Journal of Chromatography, 602 (1992) 197–204
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2523

Liquid chromatography and postcolumn indirect detection of glyphosate

Michael J. Lovdahl and Donald J. Pietrzyk*

Department of Chemistry, University of Iowa, Iowa City, IA 52242 (USA)

ABSTRACT

Glyphosate [N-(phosphonomethyl)glycine] and its metabolite aminomethylphosphonic acid (AMPA) were separated and detected by a postcolumn indirect detection strategy. Separation can be done on a cation-exchange column, where glyphosate elutes before AMPA, or on an anion-exchange column, where the elution order is reversed. Detection was achieved by using a fluorescent Al^{3+}–morin postcolumn reagent. When the postcolumn reagent combines with the column effluent in a mixing tee, the fluorescence decreases in the presence of both analytes. Variables affecting the postcolumn indirect fluorescence detection were established and optimized; the major factors were postcolumn pH and volume and temperature of the postcolumn reaction coil. Detection limits, defined as three times the background noise, for glyphosate and AMPA separated on an anion-exchange column were 14 and 40 ng, respectively.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine) (**I**), is a widely used broad-spectrum, non-selective, post-emergence herbicide and there is great interest in and a need to determine the herbicide and its metabolite aminomethylphosphonic acid (AMPA) (**II**), in physiological, water, plant, food and soil samples.

$HO_2CCH_2NHCH_2P(O)(OH)_2$

I

$H_2NCH_2P(O)(OH)_2$

II

Procedures for their determination have been reviewed [1]. Although gas chromatographic procedures [1,2–4] continue to be of interest, in general they suffer from tedious sample preparation because of the need to convert the analytes into volatile derivatives. For this reason and the requirement for a better detection limit, liquid chromatographic (LC) procedures have been developed [5–13].

Three LC approaches have been used to achieve the separation. As glyphosate and AMPA are acidic, they can be separated by anion exchange [5,8,10,11]. However, they are not easily detected without derivatization except at low UV wavelength (<200 nm) [5], where the detection limits are not favorable. Postcolumn derivatization at the amine function using *o*-phthalaldehyde (OPA) [6,8,11] can be applied, but glyphosate must first be chemically modified on-line following the separation prior to forming the OPA derivative. On the other hand, reversed-phase liquid chromatography (LC) [12,13] and ion interaction LC [9] can be used, provided that glyphosate and AMPA are derivatized precolumn. Other reagents used to derivatize the amine function are 9-fluorenylmethyl chloroformate [7,10], 1-fluoro-2,4-dinitrobenzene [9] and *p*-toluenesulfonyl chloride [12,13]. In each instance detection is possible at a high UV wavelength. The derivatization reactions will provide favorable detection limits. However, the reactions can be more complex, particularly if done precolumn, and can suffer from a lack of reproducibility.

Morin (3,5,7,2′,4′-pentahydroxylflavone) will complex with Al^{3+} and many other metal ions to form a highly fluorescent solution which is the basis for the fluorimetric determination of the metal ions [14]. In the presence of phosphate or F^- the emitted fluorescence of the Al^{3+}–morin decreases and the decrease can be correlated with the amount of

0021-9673/92/$05.00

phosphate [15] or F^- [14] in the sample. This indirect fluorimetric chemistry can be used postcolumn to detect phosphate [16] and F^- [17] following a liquid column chromatographic separation with detection limits of 15 and 2 ng, respectively, at a signal-to-noise ratio of 2:1.

The use of Al^{3+}–morin as a postcolumn reagent is an indirect detection (ID) because the decrease in Al^{3+}–morin fluorescence in the analyte band relative to the fluorescent background is being monitored. This differs from other ID strategies used in LC because in these instances equilibrium effects that occur within the column between the analyte, a detector-active component in the mobile phase and the stationary phase are the basis for the ID [18–20]. A postcolumn ID and the effects of the experimental and detector variables have only rarely been used and/or evaluated [16,17,21].

In the procedure described here, which can be used for the determination of both glyphosate and AMPA, an Al^{3+}–morin solution is combined with the column effluent and the decrease in fluorescence is recorded when either analyte, both of which compete favorably with morin to form the Al^{3+} complex, passes through the column. There were four major aims: to demonstrate that postcolumn ID is a viable and sensitive detection strategy in LC separations, to establish the parameters that influence postcolumn ID particularly as they apply to the use of Al^{3+} morin as a postcolumn reagent, to show that the postcolumn ID with Al^{3+}–morin is easily carried out and provides low detection limits for the determination of glyphosate and AMPA and to demonstrate that the Al^{3+}–morin reagent is a selective reagent for ID.

