mL glyphosate standard is 0.001 μg; 0.001 μg in a total of 2 mL derivatizing solution yields a 0.0005 μg/mL glyphosate-FMOC solution; a 0.2 mL injection of that solution is 0.0001 μg or 0.1 ng). The 20-fold concentration factor achieved in sample preparation allows ppb method detection limits in natural water samples.

For analysis of the natural waters examined, sample preparation was minimal. Sample preparation by filtration, rotary evaporation, and a final filtration took about 1.5 h/sample and achieved a 20-fold concentration factor. In contrast, the ion exchange cleanup used previously (11) would take much longer to realize a similar concentration factor in addition to the expense and preparation time of the ion exchange resin. Furthermore, Glass (11) did not determine if AMPA was quantitatively recovered by the ion exchange method.

During the evaluation of our preparation procedure, recoveries of glyphosate from fortified deionized water were inconsistent. Since glyphosate is known to adsorb strongly to soils (5, 13), we believe that similar adsorption to glass surfaces was responsible for irregular recoveries. Subsequently, we found that addition of phosphate buffer to the water sample before concentration resulted in higher and more reproducible recoveries (see Table 2). It appears that inorganic phosphate competes with glyphosate for binding sites on glass, thereby minimizing adsorption of the analyte.

All determinations were considered to be "free" glyphosate and AMPA since filtration would remove the sorbed fraction. The waters reported here had small amounts of particulates and recoveries were good, suggesting that sorption was minimal. Waters with high levels of suspended matter, especially clays, would probably lose significant amounts of glyphosate and AMPA through filtration.

Also, the temperature of the sample may have been affecting recoveries during the rotary evaporation step. However, concentration of duplicate solutions fortified with glyphosate (plus phosphate) at 30, 40, and 50°C showed no significant differences in recovery, indicating that temperature over this range is not critical to good recovery. It should be noted that the rate of concentration was fastest at 50°C; this temperature was used throughout the course of this study.

Recovery of glyphosate and AMPA from fortified natural waters was good at all levels tested (Table 2). Standard devia-

**Table 2. Recovery of AMPA and glyphosate from fortified forest water samples, using phosphate buffer addition before concentration**

| Level of spike, ppm | Av. rec., % AMPA | Av. rec., % Glyphosate |
|---|---|---|
| 0.010 ($n$ = 3) | NA[a] | 111 (RSD 6%) |
| 0.050 ($n$ = 8) | 80 (RSD 15%) | 76 (RSD 16%) |
| 0.50 ($n$ = 3 AMPA) ($n$ = 6 GLYPH) | 100 (RSD 24%) | 91 (RSD 7%) |
| 5.0 ($n$ = 3) | 97 (RSD 7%) | 96 (RSD 10%) |

[a]NA = Not analyzed in triplicate.

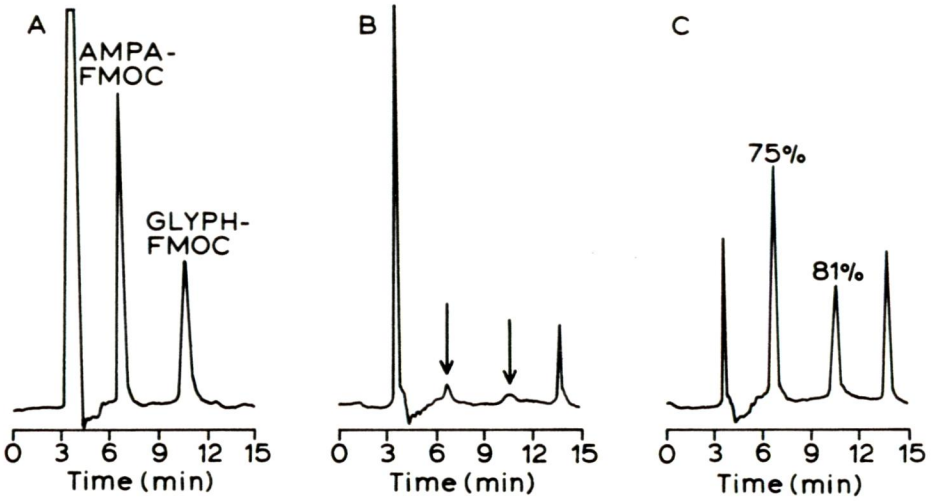


**Figure 3. Chromatograms of (A) 25 ng each AMPA-FMOC and glyphosate-FMOC, (B) forest water sample (ca 2 ppb glyphosate and AMPA), and (C) same forest water fortified with 50 ppb glyphosate and AMPA (percent recoveries are listed above peaks).**

tions were acceptable with the exception of AMPA at the 0.50 ppm level. In many water samples analyzed, interferences eluted at or near the AMPA-FMOC retention time, which made quantitation difficult, especially at lower AMPA concentrations. No interferences were observed for glyphosate-FMOC in the samples analyzed, but many samples had a peak that eluted after glyphosate-FMOC (ca 15 min; see Figure 3).

Propionic acid can act as a bactericide and we evaluated its action as a preservative for glyphosate and AMPA in water. Fortified samples that were treated with propionic acid and stored in a refrigerator (4°C) for 3 months showed significant loss of glyphosate and AMPA. For triplicate natural water samples fortified at 0.05 ppm, recoveries averaged 45% (RSD 29%) for glyphosate and 109% (RSD 32%) for AMPA. These results suggest that glyphosate was degraded to AMPA. For triplicate natural water samples fortified at 0.50 ppm, recoveries of glyphosate averaged 73% (RSD 20%) while AMPA recoveries averaged 30% (RSD 68%). The results of this storage study indicate that addition of propionic acid and refrigeration of water samples at 4°C is not sufficient to retard degradation of glyphosate for long periods.

Sometimes sample concentrates could not be analyzed immediately and were refrigerated for up to 2 weeks. To ensure stability of glyphosate, spiked samples were reanalyzed periodically. No significant decrease was observed in spiked water sample concentrates stored in the refrigerator for up to one month.

Subsequent experiments where glyphosate was fortified into a natural water (no propionic acid) and frozen at 0°C for 3 months showed that it was not significantly degraded. For duplicate natural water samples fortified at 0.05 and 0.50 ppm, recoveries of glyphosate averaged 81% (RSD 11%) and 86% (RSD 3%), respectively. Thus, freezing water samples as soon as possible after collection is suggested to ensure the stability of glyphosate in samples to be analyzed at a later date.

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