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Integrated pulsed amperometric detection of glufosinate, bialaphos and glyphosate at gold electrodes in anion-exchange chromatography

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Abstract

A rapid and practical method for direct detection of the herbicides (glufosinate, bialaphos and glyphosate) in anion-exchange chromatography has been developed with integrated pulsed amperometric detection (IPAD). The electrochemical behavior of these herbicides showed catalytic currents based on the oxidation of amines in their structures. Waveform in IPAD was similar to that for amino acids, which exhibited adsorption/desorption catalytic features at gold electrode surface in alkaline solution. Under optimized conditions, detection limits (signal-to-noise ratio of 3) for glufosinate, bialaphos and glyphosate were 20, 65 and 50 ng ml^{-1}, respectively, with correlation coefficients of 0.995, 0.997 and 0.996 over concentration ranges of 0.1–45, 0.3–32 and 0.1–50 μg ml^{-1}, respectively. The relative standard deviations ($n=5$) were 1.7–3.0%. The present method was successfully applied to the determination of glyphosate in urine and serum.

Keywords: Gold electrodes; Integrated pulsed amperometry; Glufosinate; Bialaphos; Glyphosate; Pesticides

1. Introduction

Glufosinate [DL-homoalanine-4-yl(methyl) phosphinic acid], bialaphos [L-2-amino-4-(hydroxy-)(methyl)phosphonoyl]butyryl-L-alanine and glyphosate [*N*-(phosphonomethyl)glycine] are phosphorus-containing amino acid-type herbicides (see Fig. 1). As they are of comparatively low toxicity to humans and animals, these herbicides have been extensively used. However, because their effects on non-target

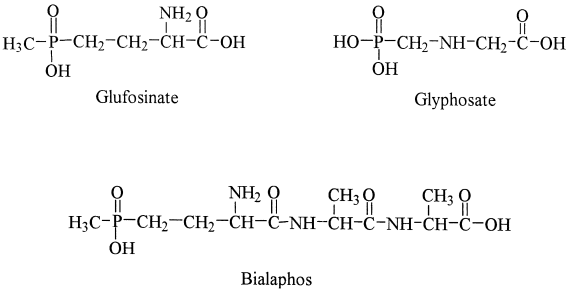

Fig. 1. Structures of glyphosate, glufosinate and bialaphos.

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organisms and the environment have not been entirely investigated, questions regarding the environmental safety with their increasing use have to be addressed. Therefore, the monitoring of trace levels of these compounds in environmental and biological samples has gained increasing importance.

The determination of glufosinate, bialaphos and glyphosate by gas chromatography (GC) and high-performance liquid chromatography (HPLC) is well documented. However, the detection of these compounds in HPLC is not very simple because they lack an inherent chromophore or fluorophore for photometric and fluorometric detection. On the other hand, gas chromatographic separations are difficult due to the high polarity of these compounds. Therefore, a pre- or post-column derivatization procedure is required to improve both the chromatographic behavior and the detection ability in GC and HPLC analysis. For example, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide [1] and isopropyl chloroformate [2] were employed in the pre-column derivatization procedures in GC, whereas 9-fluorenylmethylchloroformate was used for high sensitivity fluorescence detection [3,4] and mass spectrometry (MS) [5] in HPLC analysis. Although, the detection of derivatives in GC or HPLC exhibited high sensitivity and selectivity, the preparation of derivatives is a time-consuming procedure. In addition, the derivatization frequently generates products that are unstable. Recently, some detection methods without derivatization, such as electrogenerated chemiluminescence detection [6], ion chromatography coupled with MS [7], conductivity detection [8] or inductively-coupled plasma (ICP) MS [9], have been reported. As poisoning incidents caused by these herbicides gradually increase in Japan, a simple and rapid quantitative method is desired from the viewpoint of forensic and emergency toxicology.