EXPERIMENTAL

Reagents

Morin hydrate and $Al(NO_3)_3$ were obtained from Aldrich, glyphosate and AMPA from Sigma and all other acids, bases and salts from EM Science. LC-grade water was prepared by passing laboratory distilled, deionized water through a Milli-Q Plus system (Millipore). USP-grade 95% ethanol was used. Prepacked 150 mm × 4.1 mm I.D. PRP-X100 (10 μm) anion-exchange columns, and 250 mm × 4.1 mm I.D. PRP-X400 (7 μm) cation-exchange columns were obtained from Hamilton.

Instrumentation

The LC system consisted of a Spectra-Physics M 8800 pump, a Rheodyne Model 7125 injector and a Kratos Model 9000-9501 fluorescence detector equipped with a Kratos FSA 113 coated mercury lamp, a Kratos FSA 404 excitation (400–470 nm) filter and a Kratos high-pass emission filter (50% transmission at 480 nm). The detector response was recorded on a strip-chart recorder and collected on a Spectra-Physics Model 4270 integrator coupled with a Spectra-Physics WINner software package. Peak areas are reported as relative integrator units. The postcolumn system consisted of a Varian M 2010 pump to deliver the Al^{3+}–morin solution through a pulse damper made from an empty 250 mm × 8.0 mm I.D. column, a 150 mm × 4.6 mm I.D. column containing 80-mesh glass beads and a coil of 2 m × 0.508 mm I.D. stainless-steel tubing. The Al^{3+}–morin reagent stream and the column effluent were combined through a Lee Visco mixing tee (No. 344790 SN152). The connection between the tee and the detector was a reaction coil made from 4.57 m × 0.76 mm and 1.52 m × 1.02 mm I.D. PEEK tubing (Upchurch Chromatography), which provided an internal volume of 3300 μl and was woven to minimize postcolumn peak broadening. The coil temperature was maintained at 55°C with a DuPont M 851201-901 temperature-controlled column oven.

Procedures

Analyte stock standard solutions (1 mg/ml) were prepared by dissolving known amounts in LC water. Serial dilutions of the stock standard solutions were done to obtain calibration standard solutions. The mobile phases were aqueous 25 m*M* $NaNO_3$ (pH 9.5, adjusted with dilute NaOH) for anion-exchange separations and 10 m*M* HNO_3 (pH 2.0) for cation-exchange separations at flow-rates of 1.0 and 0.50 ml/min, respectively. Sample aliquots were injected by syringe in amounts of 10 μl or less.

The postcolumn Al^{3+}–morin solution was prepared by pipetting aliquots of morin, $Al(NO_3)_3$ and acetic acid from stock solutions and combining these with 95% ethanol and LC water and aged overnight to ensure reproducible formation of the Al^{3+}–morin complex. The reagent was stable for at least 2 months when stored in a closed container. The solution contained 4.0 μ*M* $Al(NO_3)_3$, 21 μ*M* morin,

27.5 μM acetic acid and ethanol–water (4:1) and was delivered to the tee at 0.50 ml/min. Dilute NaOH or NHO_3 was added to the Al^{3+}–morin solution to produce a pHof 4.3 when the column effluent and the postcolumn Al^{3+}–morin solution were combined in the reaction coil.

RESULTS AND DISCUSSION

Glyphosate and AMPA are readily separated by ion exchange. If the mobile phase pH is about 9.5, glyphosate will be more highly retained on an anion exchanger [5,8,10,11] because of its additional acidic site. As the pH is decreased into the acidic range, both are converted into cations, as both contain a basic amino group, and on a cation exchanger the AMPA cation is more highly retained than the glyphosate cation. Therefore, it is possible to carry out the separation so that the glyphosate is eluted either first (by cation exchange) or second (by anion exchange). This choice is particularly important if the primary objective of the analysis is to determine either AMPA or glyphosate in the presence of the other as a trace component.

The other variables affecting the retention of glyphosate and AMPA on ion exchangers are typical of ion-exchange separations. Thus, increasing the mobile phase counter-ion concentration or switching to a counter ion of higher ion-exchange selectivity decreases the retention. Under the recommended separation conditions outlined in the following discussion, mobile phase conditions were optimized for separations on the anion- and the cation-exchange analytical columns to achieve baseline resolution and to minimize mobile phase component effects on the postcolumn detection chemistry.

