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Analysis of glyphosate, glufosinate and aminomethylphosphonic acid by capillary electrophoresis with indirect fluorescence detection

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Abstract

A capillary electrophoresis (CE)–indirect fluorescence detection method is described for the simultaneous determination of glufosinate, glyphosate and aminomethylphosphonic acid. The three analytes were separated by CE in 5 min with a 1 m*M* fluorescein solution at pH 9.5. Fluorescein also functioned as a background fluorophore for the indirect detection of these nonfluorescent species. Linearity of more than two orders of magnitudes was generally obtained. The concentration limits of detection were in the μ*M* range. Precisions of migration times and peak areas were less than 1.7% and 7.4%, respectively. Quantitation of glyphosate and glufosinate in commercial herbicides is demonstrated. In addition, the applicability of the method for the analysis of ground water was examined.

Keywords: Indirect detection; Detection, electrophoresis; Glyphosate; Glufosinate; Aminomethylphosphonic acid; Phosphoric acids; Pesticides; Organophosphorus compounds

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine] (GLYP) and glufosinate [DL-homoalanine-4-yl-(methyl)phosphinic acid] (GLUF) are non-selective herbicides for control of long grasses and broad-leaved weeds. As shown in Fig. 1, GLYP and GLUF have similar chemical structures. Once applied to agriculture, it is absorbed and translocated throughout the plant tissues. They will interfere with the formation of amino acids and other chemicals in plant [1]. Photosynthesis and respiration are also affected. The treated plants will die in 1–3 weeks. These herbicides are widely used all over the world for different applications. Therefore, there is a need to develop a rapid and sensitive analytical method for the determination of GLUF, GLYP and aminomethylphosphonic acid (AMPA), the main metabolite of GLYP, in a variety of sample matrices.

The analytical methods of GLYP, GLUF and related compounds have been reviewed by Stalikas [2]. The difficulties in establishing analytical methods for the determination of these compounds at residue level are mainly due to their properties: relatively high solubility in water, insolubility in organic solvents and favoured complexing behavior. In GC analysis [3], sample derivatization is necessary to enhance the volatility of analyte. Typical derivatization agents used includes trifluoroacetic anhydride in conjunction with trifluoroethanol [4–7] and heptafluorobutanol [8]. Several LC methods also

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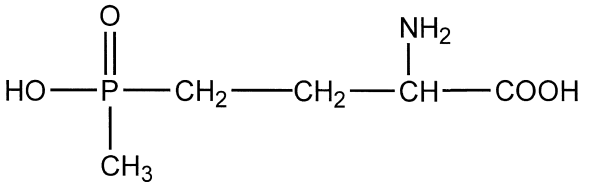
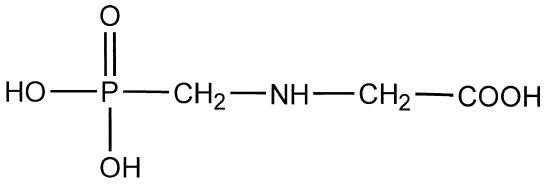
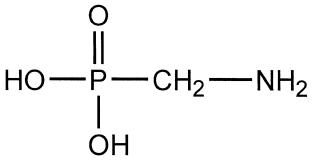


GLUF



GLYP



AMPA

Fig. 1. Structural formula of GLUF, GLYP and AMPA.

have been used to achieve the separation [9,10]. Since they do not have a chromophore or a fluorophore in their structure, it is not easy to detect them with UV–Vis detection except at low UV wavelengths, where the detection limits are not favorable. Lengthy extraction and clean-up procedures are required for the analysis of real sample. High-performance liquid chromatography (HPLC) techniques with pre- or post-column derivatization offer more variability. 9-Fluorenylmethyl chloromate [11–13], *p*-toluenesulphonyl chloride [14] and *o*-phthalaldehyde–mercaptoethanol (OPA–MERC) [15–18] have been commonly used as derivatizing agents with fluorescence detection. Post-column indirect fluorescence detection [19] was also reported where the background fluorescence is provided by an Al^{3+}-Morin (3,5,7,2′,4′,-pentahydroxyl flavone) complex. In addition, LC–electrospray mass spectrometry [20,21] was reported for the determination of GLYP and AMPA. In recent years, capillary electrophoresis (CE) has been an important separation techniques due to its high resolving power and speed. *p*-Toluenesulfonyl chloride [22] was used to derivatize GLYP and AMPA prior to separation by CE, followed by detection with an UV absorbance detector. Ribonucleotides [23] and phthalate [24] also have been employed to provide the background signal for indirect UV detection in CE analysis of GLYP and AMPA.