Pulsed electrochemical detection at gold or platinum electrodes has become one of the most important detection techniques in anion-exchange chromatography (AEC). With a pulsed waveform of positive and negative potential excursions for ''on-line'' cleaning and reactivation of the electrode surface, it is possible to detect compounds containing amine, alcohol, or sulfur moieties directly in alkaline medium [10–12]. Because, glufosinate, bialaphos and glyphosate are amphoteric compounds, they are present as anions under alkaline conditions. Therefore, they are amenable to separation by AEC. Moreover, these compounds contain primary or secondary amine groups, they are therefore expected to be directly oxidized at gold electrodes, resulting in analytical signals under alkaline conditions. In this study, integrated pulsed amperometric detection (IPAD) was applied to detect these compounds in AEC. The principal experimental parameters governing its performance were characterized. It was demonstrated that AEC–IPAD has the advantages of a rapid and simple method for the determination of glufosinate, bialaphos and glyphosate in real samples.

2. Experimental

2.1. Reagents

Ammonium salt of glufosinate (99.2%), sodium salt of bialaphos (97.1%), glyphosate (99.6%) and isopropylamine salt of glyphosate (62% aqueous solution) were provided by Aventis CropScience Japan (Chiba, Japan), Meiji Seika Kaisha (Tokyo, Japan), Zeneca (CA, USA) and Monsanto Japan (Tokyo, Japan), respectively. Sodium hydroxide (NaOH) and amino acids were purchased from Wako (Tokyo, Japan). Water was purified by a Milli-Q system (Millipore, France). The reagents were used without further purification. The samples were made freshly by dilution with water prior to the chromatographic experiments.

2.2. Apparatus

An ALS/CHI Model 802 Electrochemical Analyzer (ALS/CH Instruments, Tokyo, Japan) was used for voltammetric measurements. All experiments employed a three-electrode electrochemical cell with a working electrode, an Ag/AgCl reference electrode (Model RE-1, BAS Japan, Tokyo, Japan) and a platinum wire auxiliary electrode. The electrochemical cell was housed in a Faraday cage (Model C-1, BAS Japan) to minimize the interference from external electric noise. The working electrode for voltammetric measurements was made by sealing a gold rod of 1-mm diameter into a Plexiglas tube with

epoxy resin. It was first sanded using Emery papers (Nihonkenshi, Japan) in successive order of grit size 400, 600 and 2400, and then polished with alumina powders of 0.3- and 0.05-μm size (Refine Tech, Yokohama, Japan) on a cloth pad (No. 53-108, Refine Tech). Finally, the electrode was sonicated and rinsed thoroughly with purified water.

Chromatographic experiments were performed with a Dionex ion chromatography system (DX-500) consisting of a GP50 gradient pump, a Rheodyne injector with a 25-μl sample loop and an IonPac AS15 anion-exchange column (250×4 mm I.D., Dionex) coupled with an IonPac AG15 (50×4 mm I.D.) guard column. The mobile phases were Milli-Q water and 0.20 *M* NaOH, which were kept under nitrogen and passed through an on-line degasser in the HPLC pump (GP50, Dionex). Anion-exchange chromatographic separation of the analytes was carried out at a flow-rate of 1.0 ml min^{-1}. For the amperometric measurements, a thin-layer flow cell with a 25-μm thick PTFE gasket (the volume of the cell was 0.3 μl) connected to the ED 40 electrochemical detector (Dionex Japan, Tokyo, Japan) was used. The flow cell consisted of an Au working electrode, an Ag/AgCl reference electrode and a titanium cell body as an auxiliary electrode. The working electrode was constructed by embedding an Au rod (1-mm diameter) into a Kel-F block. The experiments were carried out at room temperature (ca. 25°C).

2.3. Real sample preparations

Human blood and urine samples were obtained from healthy volunteers in our laboratory. The human serum was prepared by centrifuged human whole blood for 10 min at 3000 *g*. After human serum was diluted 1:100 in Milli-Q water, and then transferred to a centrifugal filtration tube (ultrafiltration membrane nominal molecular weight limit (NMWL) 30 000, Ultrafree-MC, Millipore) centrifuging for 15 min at 3000 *g*. The filtrate was used as the sample solution for analysis.

The human urine was diluted 1:1000 in Milli-Q water, and was transferred to a centrifugal filtration tube (0.22-μm pore size microporous membrane, Ultrafree-MC, Millipore). After being centrifuged for 5 min at 3000 *g*, the filtrate was used as the sample solution for analysis.