Although glyphosate and AMPA are readily separated, detection with a favorable detection limit is not easily done without derivatization. When highly fluorescent Al^{3+}–morin solution and either glyphosate or AMPA are combined, a decrease in the fluorescence of the Al^{3+}–morin is observed. Further, as the amount of either analyte is increased, the fluorescence of the Al^{3+}–morin decreases in proportion to the amount of analyte added, provided that the Al^{3+}–morin is in excess. The decrease in fluorescence occurs occurs because the analyte competes favorably with morin to form the Al^{3+}–analyte complex over the Al^{3+}–morin complex, as shown in the equation

$$Al^{3+}\text{–(morin)}_n + mG \rightleftharpoons Al^{3+}\text{–}G_m + n\text{morin} \quad (1)$$

using glyphosate, G, as the example. The stoichiometry of the two complexes, which is not identified in eqn. 1, will also determine the degree of change in fluorescence. Further, the change in fluorescence is selective as only analytes that form Al^{3+} complexes will cause a fluorescence decrease.

By combining an Al^{3+}–morin solution with the column effluent from either an anion- or a cation-exchange separation, it should be possible to detect when glyphosate or AMPA (also phosphate) emerges from the column because within these analyte bands the fluorescence should decrease. Further, the fluorescence decrease should be proportional to the amount of glyphosate or AMPA (also phosphate) present in the separated bands. To achieve postcolumn indirect fluorescence detection (PCIFD), the detector is set at the wavelength of fluorescence and the detector electronic offset is used to zero the detector signal as the postcolumn fluorescent solution passes through the detector. Therefore, the analyte, when it appears, is indicated by a negative peak. The postcolumn concentration of the Al^{3+}–morin solution must be in excess to provide a decrease in fluorescence that occurs rapidly and with an appreciable change but yet low enough that the background fluorescence does not exceed the offset capability of the detector and/or provide a background noise level that limits the ability to detect the change in fluorescence with appropriate sensitivity and accuracy.

The variables affecting PCIFD can be divided into two types. One group, which primarily affects the background fluorescence, includes the pH for the postcolumn reaction, the buffer concentration, the postcolumn solvent composition, the Al^{3+} to morin mole ratio and the concentration of the Al^{3+}–morin. The second group will have a major effect on the rate of the postcolumn reaction; these include the postcolumn reaction temperature and volume. After a series of preliminary experiments designed to establish the qualitative effect of each variable, each was carefully evaluated over a defined range while all other factors were held constant. The preliminary experiments also demonstrated that a column flow-rate of 1.0 ml/min and a postcolumn

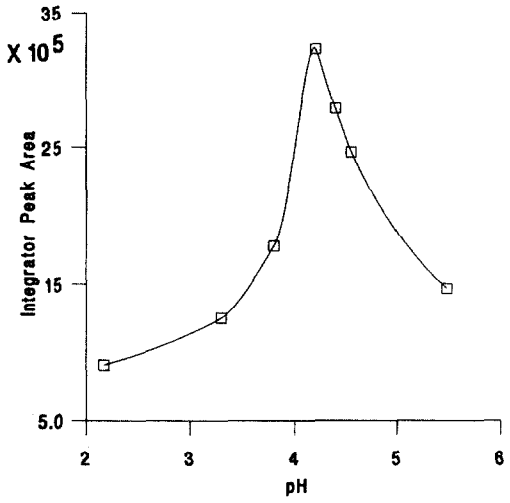

Fig. 1. Effect of postcolumn reaction pH on glyphosate peak area. Mobile phase, 20 m*M* $NaNO_3$ (pH 9.5); PRP-X100 anion-exchange column. The postcolumn solution contained 4.0 μM Al^{3+}, 21 μM morin, 25 m*M* acetate buffer and ethanol–water (4:1) and the temperature was 26°C.

