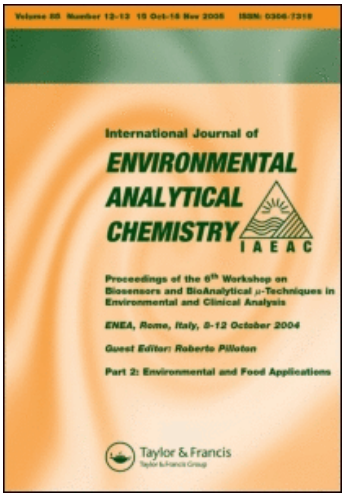

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Sensitive and Selective Fluorescence Detection of Phenoxyacid Herbicides by Liquid Chromatography with On-Line Ion-Pair Extraction

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To cite this Article De Ruiter, C. , Minnaard, W. A. , Lingeman, H. , Kirk, E. M. , Brinkman, U. A. Th. and Otten, R. R.(1991) 'Sensitive and Selective Fluorescence Detection of Phenoxyacid Herbicides by Liquid Chromatography with On-Line Ion-Pair Extraction', International Journal of Environmental Analytical Chemistry, 43: 2, 79 — 90
To link to this Article: DOI: 10.1080/03067319108026968
URL: http://dx.doi.org/10.1080/03067319108026968

Intern. J. Environ. Anal. Chem., Vol. 43, pp. 79–90
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SENSITIVE AND SELECTIVE FLUORESCENCE DETECTION OF PHENOXYACID HERBICIDES BY LIQUID CHROMATOGRAPHY WITH ON-LINE ION-PAIR EXTRACTION

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(*Received 24 January 1990*)

Fluorescent probes, especially a newly synthesized N-substituted 1-cyanobenz[f]-isoindole quaternary ammonium fluorophore, were used as counter ions in a reaction detector for on-line ion-pair extraction of phenoxyacid herbicides. The probe was used in an on-line post-column set-up coupled to a reversed-phase chromatographic system. After separation on an C-8-bonded silica column using an aqueous methanol (pH 2.5) mobile phase, the herbicides were on-line deprotonated by post-column addition of a 10 mM sodium phosphate buffer (pH 8.0), in which the probe was dissolved. Subsequently, the ion-pairs were extracted on-line with chloroform – 1-butanol (80:20, v/v) and were monitored by fluorescence detection. Using this system, at least seven herbicides could be separated. The detection limits of 2,4-dichlorophe xyacetic acid and 2,4,5-trichlorophenoxyacetic acid were 400 pg (S/N = 3). The repeatability, based on peak height measurements, for 100 ng injections was about 0.5%. Calibration curves were linear over the investigated range of 1–100 ng, with correlation coefficients of 0.999 for the two analytes. Application to a drinking water sample is presented.

KEY WORDS: Phenoxyacid herbicides, on-line ion-pair extraction, water analysis, fluorescence detection.

INTRODUCTION

For the fluorescence derivatization of carboxylic acids in column liquid chromatography (LC), many labels are available, e.g. aryl bromides[1–6] and diazo compounds.[7–9]. Labelling reactions for carboxylic acids are generally slow, even at elevated temperatures and, as a result, many side-products of the reaction are usually formed. More selectivity can be obtained by indirect labelling methods, for example, after activation of the carboxylic acid function with 2-bromo-1-methylpyridinium iodide and subsequent reaction with a fluorophoric amine or alcohol.[10] In general, however, labelling of carboxylic acids has to be carried out

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in aprotic solvents, because the reactivity of carboxylic acids in water, or other nucleophilic solvents, is rather low. In other words, when analysing aqueous samples, some form of phase transfer of the analytes is necessary. This causes LC methods to be time-consuming and laborious.

An elegant alternative is the use of on-line post-column ion-pair extraction of carboxylic acids with a cationic dye, e.g. methylene blue, as was recently demonstrated by Lawrence and Charbonneau[11] for the LC determination of free fatty acids in butter, margarine and orange juice. Although excellent selectivity was obtained, the detection limits (20–80 ng) were not acceptable for trace analysis ($\leqq$1 ppb) of carboxylic acids in environmental samples. Therefore, the application of highly fluorescent cations for the on-line post-column ion-pair extraction of carboxylic acids in LC is evaluated in this study. Recently, Stewart *et al.*[12] presented the use of a fluorescent acridinium cation. In this case the ion-pair extraction detector was coupled to a reversed-phase (RP) LC system, using 10 mM phosphate buffer (pH 7)-methanol (90:10, v/v) as the mobile phase. As a result, the carboxylic acids were chromatographed as their respective anions and all eluted at or near the column void volume. Therefore, it will be difficult to use this system for the determination of carboxylic acids in real environmental samples.