Isopropylamine salt of glyphosate was administered orally to a male mouse (37 g) at a level of 750 mg kg^{-1}. After 6 h, the mouse urine was collected, and was prepared in the same way as the human urine.

3. Results and discussion

3.1. Voltammetric responses at gold electrodes

The voltammetric responses for glyphosate, glufosinate and bialaphos at gold electrodes in NaOH solution were examined by cyclic voltammetry. Fig. 2 shows the cyclic voltammograms of glyphosate at gold electrodes in 0.1 *M* NaOH solution (air-saturated). The dashed line is the voltammogram in the absence of the analyte. During the positive scan, a broad anodic peak in the vicinity of +0.30 V was attributed to the oxide formation (AuOH and AuO) on the electrode surface. On the reserve scan, the cathodic peak at +0.10 V was due to the reduction of the surface oxide, whereas the peak at −0.2 V was caused by the reduction of dissolved oxygen. In the presence of 5 m*M* glyphosate (solid line), an in-

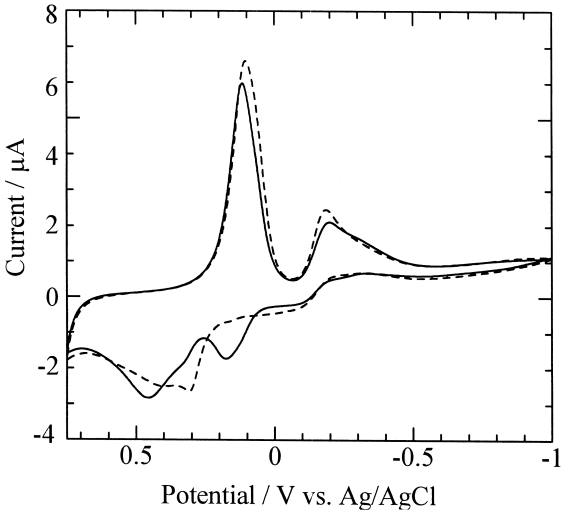

Fig. 2. Cyclic voltammograms of glyphosate at gold electrodes in 0.10 *M* NaOH. Solid line: 5 m*M* glyphosate; dashed line: 0 m*M* glyphosate. Scan rate: 100 mV s^{-1}.

creased anodic current was observed near +0.20 V at the positive scan. It was found that the anodic wave was proportional to the concentration of glyphosate. Similar catalytic currents were also observed for glufosinate and bialaphos (data not shown). The electrochemical behavior of some amines and sulfur-containing compounds on gold electrode had been well documented by Johnson and LaCourse [10]. It is believed that amines would adsorb on an oxide-free gold surface with their nonbonded electron pair on the N-atom. Under alkaline conditions, the adsorbed species can be oxidatively desorbed simultaneously with the gold oxide formation process to give useful analytical signals. The intermediate products in the oxide formation (AuOH) may act as an active site to catalyze the electrochemical oxidation of amines at gold electrodes. Recently, Clarke et al. applied a rotated ring-disk voltammetric technique to study the oxidation and adsorption potentials for various amino acids on gold electrodes, and used their results to optimize a waveform in IPAD [12]. The adsorption/desorption phenomena were confirmed. As the structural features of glyphosate, glufosinate and bialaphos are very similar to amino acids, which contain primary or secondary amine groups, the responses for glyphosate, glufosinate and bialaphos at gold electrodes were considered to be similar to the catalytic oxidation of amines [10,13] and amino acids [10–12].

In this study, potential preconditioned cyclic voltammetry was used to examine potential dependence adsorption behavior for glyphosate, glufosinate and bialaphos on a gold electrode in 0.1 *M* NaOH. When the electrode was preconditioned at −0.3 V for 5 s, relative larger catalytic oxidation currents were observed for these compounds. At −0.3 V, the surface oxides were completely reduced, and therefore, the analytes were considered to be adsorbed on an oxide-free electrode surface. During the anodic scan, an oxide-catalyzed wave was observed near +0.2 V. On the contrary, when the electrode was preconditioned at +0.3 V for 5 s, the resulting oxidation currents at +0.2 V decreased significantly. This is because a stable oxide film (AuO) will form on the electrode surface at this potential. The presence of oxide film would inhibit the adsorption of the analytes, and consequently, cannot support the oxidation mechanism as described above. These results are consistent with the conclusion that preadsorption of the analytes on oxide-free surface is required for the detection.