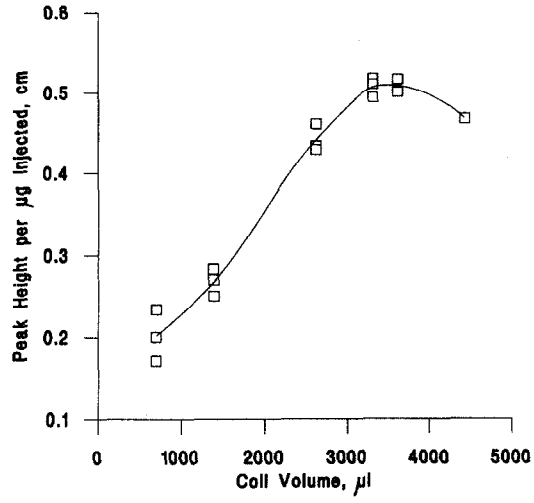

Fig. 2. Effect of postcolumn reaction coil volume on glyphosate peak area. Conditions as in Fig. 1.

Al^{3+}–morin solution flow-rate of 0.50 ml/min were optimum for the glyphosate and AMPA sample sizes being separated and detected. The purpose in optimizing each variable was to obtain an accurate, reproducible change in fluorescence that would correspond to the best detection limits for glyphosate and AMPA.

Of the seven postcolumn variables, those which had the greatest effect on fluorescence change and therefore require careful control over narrow limits in order to achieve the lowest detection limits were reaction pH, postcolumn reaction temperature and postcolumn volume. Fig. 1 shows that a maximum peak area (largest change in fluorescence decrease) using glyphosate as the test analyte is obtained when the postcolumn pH is about 4.3. In these experiments all other postcolumn conditions, including the Al^{3+}–morin concentration reaction coil length, volume and temperature and flow-rate are held constant while the pH of the acetate buffer is changed. The optimum pH remains the same when the postcolumn mixing volume and/or temperature is elevated. As the pH is shifted above or below 4.3, the peak area (degree of fluorescence decrease) decreases sharply. It is essential that the postcolumn Al^{3+}–morin solution contains a buffer of sufficient capacity to overcome the mobile phase pH if the latter is significantly different from 4.3. When the mixing coil beyond the mixing tee is increased in length, while other variables are held constant, the reaction of the analyte with the Al^{3+}–morin is more complete, and the decrease in fluorescence (increase in peak area) becomes larger. However, the increased volume of the tubing becomes a factor, and band-broadening effects start to reduce the peak area. As shown in Fig. 2, when the postcolumn coil volume is 3300 μl, the reaction temperature is 26°C and the flow-rate of the combined column effluent and postcolumn reagent solution is 1.5 ml/min, the maximum peak area is obtained. In this study, two lengths of PEEK tubing of 0.76 and 1.02 mm I.D. were connected in order to adjust the coil length easily. For routine appliations a single PEEK tube of small I.D. yielding 3300 μl should be used. The PEEK tube should also be woven to increase mixing and reduce band broadening. Using the same conditions, but raising the temperature, indicates (see Fig. 3) that the optimum reaction coil temperature for glyphosate and AMPA is about 55°C. At higher temperatures the peak area begins to decrease because the fluorescence intensity of the Al^{3+}–morin starts to decrease at higher temperatures.

When the effects of postcolumn reaction solvent composition was evaluated, the maximum peak area for glyphosate and AMPA was obtained when the ethanol to water ratio was about 2:3. Similarly, the

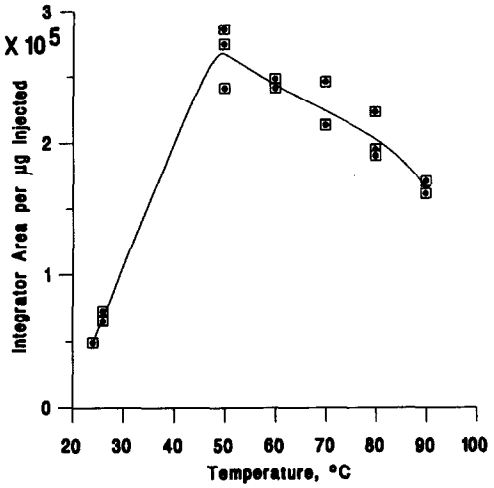

Fig. 3. Effect of postcolumn reaction temperature on glyphosate peak area. Conditions as in Fig. 1 with a reaction coil of 3300 μl and a postcolumn pH of 4.3.

maximum peak area was produced when the Al^{3+} to morin ratio was 1:6 and the Al^{3+} concentration was about 1.4 μM in the mixing tee. As the acetate buffer concentration was increased from 10 to 100 mM in the postcolumn Al^{3+}–morin solution, the peak area decreased by about 40%. However, at about 10 mM after mixing in the tee, a modest change in the buffer concentration produced only minor effects on the peak area. In general, the effects of postcolumn solvent composition, Al^{3+}–morin ratio and concentration and buffer concentration on glyphosate and AMPA are similar to those on peak areas for other analytes detected by the PCIFD stategy [16,17].