In this paper, two commercially available fluorescent cations, as well as newly synthesized quaternary ammonium fluorophores, are evaluated for their synthesized use in a post-column ion-pair extraction detector.[13–15] Phenoxyacid herbicides were chosen as model compounds, because they are used worldwide as agricultural and forestry herbicides. In addition, the extreme toxicity and potential hazard of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD), often present as an impurity in 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) demand the development of sensitive and selective analytical methods for these herbicides.[16,17] Next to phenoxyacid herbicides, chlorinated benzoic acids also are used as herbicides and both classes of compounds can be used in combination. In this study, 2,3,6-trichlorobenzoic acid was used as a representative of the latter group.

EXPERIMENTAL

Chemicals

Methanol, acetonitrile (HPLC grade) and chloroform, 1-butanol, 1-pentanol, 1-hexanol, isopropanol and phosphoric acid (85%, w/v) (analytical grade) were obtained from J. T. Baker (Deventer, The Netherlands). All aqueous solutions were prepared with demineralized water, obtained with a Milli Q (Millipore, Bedford, MA, USA) ultrafiltration system.

1-Pyrenebutyrylcholine bromide (PBC^+), 1-pyrenesulfonamidoethyl-trimethylammonium iodide ($PSTA^+$), naphthalene-2,3-dicarboxaldehyde (NDA) and anthracene-2,3-dicarboxaldehyde (ADA) were obtained from Molecular Probes (Eugene, OR, USA). 2,3,6-trichlorobenzoic acid (2,3,6-TBA), 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-D and 2,4,5-T were purchased as certified

reference materials from the National Physical Laboratory of the UK. 2-(4-Chloro-2-methylphenoxy)-propionic acid (MCPP), (4-(4-chloro-2-methylphenoxy) butyric acid (MCPB) and 4-(2,4-dichlorophenoxy)-butyric acid (2,4-DB) (all at 99% purity), were obtained form Dr S. Ehrenstorfer (Augsburg, FRG). 2-Aminoethyltrimethylammoniumchloride hydrochloride (analytical grade) was purchased from Merck (Darmstadt, FRG).

All other chemicals used were analytical grade and were obtained from Baker.

Synthesis of Quaternary Ammonium Fluorophores

Based on the recently reported reaction of NDA with primary amines, in the presence of cyanide, to form highly fluorescent N-substituted 1-cyanobenz[f]-isoindoles,[18,19] a quaternary ammonium analogue was synthesized. To this end, 9.5 mg NDA (51 μmol) were dissolved in 100 ml methanol, using ultrasonication for 10 min (solution A). Then, 9 mg 2-aminoethyltrimethylammoniumchloride hydrochloride (51 μmol) were dissolved in 100 ml aqueous 10 mM sodium borate buffer (pH 9.5), and 5.5 ml of a 10 mM sodium cyanide solution were added (solution B). All solutions were prepared in brown flasks. Subsequently, solution A was mixed with solution B, the reaction mixture was shaken for 20 s and allowed to react (Eq. 1) for 2 h at room temperature.

Thereafter, the reaction mixture was extracted twice with 200 ml of chloroform, to remove fluorescent side-products, the resulting aqueous layer was used as the stock solution and was kept in the dark at 20 °C. This solution was stable for at least one week. For use in the post-column ion-pair extraction detection system, it was diluted with 19 volumes of a 10 mM sodium phosphate buffer (pH 8.0), to reach a final NDA^+ concentration of approx. 12.5 μM (12.5 μM will be obtained at a 100% reaction yield).

In the same way, the quaternary ammonium analogue (ADA^+) was synthesized, using 12.1 mg (51 μmol) ADA instead of NDA in the method described above.

Apparatus and Methods

A scheme of the set-up used is shown in Figure 1. Injection of the samples was performed with a Valco (Houston, TX, USA) six-port valve, equipped with a 10 μl loop. The eluent, aqueous reagent, and organic extractant were delivered by three Gilson (Villiers-le-Bel, France) Model 302 pumps with home-made membrane pulse dampers. The column was a 150 mm × 3.1 mm I.D. stainless-steel column, packed with Hypersil MOS (Shandon, Runcorn, UK) 3 μm C-8 material. The post-column ion-pair extraction detector consisted of two Valco T-pieces with 0.25 mm bores, a 250 mm × 0.5 mm I.D. stainless-steel mixing capillary coiled from 1 mm to 25 mm O.D.,[20] a 150 cm × 0.5 mm I.D. stainless-steel extraction capillary (helix diameter, 40 mm), and a home-made sandwich phase separator.[21] The detector used was an Applied Biosystems (Ramsey, NJ, USA) Model 980 fluorescence detector, set at 260 nm for the excitation (slit width, 10 nm) an emission cut-off filter (with a 90% transmittance at 470 nm), a photomultiplier voltage of 935 V, and a rise time of 5 s. For peak height measurements, chromatograms were recorded on a Kipp (Delft, The Netherlands) BD-8 recorder.