3.2. Waveform optimization in IPAD

On the basis of the voltammetric results described above, it is apparent that amperometric detection of glufosinate, bialaphos and glyphosate at gold electrodes is possible in flow streams. However, the oxide film must be cathodically dissolved by a negative potential excursion to regenerate the clean electrode surface. To solve this problem, multi-step potential waveforms are applied to prepare clean and reactive electrode surfaces reproducibly. As can be seen in Fig. 2, the maximum oxidation current was observed at nearby potential of surface oxide formation. Hence, a large baseline signal is always encountered in such pulsed detection mode. To enhance the detection signal generated by the oxidation of analyte and to suppress the signal from the oxidation of gold, Johnson and co-workers developed a new technique – IPAD [10,11]. Although similar to pulsed amperometry, in which repeating multi-potential waveform is applied to a working electrode, the potential during current sampling is not held constant but varied between a high and low value. For example, the oxidation of glufosinate and gold occurs simultaneously at the higher potential. The gold oxide formed during the higher potential is reduced during the lower potential. Since the oxidation of gold is reversible and the oxidation of glufosinate is irreversible, the anodic charge for the formation of oxide on the anodic scan tends to be compensated by the corresponding cathodic charge (opposite polarity). As a result, the net signal will be only contributed from the oxidation of analyte.

In the applications of IPAD in flow systems, a choice of the operational waveform is important. Recently, Clarke et al. modified the pulsed waveform in IPAD with six-step potentials for the detection of amino acids in anion-exchange chromatography [12]. The waveform has the features of minimized baseline drift during the gradient and improved signal-to-noise ratio. In this study, we applied a similar waveform for the detection of glufosinate, bialaphos and glyphosate because their structures and

electrochemical behaviors at gold electrodes are similar to amino acids. The waveform consists of three distinct regions divided by adsorption/initiation, E1, E2; current integration, E3, E4; and cleaning/activation, E5, E6, as is shown in Fig. 3. E1 and E3 are factors affecting the detection sensitivity, and therefore were especially examined. Values for E2, E4, E5, E6 and the duration of six potentials were similar to those described in the literature [12]. Fig. 4 shows the IPAD responses of 50 μ*M* each of glufosinate, bialaphos and glyphosate as a function of E1 (A) and E3 (B) obtained in anion-exchange chromatographic experiments. Based on chromatographic conditions in the subsequent section, 0.04 *M* NaOH was used as mobile phase for glufosinate and bialaphos, and 0.10 *M* NaOH for glyphosate at a flow-rate of 1.0 ml min^{-1}. As can be seen in Fig. 4A, a preferred value for E1 was −0.4~−0.3 V, and −0.3 V was chosen for E1. In Fig. 4B, the maximum responses for glufosinate and bialaphos were +0.23~+0.25 V, whereas the maximum response for glyphosate was about +0.20 V. A potential value of +0.23 V gave the best compromise and was chosen as E3. As a result, the waveform for IPAD in this study was optimized as shown in Table 1.

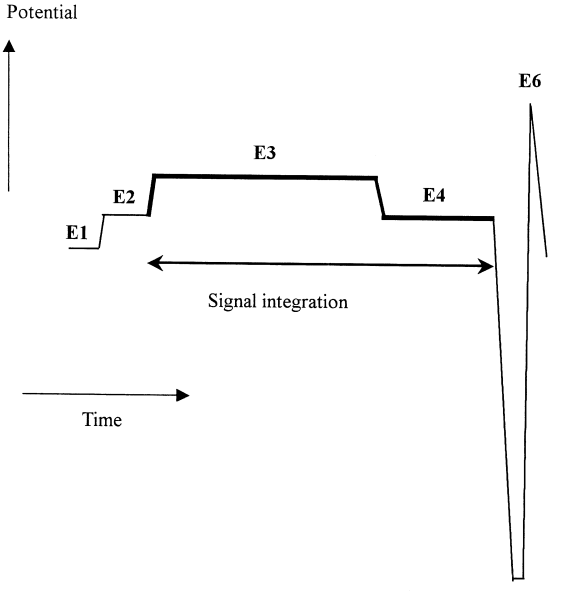

Fig. 3. Waveform of IPAD.