Fig. 4 illustrates the separation of a mixture of about 50 ng of phosphate, 400 ng of glyphosate and 540 ng of AMPA by cation exchange and a nitric acid eluent with PCIFD using the Al^{3+}–morin reagent. Because of the acidic mobile phase, the postcolumn Al^{3+}–morin pH was adjusted with dilute NaOH so that a pH of 4.3 was obtained when the column effluent and postcolumn solution were combined in the mixing tee. The phosphate, which was included in the sample because phosphate would be present in physicological samples and is detected by PCIFD [16], is not retained on the cation

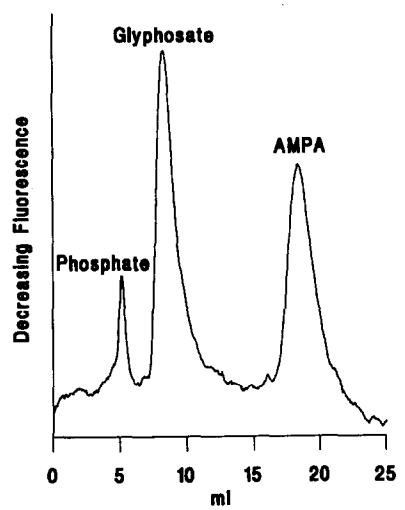

Fig. 4. Separation on a cation exchanger. Mobile phase 10 mM HNO_3 and PRP-X400 cation-exchange column using the postcolumn reaction conditions as in Fig. 3 with a postcolumn reaction temperature of 55°C. Flow-rate, 0.50 ml/min.

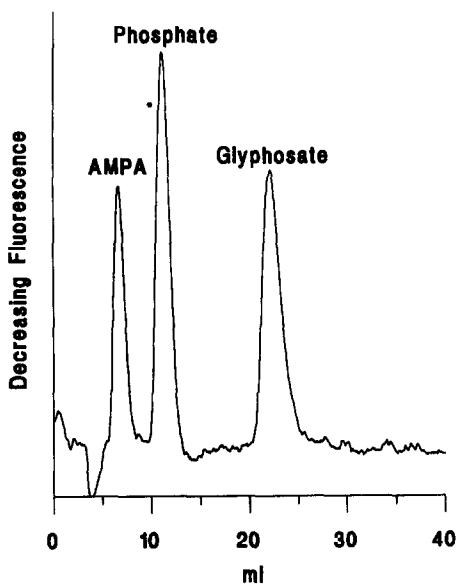

Fig. 5. Separation on an anion exchanger. Mobile and stationary phase conditions as in Fig. 1 except 25 mM $NaNO_3$ and postcolumn reaction conditions as in Fig. 4. Flow-rate, 1.0 ml/min.

exchanger and it is detected in the void volume where other common anions also appear. A commercially available 250-mm long cation-exchange column was used and baseline separation of glyphosate and AMPA is possible with a shorter column.

When an anion-exchange column is used for the separation, the elution order is reversed and AMPA appears before phosphate. This is illustrated in Fig. 5 where a mixture of 900 ng of AMPA, 313 ng of phosphate and 340 ng of glyphosate are separated. AMPA and glyphosate are readily resolved over the pH range 8–10; above pH 10 the retention of both increases sharply. The location of the phosphate peak is also sensitive to the mobile phase pH and at pH 9.5 (see Fig. 5) the peak appears between those of AMPA and glyphosate. If the pH is decreased the phosphate peak shifts towards the AMPA peak. Other common inorganic anions, such as Cl^- and NO_3^-, that may be present in the sample will not interfere as they are not detected by PCIFD. Fluoride, if present, would be detected [17] in the void volume, where it is eluted, whereas SO_4^{2-}, which causes a fluorescence decrease, does not interfere.

PCIFD is both a sensitive and a selective detection strategy. This is illustrated in Fig. 6 using an anion-exchange column and mobile phase conditions where F^- and Cl^- are retained on the column. The F^- peak, which corresponds to 0.33 μg of NaF, is barely detected by conductance whereas Cl^- as NaCl (11.5 μg), which is 35 times larger than F^- as NaF, is readily detected. PCIFD, with the detector connected in series with the conductance detector, provides a significant F^- peak area by comparison and is also selective as it does not respond to Cl^-. In addition, the large Cl^- excess does not affect the elution time of F^- on the anion-exchange column.