In this paper, a scheme for the separation and detection of the GLUF, GLYP and AMPA using CE coupled with indirect fluorescence detection is described. Fluorescein was employed as the buffer fluorophore and an argon-ion laser was used to induce the fluorescence background. Linearity, reproducibility and detection limits were examined. The feasibility of this method for the analysis of real sample was also investigated.

2. Experimental

2.1. Chemicals

Glufosinate ammonium and sodium fluorescein were purchased from Riedel-de Haen (Milwaukee, WI, USA). Glyphosate and aminomethylphosphonic acid were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade. Water purified with a Barnstead NANOpure system (Dubuque, IA, USA) was used for all solutions. Stock solutions of GLUF, GLYP and AMPA at a concentration of 1 m*M* were prepared in deionized water and kept at 4 °C. Working standard solutions of lower concentrations were prepared by dilution with the running buffer. The running buffer containing fluorescein was prepared with deionized water. The

pH of the running buffer was adjusted by addition of NaOH.

2.2. Instrumentation

The capillary electrophoresis–laser-induced fluorescence (CE–LIF) system was assembled in-house. A 0–30 kV power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) provided the separation voltage. The capillary used for separation was 60 cm total length (50 μm I.D.×360 μm O.D.) (Polymicro Technologies, Phoenix, AZ, USA). The effective length of capillary is 40 cm. The 488 nm beam (10 mW) from an argon-ion laser (Uniphase, San Jose, CA, USA) was used for excitation. The laser light was focused into the capillary with a 1.4 cm focal length lens. Background fluorescence emitted from the fluorescein in the CE buffer was collected with a 10× microscope objective and passed through a 520 nm interference filter (Edmund Scientific, Barrington, NJ, USA). The collected fluorescence was detected by a photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). Recording of electropherograms and quantitative measurements of peak area were performed with a computer connected to a Turbochrom data acquisition interface (Perkin-Elmer, San Jose, CA, USA).

2.3. Procedure

The capillary was rinsed daily with methanol for 10 min, followed by a 5 min rinse with water and a 5 min flush with the running buffer. The capillary was then equilibrated with the running buffer under an electric field of 250 V/cm for 30 min. Samples were injected at the anodic end of the capillary by hydrostatic injection. The sample was injected by raising the anodic end 14 cm above its normal position for 5 s.

3. Results and discussion

3.1. Separation and detection

In indirect fluorescence detection, a fluorescing ion is added to the running buffer to create a constant fluorescence background. A nonfluorescent analyte ion then displaces the fluorescent ion of the same charge due to local charge neutrality, resulting in a decreased background signal. Fluorescein was employed as the background fluorophore which can be excited by the 488 nm beam of an argon-ion laser. However, the fluorescence intensity of fluorescein is pH dependent. With increasing solution pH, fluorescence intensity increases rapidly and reaches a constant level at pH≥8 [25]. In addition, the ionization capability of analytes is affected by the pH of buffer solution. The influence of buffer pH on the separation of these analytes was studied in the pH range 8–11. Fig. 2 compares the electropherograms obtained under different buffer pH. The general trend observed indicates that the apparent mobility increases slightly for all the species when the pH is raised from 8 to 10. At this pH range, GLYP has two negative charges while GLUF and AMPA are single-charged ions. The increased mobility is due to the increased electroosmotic mobility with increasing pH. At pH 10, the migration times for all species slightly delayed. This is due to the partial changes in analyte charges. The pK_4 of GLYP is 10.2 [24], therefore double- and triple-charged forms of GLYP co-exist at pH 10. For GLUF and AMPA, since their