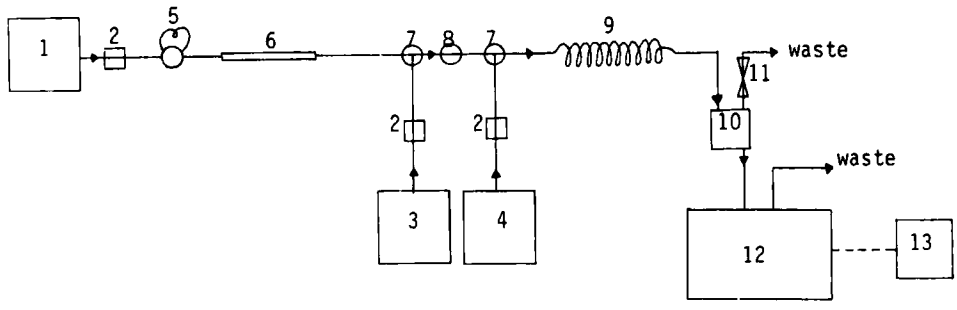

Figure 1 Scheme of the apparatus used: 1, mobile phase pump; 2, membrane pulse damper; 3, aqueous reagent pump; 4, organic extractant pump; 5, injection valve; 6, analytical column; 7, T-piece; 8, mixer; 9, extraction coil; 10, sandwich phase separator; 11, restrictor; 12, fluorescence detector; 13, recorder.

The eluent used was methanol-water (acidified to pH 2.5 with phosphoric acid) (55:45, v/v) at a flow rate of 0.6 ml/min. The aqueous reagent was a NDA^+ solution ($\leqq 12.5\,\mu M$) in 10 mM sodium phosphate buffer of pH 8.0 (containing 2.5% methanol) at a flow rate of 0.6 ml/min. The organic extraction solvent was chloroform – 1-butanol (80:20, v/v) at a flow rate of 1.0 ml/min. The flow rate of the organic extractant through the detector was about 0.3 ml/min, and could be adjusted using a simple restrictor around a PTFE capillary, connected to the aqueous outlet of the phase separator.

For preconcentration of the herbicides from aqueous samples, a 10 mm × 2 mm I.D. Chrompack (Middelburg, The Netherlands) precolumn was used, packed with 15–25 μm a PLRP-S divinylbenzene-styrene copolymer (Polymer Laboratories, Shropshire, UK), which was installed instead of the 10 μl loop. Elution with the LC eluent was carried out in the backflush mode. Breakthrough volumes were measured using an Applied Biosystems Model 957 UV detector, set at 280 nm and connected to the outlet of the precolumn. Solutions were delivered with a Kontron (Zürich, Switzerland) Model 410 LC pump, at a flow rate of 2.5 ml/min.

Optimisation of the on-line ion-pair extraction system was performed using the system described in Figure 1, without the mobile phase pump and the analytical column, while the injection valve was placed in the aqueous reagent stream. In such a flow injection analysis (FIA) system, standard solutions of the herbicides in the aqueous phase, i.e., a 22 μM solution of the fluorescent probe in 2.5 mM sodium phosphate buffer (pH 8.0)—methanol (90:10, v/v), were used. The flow rates of the aqueous reagent and the organic extraction solvent were 0.5 ml/min and 1.0 ml/min, respectively. The flow rate of the organic extractant through the detector was about 0.3 ml/min.

RESULTS AND DISCUSSION

Development of the Post-Column Ion-Pair Extraction Detector

Optimisation of an on-line ion-pair extraction system for phenoxyacid herbicides,

was carried out using a FIA system as described under "Experimental". A 2.5 mM sodium phosphate buffer (pH 8.0)—methanol (90:10, v/v) mixture was used as the aqueous phase. Chloroform was used as the organic phase, because of its good extraction and phase separation properties shown in an earlier study.[15] During these experiments, PBC^+ was used as the fluorescent probe. In this FIA system, however, the extraction yields for the various herbicides were quite different. For instance, the extraction yields for 2,3,6-TBA and MCPB were very low, resulting in detection limits of about 50 and 100 ng, respectively, whereas for 2,4,5-T a detection limit of 1 ng was observed. Because much lower detection limits are generally observed for highly fluorescent compounds, the latter observation indicates that also for 2,4,5-T the extraction yield is far from 100%. The use of another extraction coil with equal dimensions, but with a length of 5 m instead of 1.5 m, or the use of an extraction coil with similar dimensions made from PTFE instead of stainless-steel, did not improve the extraction recoveries. This means that the extraction equilibria are fast and that the installed extraction coil has the proper dimensions.