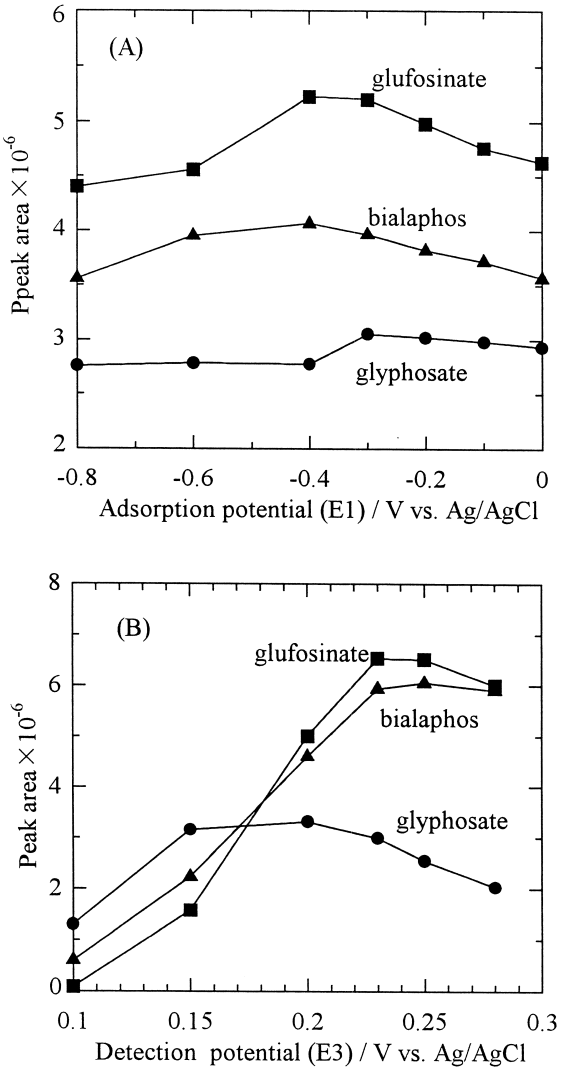

Fig. 4. Effects of (A) E1 (adsorption potential) and (B) E3 (detection potential) on the peak area of glufosinate (■), bialaphos (▲) and glyphosate (●). Concentration of the analytes: glufosinate 9.1 μg ml^{-1}, bialaphos 16.2 μg ml^{-1}, glyphosate 8.5 μg ml^{-1}; mobile phase: 0.04 *M* NaOH for glufosinate and bialaphos, 0.10 *M* NaOH for glyphosate; flow-rate: 1.0 ml min^{-1}; sample injection volume: 25 μl.

3.3. Anion-exchange chromatography with IPAD

Glufosinate, bialaphos and glyphosate exist as anions in NaOH solution. Therefore, they were separated by an anion-exchange column (Dionex

Table 1
Waveform specifications for IPAD

Time (s)	Potential (V) vs. Ag/AgCl		Integration
0.00	E1	−0.30	
0.04		−0.30	
0.05	E2	−0.05	
0.11		−0.05	Begin
0.12	E3	+0.23	
0.41		+0.23	
0.42	E4	−0.05	
0.56		−0.05	End
0.57	E5	−2.00	
0.58		−2.00	
0.59	E6	+0.63	
0.60		−0.30	

IonPac AS15) using NaOH solution as mobile phase, and detected by IPAD. The employed waveform and the specifications are shown in Fig. 3 and Table 1.