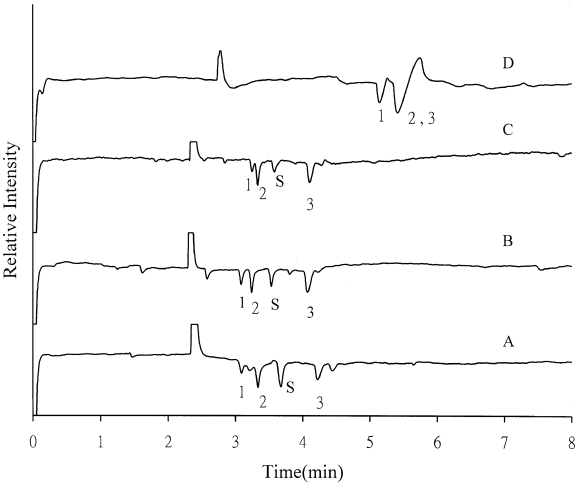


Fig. 2. Effect of buffer pH on the CE–indirect LIF detection of GLUF, GLYP and AMPA. Buffer composition: 1.0 m*M* fluorescein and (A) pH 8.0, (B) pH 9.0, (C) pH 10.0 and (D) pH 11.0. Peak identities: 1, GLUF (4.2×10^{-5} *M*); 2, AMPA (9.9×10^{-5} *M*); 3, GLYP (5.33×10^{-5} *M*); s, system peak. Conditions: fused-silica capillary, 60 cm (40 cm to the window) 50 μm I.D.×360 μm O.D.; applied voltage, 15 kV; hydrostatic injection, 5 s (14 cm); LIF detection, $\lambda_{ex}=488$ nm, $\lambda_{em}=520$ nm.

pK_3 are between 10 and 11, they are single and double-charged ions at pH 10. When the pH is raised to 11, the migration times for all species seriously delayed, and AMPA was not separated from GLYP. GLYP has three negative charges while GLUF and AMPA have two negative charges. Therefore, they migrate slower toward cathode. Since AMPA and GLUF have same charges, they cannot be separated at pH 11.

The effect of the concentration of fluorescein on migration time and detection sensitivity is illustrated in Fig. 3. With decreasing fluorescein concentrations, the effective mobility of these three species increased and migration time decreased. This is mainly due to the increasing ξ-potential of the capillary which increases the electroosmotic flow (EOF) velocity. In addition, the detector response of the system for the three species has a maximum at 1 m*M* fluorescein (Fig. 3B). At lower concentrations of fluorescein, sensitivity decreases due to low dynamic reserve (DR is equal to the S/N ratio of the background signal), which is caused by serious baseline shifting and high noise level [26]. At concentrations above 1 m*M*, the response gradually deteriorates because more light reaches the photomultiplier tube (PMT), which reduces ability to measure a small change on top of a large background signal.

In order to obtain a good compromise between detection and separation of all species, 1 m*M* fluorescein (pH 9.5) buffer was adopted for the indirect fluorescence detection of GLYP, GLUF and AMPA. Fig. 4 shows the electropherogram of a mixture of GLUF, GLYP and AMPA. All these species were efficiently detected and baseline separation was achieved within 5 min. Good peak shapes and high efficiencies were obtained. The peak marked as ''s'' is a system peak. This system peak was confirmed by injecting a blank solution of water.