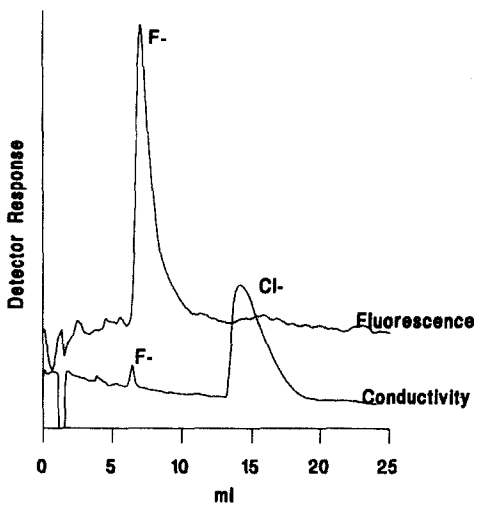

Fig. 6. Sensitivity and selectivity of postcolumn indirect fluorescence detection using Al^{3+}–morin compared with conductivity detection. Mobile phase 4.0 m*M* NaOH and postcolumn indirect detection conditions as in Fig. 4 except for a postcolumn reaction temperature of 26°C. Column, PRP-X100; flow-rate, 1.0 ml/min.

When a woven stainless-steel tube of similar inside diameter and volume as the PEEK tube was used as the reaction coil, the peak areas were similar but the peak heights were reduced and the peaks were broad and tailed. This is illustrated by comparing Figs. 4 and 5. In Fig. 4 where a stainless-steel coil was used the peaks are broader than in Fig. 5 where a PEEK coil was used. Apparently, glyphosate and AMPA undergo adsorption and/or react with the stainless-steel coil at elevated temperatures. Both are capable of exhibiting ligand properties and can form stable metal ion ligand complexes [22] which would contribute to broadening.

Calibration graphs were constructed with glyphosate and AMPA standards using an anion-exchange column, 10-μl injections and the mobile phase conditions outlined in Fig. 5. For glyphosate the straight line corresponded to the equation integrator area counts = 14.1 + 13 300 (nmol injected) with a correlation coefficient of 1.00; for AMPA it was integrator area counts = 3600 + 2560 (nmol injected) with a correlation coefficient of 0.99. No attempt was made to determine the upper limit of linearity. The slope for glyphosate is almost five times greater and the increased sensitivity for the glyphosate determination over AMPA arises because more Al^{3+}–morin undergoes a reaction with glyphosate under the postcolumn reaction conditions than for an equivalent amount of the AMPA. Hence, the fluorescence decrease for glyphosate is larger. Another factor contributing to the increased sensitivity is that glyphosate is probably a better ligand than AMPA for Al^{3+}. The precision for the data used to establish the glyphosate calibration graph, for example, was better than 3% (relative standard deviation) at each experimental point on the graph. The detection limits for the anion-exchange separation where the decrease in the

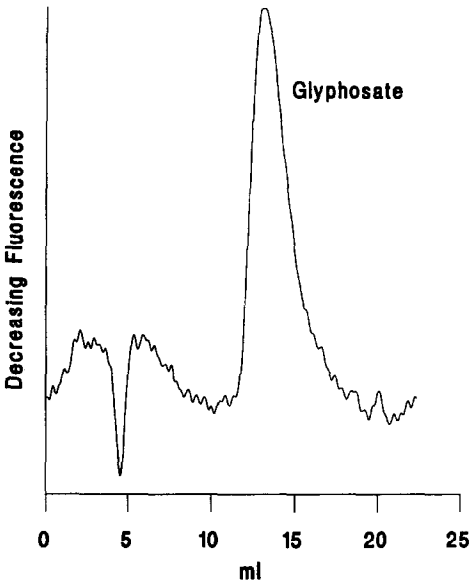

Fig. 7. Separation of glyphosate in a commercial herbicide. Conditions as in Fig. 5 except 40 m*M* $NaNO_3$.

fluorescence signal was three times the noise were found to be 14 ng of glyphosate and 40 ng of AMPA. For glyphosate separated on the cation exchanger according to the conditions in Fig. 4, the detection limit as defined above was about 28 ng of injected analyte.