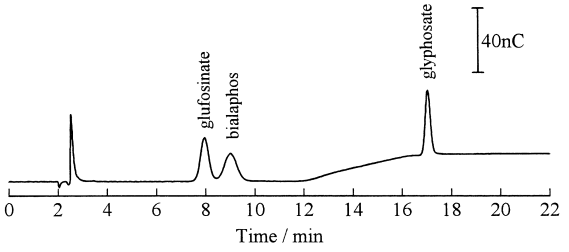

Fig. 5. Chromatogram of a standard mixture of glufosinate, bialaphos and glyphosate detected at gold electrodes by IPAD. Concentration of the analytes: glufosinate 9.1 μg ml^{-1}, bialaphos 16.2 μg ml^{-1}, glyphosate 8.5 μg ml^{-1}. Mobile phase condition: isocratic elution with 0.04 *M* NaOH for 9 min, linear gradient elution to 0.10 *M* NaOH for the next 4 min, and then isocratic elution with 0.10 *M* NaOH. Column: IonPac AS15 (250×4 mm I.D.). Flow-rate: 1.0 ml min^{-1}. The waveform and the specifications for IPAD are shown in Fig. 3 and Table 1. Sample injection volume: 25 μl.

Binary gradient elution by Milli-Q water and 0.20 *M* NaOH was executed at a flow-rate of 1.0 ml min^{-1}. Fig. 5 shows a typical chromatogram for glufosinate, bialaphos and glyphosate. The retention behavior of glufosinate, bialaphos and glyphosate depended on the hydroxide concentration. The higher the concentration of NaOH solution is, the shorter the retention times for glufosinate, bialaphos and glyphosate become. Glufosinate and bialaphos could not be separated completely when hydroxide concentrations were higher than 0.04 *M* NaOH. Therefore, 0.04 *M* NaOH was used as mobile phase. As the retention time for glyphosate became too large in this condition, a linear gradient elution mode was employed in order to make it elute faster. Thus, in this study, the separations were conducted by using isocratic elution with 0.04 *M* NaOH for 9 min, linear gradient elution to 0.10 *M* NaOH for the next 4 min, and then isocratic elution with 0.10 *M* NaOH.

The peak at 2.5 min was most likely due to the ammonium ion dissociated from the ammonium salt of glufosinate. The slight baseline drift after 12 min was attributed to the changes in pH and ionic strength by the gradient of hydroxide concentration. The detection limit, retention time and dynamic range of this approach are summarized in Table 2. The detector exhibited a linear response up to at least 32 μg ml^{-1}. The relative standard deviations (RSDs) (n=5) were 1.7–3.0%, and the detection limits were 20, 65 and 50 ng ml^{-1} for glufosinate, bialaphos and glyphosate, respectively. Although the detection limits are much higher than in the methods using MS [5,7,9], AEC–IPAD has its own merit of simple, rapid and good selectivity. Actually, with poisoned or deceased persons, the concentrations of these compounds in serum and urine are often very high

Table 2
Determination of glufosinate, bialaphos and glyphosate by AEC–IPAD[a]

Sample	Retention time (min)	Detection limit[b]		Linear range (μg ml^{-1})	r^2	RSD[c] (%)
		ng	μg ml^{-1}			
Glufosinate	8.0	0.5	0.018	0.1–45.3	0.995	3.0
Bialaphos	9.0	1.6	0.065	0.3–32.3	0.997	2.7
Glyphosate	17.0	1.3	0.051	0.1–50.7	0.996	1.7

[a] The chromatographic conditions are as in Fig. 5.
[b] Detection limits are estimated at S/N ratio ≥3.
[c] Relative standard deviation obtained from five repetitive injections of samples.

(ca. 1 mg ml^{-1} level, see Ref. [4] and references therein). So, AEC–IPAD provides sufficient sensitivity for determination of these compounds in real sample in the situations of forensic medicine and emergency toxic analysis.

In the application to the biological samples, the interferences from the amino acids should be taken account. In this study, 17 amino acids, arginine, asparagine, glutamine, serine, glycine, threonine, alanine, histidine, lysine, proline, valine, glutamic acid, aspartic acid, cysteine, methionine, isoleucine, and leucine, were examined in the same system. Although they are detectable by IPAD, most amino acids were found separated from these herbicides.