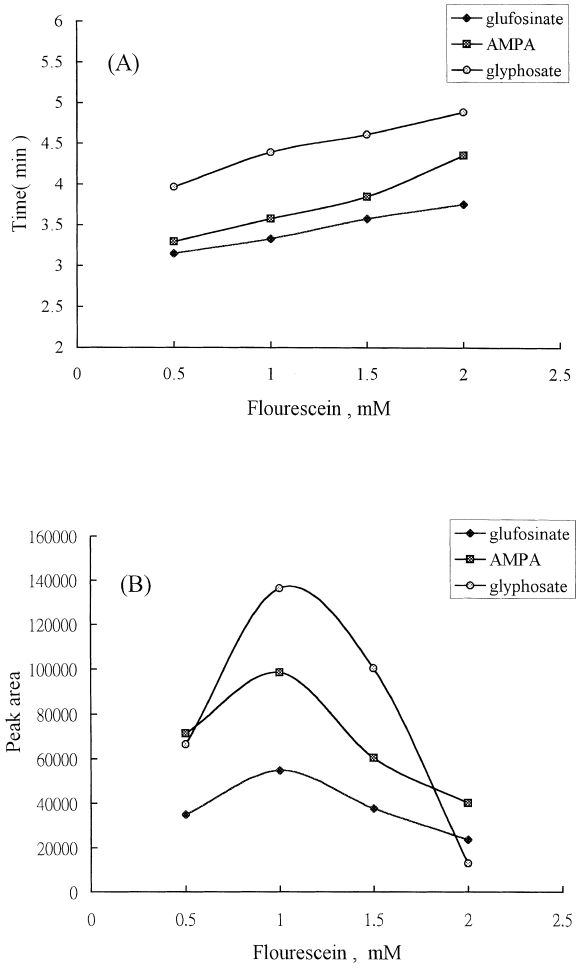


Fig. 3. Effect of fluorescein concentration on (A) migration times of GLUF, GLYP and AMPA, and (B) peak areas in CE separation. Conditions as in Fig. 2, buffer pH 9.5.

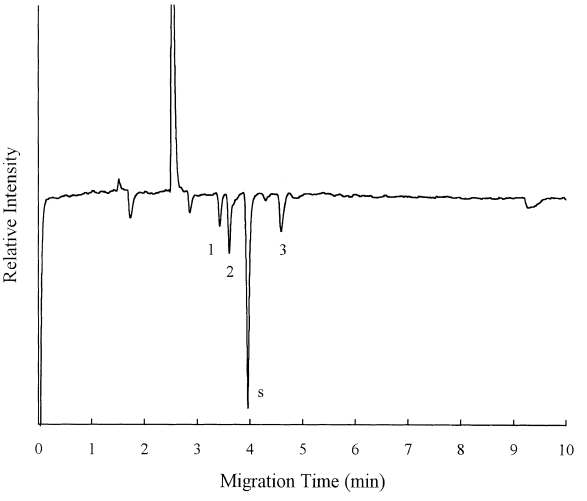


Fig. 4. Electropherogram of a mixture of GLUF (4.2×10^{-5} *M*), GLYP (5.33×10^{-5} *M*) and AMPA (9.9×10^{-5} *M*). Buffer composition: 1.0 m*M* fluorescein at pH 9.5. Peak identities and other conditions as in Fig. 2, pH 9.5.

Table 1
Calibration data and relative standard deviations of migration times and peak areas for GLUF, GLYP and AMPA

Species	Linear range (μM)	Slope	Intercept	r^a	RSD (%)[b]	
					Migration time	Peak area
GLUF	6–1970	1466	+17	0.9996	1.7	5.1
GLYP	13–1600	1667	+95	0.9906	0.8	2.2
AMPA	38–1890	626	−4	0.9982	1.3	7.4

[a] Correlation coefficient.
[b] Relative standard deviation, based on 10 measurements with replicate injections of each species at the conditions as described in Fig. 4.

3.2. Calibration

A series of solution mixtures containing a known amount of GLUF, GLYP and AMPA were prepared using the running buffer as the solvent. These standard solutions were used for the construction of calibration curves. The results are summarized in Table 1. Within the concentration range studied, a good linear correlation ($r>0.99$) between peak areas and concentrations was obtained for each analyte. The linear dynamic range covered more than two orders of magnitude of concentration. The concentration limits of detection (LODs) were calculated based on an S/N ratio of 3, which are 2.5, 7.7, 15.9 μM for GLUF, GLYP and AMPA, respectively. The repeatability of migration times and peak areas was evaluated by ten replicate injections of each analyte. Results expressed in RSD range from 0.8 to 1.7% and from 2.2 to 7.4% for migration times and peak areas.