Fig. 7 illustrates the separation and detection of glyphosate in a commercially available herbicide sample on an anion-exchange column. The glyphosate was listed in the product as 0.96% as the isopropylamine salt. A small aliquot of the commercial sample was passed through a Millipore Sep-Pak C_{18} cartridge and a Millipore 0.2-μm filter to remove the sample matrix. When a 3-μl neat aliquot of this sample was injected, the glyphosate, which overloaded the column, was readily detected. Diluting the sample 1:10 with water and injecting a 4-μl aliquot provided the chromatogram shown in Fig. 7. Comparison of peak area with the calibration graph indicated that the glyphosate concentration in the sample was about 0.96%.

CONCLUSIONS

Postcolumn indirect detection is a viable detection strategy and other postcolumn reactions should be adaptable to indirect detection. Like other indirect detection strategies, the background signal developed in the postcolumn reaction must be low enough that the signal can be zeroed by the detector offset electronics. When using an Al^{3+}–morin solution as a postcolumn reagent, a decrease in fluorescence is used to indicate the amounts of glyphosate, AMPA and phosphate in the sample. The elution order can be reversed depending on whether the separation is carried out on an anion or a cation exchanger. The detection is sensitive and simple, and the procedure does not suffer from the experimental problems usually associated with postcolumn derivatization reactions. The Al^{3+}–morin indirect detection of glyphosate and AMPA is selective, as few other analytes will cause a fluorescence decrease, and therefore even if they are not separated from glyphosate and AMPA they will not interfere in the detection.

ACKNOWLEDGEMENT

Part of this work was supported by Grant DE 8613 awarded by the National Institute of Dental Research.

REFERENCES

1 P. C. Bardalaye, W. B. Wheeler and H. A. Moye, in E. Grossbard and D. Atkinson (Editors), *The Herbicide Glyphosate*, Butterworths, London, 1984, p. 263.
2 R. A. Guinavan, N. P. Thompson and W. B. Wheeler, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 35.
3 J. N. Seiber, M. M. McChesney, R. Kon and R. A. Leavitt, *J. Agric. Food Chem.*, 32 (1984) 678.
4 H. Kataoka, K. Horii and M. Makita, *Agric. Biol. Chem.*, 55 (1991) 195.
5 A. J. Burns and D. F. Tomkins, *J. Chromatogr. Sci.*, 17 (1979) 333.
6 H. A. Moye, C. J. Miles and S. J. Scherer, *J. Agric. Food Chem.*, 31 (1983) 69.
7 R. L. Glass, *J. Agric. Food Chem.*, 31 (1983) 280.
8 T. E. Archer and J. D. Stokes, *J. Agric. Food Chem.*, 32 (1984) 586.
9 L. N. Lundgren, *J. Agric. Food Chem.*, 34 (1986) 535.
10 C. J. Miles, L. R. Wallace, and H. A. Moye, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 458.
11 J. E. Cowell, J. L. Kunstman, P. J. Nord, J. R. Steinmetz and G. R. Wilson, *J. Agric. Food Chem.*, 34 (1986) 955.
12 S. Kawai, B. Uno and M. Tomita, *J. Chromatogr.*, 540 (1991) 411.
13 M. Tomita, T. Okuyama, S. Watanabe, B. Uno and S. Kawai, *J. Chromatogr.*, 566 (1991) 239.
14 F. J. Welcher, *Organic Analytical Reagents*, Vol. IV, Van Nostrand, New York, 1948, p. 370.

15 D. Lands and S. Edmonds, *Mikrochim. Acta*, 6 (1966) 1013.
16 S. E. Meek and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988) 1397.
17 D. J. Pietrzyk and M. J. Lovdahl, in P. Jandik and R. M. Cassidy (Editors), *Advances in Ion Chromatography*, Vol. 2, Century International, Medfield, MA, 1990, p. 255.
18 P. R. Haddad and P. E. Jackson, *Ion Chromatography*, Elsevier, Amsterdam, 1990, pp. 351–369.
19 P. G. Rigas and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988) 454.
20 E. S. Yeung, *Acc. Chem. Res.*, 22 (1989) 125.
21 R. A. Baumann, D. A. Kamminga, H. Derlagen, C. Gooijer, N. H. Velthorst and R. W. Frei, *J. Chromatogr.*, 439 (1988) 165.
22 D. D. Perrin (Compiler), *Stability Constants of Metal Ion Complexes, Part B, Organic Ligands*, Pergamon, Oxford, 1979, p. 24.