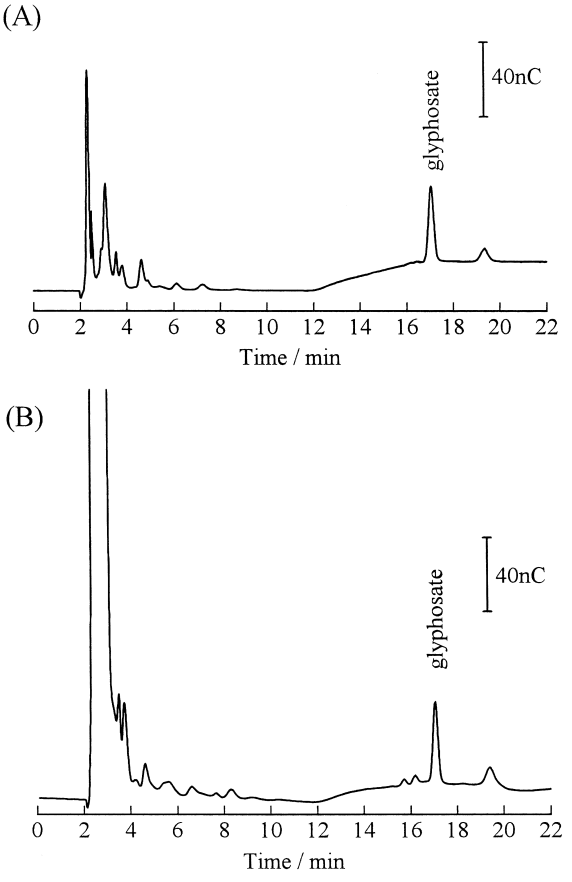

Fig. 6. Chromatograms of (A) human urine spiked with glyphosate at the 8.5 mg ml^{-1} level, and diluted 1:1000 (8.5 μg ml^{-1}) with water. (B) Human serum spiked with glyphosate at the 0.85 mg ml^{-1} level, and diluted 1:100 (8.5 μg ml^{-1} with water. Other conditions as in Fig. 5.

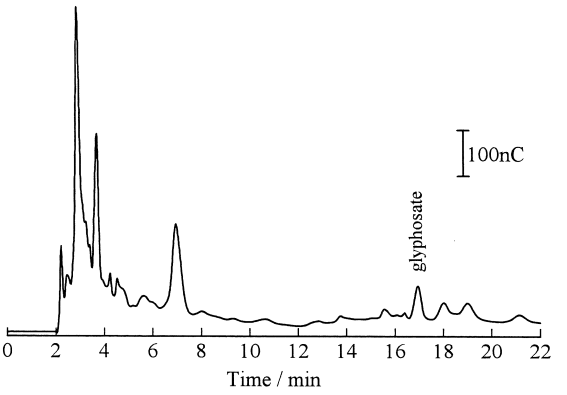

Fig. 7. Chromatogram of mouse urine administered orally with glyphosate at a level of 750 mg kg^{-1}. The sample was diluted 1:50 with water. Other conditions as in Fig. 5.

Proline and valine eluted closed to glufosinate and bialaphos. But these amino acids are rarely found to be present in the real samples.

AEC–IPAD was applied to the determination of glyphosate spiked in human urine and serum. Fig. 6(A) shows chromatograms of a human urine sample spiked by 8.5 mg ml^{-1} glyphosate and then diluted 1:1000 (8.5 μg ml^{-1}) with water, while (B) represents a human serum sample spiked by 0.85 mg ml^{-1} glyphosate and then diluted 1:100 (8.5 μg ml^{-1}) with water. The recoveries of spiked sample obtained by five repetitive injections were 98–101%.

Finally, AEC–IPAD was applied to detect glyphosate in mouse urine. In Japan, some cases show that glyphosate is frequently used to suicide, it was thus chosen as an analyte here. Fig. 7 shows a chromatogram for mouse urine. This sample was collected after glyphosate was administered orally to a male mouse at a level of 750 mg kg^{-1} (oral LD_{50}: 1568 mg kg^{-1}). Glyphosate in urine could be detected without any interference from coexisting substances, and that would demonstrate the utility of this method for detecting glyphosate in real samples.

4. Conclusions

AEC–IPAD has been demonstrated as rapid and simple method for the determination of glufosinate, bialaphos and glyphosate in serum and urine. Though the sensitivity of present method was not

good as those with MS. It is applicable for the fast quantitative analysis especially in forensic toxicology.

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