3.3. Transfer ratio and detection limit

In principle, the concentration limit of detection (cLOD) for a species under a given indirect detection scheme can be related to C_M (the concentration of background fluorophore in the running buffer that is being monitored), TR (transfer ratio) and DR (dynamic reserve) by [27]:

$$\text{cLOD} = C_M/(\text{TR}\cdot\text{DR}) \quad (1)$$

Therefore, the cLOD can be lowered by decreasing C_M. However, the three parameters are not necessarily independent. Under some circumstances as one reduces C_M, TR and DR can also decrease, causing no improvement in detection sensitivity. In the present study, 1 mM fluorescein was chosen as C_M. The background fluorescence intensity of this solution was 420 mV and the peak-to-peak noise level of the background was 0.25 mV. DR was calculated to be 1680. The TR values for all species studied were determined according to the published procedure [28]. Table 2 summarizes the results for TR, the cLODs calculated from Eq. (1) and the cLODs estimated based on an S/N ratio of 3. The cLODs estimated based on a S/N ratio of 3 are about four to nine times higher than those calculated from Eq. (1). However, the results in Table 2 follow the general trend seen in the experiments, i.e. the larger the TR value, the higher the detection sensitivity. The cLODs of GLUF and GLYP obtained in the

Table 2
Transfer ratios and concentration limits of detection

Species	Transfer ratio	cLOD (μM)	
		Calculated from Eq. (1)	Based on $S/N=3$
GLUF	1	0.6	2.5
GLYP	0.5	1.2	7.7
AMPA	0.35	1.7	15.9

present study are comparable to those obtained by indirect absorption detection coupled with stacking techniques [24].

3.4. Application

The developed method in this work was applied to the commercial herbicide analysis. Ninninchun is the trade name for a 41% GLYP solution produced by Yih Fong Chemical Corp. (Taiwan). Basida containing 13.5% GLUF was produced by BASF, Taiwan. Both them were bought locally. The samples were diluted 5000-fold with running buffer and filtered through a 0.45 μm membrane filter, followed by direct injection into the CE–LIF system. The separation was affected by the sample matrix. Owing to the low concentration (1.0 m*M*) of the buffer electrolyte applied, electroosmosis is sensitive towards impurities, pH shifts, etc. In order to separate the main ingredient from other impurities, we decreased the separation voltage to 9 kV. The electropherograms of Ninninchun and Basida are shown in Fig. 5A and B, respectively. From Fig. 5A, AMPA was not present in Ninninchun or was present at a concentration below the detection limit. The GLYP concentration obtained was in good agreement with the value claimed on the label. The relative error listed in Table 3 is 1.5%. For Basida, the GLUF concentration is lower than the value on the label and the relative error is 14.8%. This may be caused by the insufficient active ingredient in the product. These peaks marked ''i'' are other components or impurities in the herbicide products. There is no need to derivatize analyte before separation that will increase the analysis time. Therefore, the developed method could be applied for the quality control of herbicide products.

In order to assess the applicability of this method to the environmental analysis, a ground water sample was used. The sample was first filtered through a 0.45 μm membrane filter, followed by direct injection into the CE–LIF system without dilution. No detectable amount of GLYP and GLUF were found. The sample was then spiked with 1.69 μg/ml GLYP, 1.98 μg/ml GLUF and 1.11 μg/ml AMPA. The electropherograms of both unspiked and spiked water samples are shown in Fig. 6A and B, respectively. The separation voltage was decreased to 7 kV,

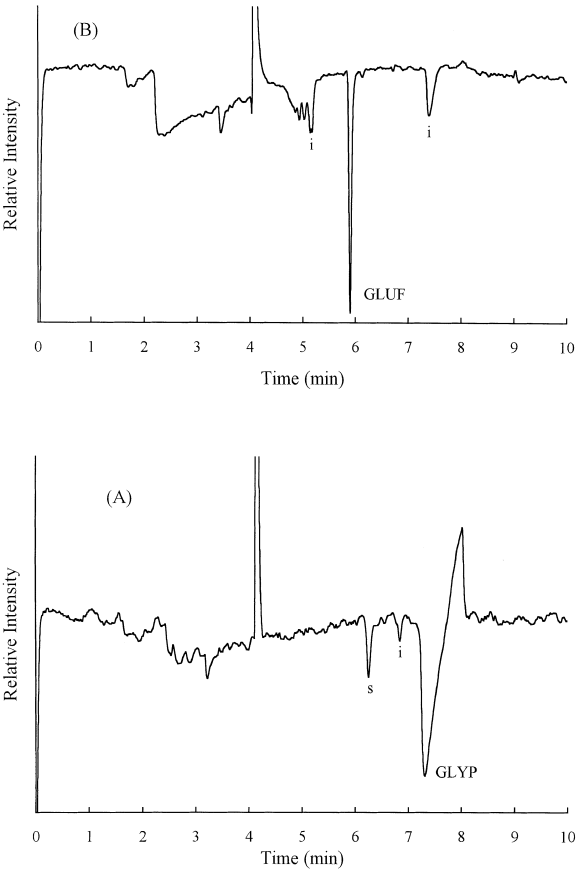


Fig. 5. Electropherograms of (A) Ninninchun herbicide solution and (B) Basida herbicide solution.

however, GLYP cannot be separated from the background signal of ground water. This is a common problem of CE with real sample matrices. The recoveries of GLUF and AMPA were determined by spiking 1 ml of ground water with 1.98 μg GLUF and 1.11 μg AMPA. Quantitation was performed with standard addition method. Based on triplicate measurements, the mean recoveries were found to be 96% and 95% for GLUF and AMPA, respectively.

Table 3
Results for the analysis of commercial herbicides

	GLYP (%)	GLUF (%)
Claimed on label	41.0	13.5
CE-indirect LIF	41.6	11.5
Relative error	1.5	14.8

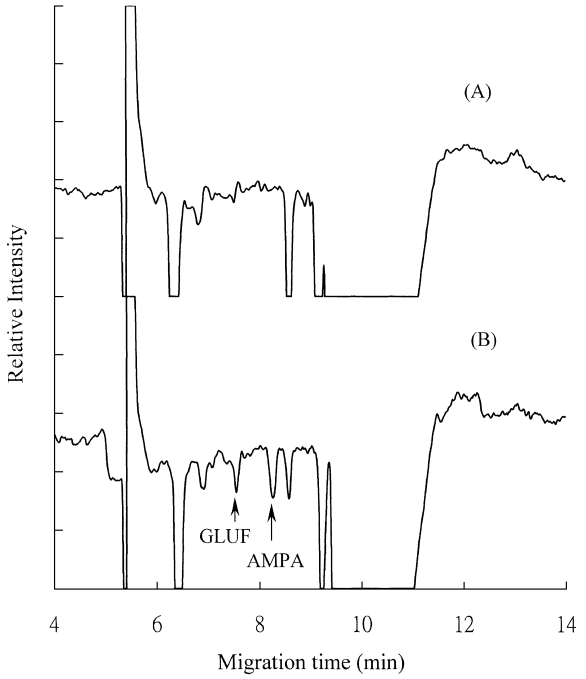


Fig. 6. Electropherograms of GLYP, GlUF and AMPA in ground water. (A) Ground water, (B) ground water spiked with GLYP (1.69 μg/ml), GLUF (1.98 μg/ml) and AMPA (1.11 μg/ml). Other conditions as in Fig. 2; applied voltage, 7 kV; buffer pH 9.5.

4. Concluding remarks

CE–indirect fluorescence detection with fluorescein as the buffer fluorophore has been demonstrated to be an efficient and sensitive method for the analysis of herbicides. The cLODs for GLUF, GLYP and AMPA with indirect LIF detection were in the μ*M* level. The direct analysis of main ingredient in commercial herbicides showed that the application of this technique to routine analysis of herbicide products is possible. The developed method can be applied to the direct analysis of GLUF and AMPA in ground water. However, application of this method to the direct analysis of GLYP in ground water is still problematic. In cooperation with an appropriate sample pretreatment procedure prior to CE analysis is probably a solution for these environmental samples of complex matrices.

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