Because the yield of the extraction depends on, e.g., the polarity and hydrogen-bonding ability of the ion-pair in the organic phase, it was expected that the extraction yields could be improved by the addition of lipophilic alcohols to the organic phase.[22] The addition of 7.5% 1-butanol to the organic phase, for example, already resulted in a 30-fold increase in the extraction recovery of 2,3,6-TBA. The influence of the addition of 1-butanol to the organic phase on the extraction yields of 2,4-D and 2,4,5-T, is demonstrated in Figure 2. With the use of 1-pentanol and 1-hexanol, similar results were obtained as for 1-butanol, but with isopropanol about 50% lower extraction yields were observed. The addition of these alcohols to the organic phase also resulted in much better peak shapes (Figure 3).

An organic phase of chloroform-1-butanol (80:20, v/v) was found to be optimal and was used in all further experiments. Under these conditions, the reaction detection system demonstrated good linearity and repeatability. A calibration curve of 2,4,5-T was linear from 2.5 to 100 ng, with a correlation coefficient of 0.9999 ($n=6$), while 8 repetitive injections of 100 ng 2,4,5-T resulted in a relative standard deviation of 1.6%.

In order to evaluate the use of the reaction detection system in combination with an LC system for separation of the herbicides, the influence of higher modifier percentages in the aqueous phase on the performance of the reactor was investigated. This was done because after the LC separation the aqueous reagent stream is mixed with the LC eluent, which usually contains a considerable amount of modifier. For PBC^+, $PSTA^+$ and NDA^+ as fluorescent counter ions, no more than about 20, 25 and 40% (v/v) methanol could be used in the aqueous phase, respectively. Using acetonitrile much lower values were even obtained. Higher modifier percentages than those quoted resulted in high fluorescence background signals and lower extraction yields. In other words, if the mixture of 2.5 mM sodium phosphate (buffer (pH 8.0) and methanol (90:10, v/v) is used as the aqueous phase and NDA^+ as the counter ion, LC eluents containing up to 70% (v/v) methanol can be used, taking into account a dilution factor of about 2, as a

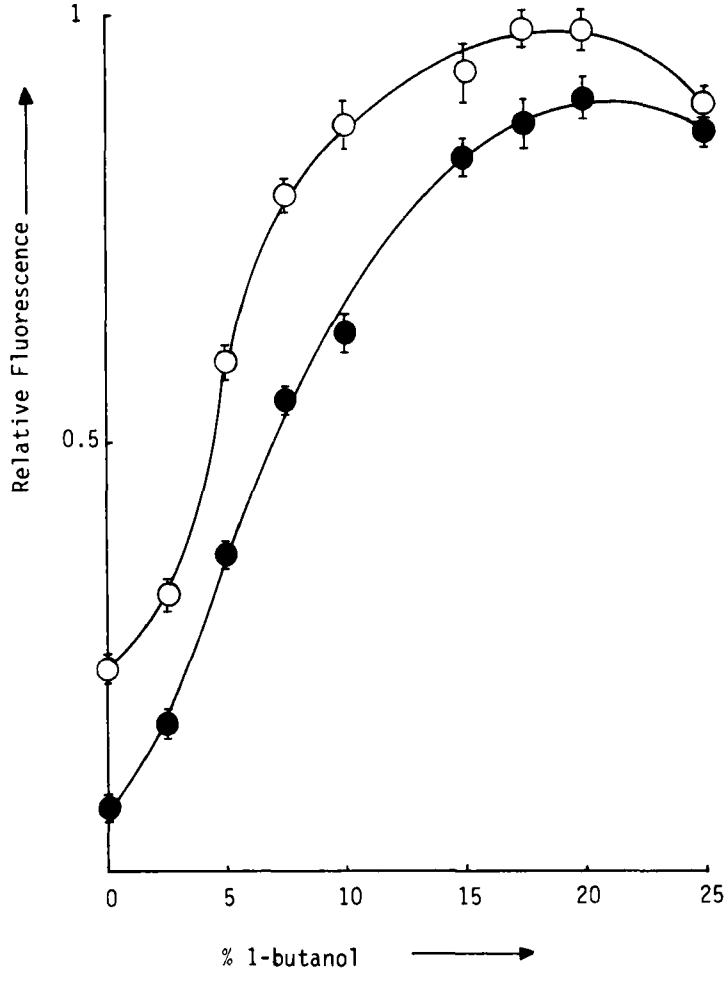

Figure 2 Influence of the addition of 1-butanol to the organic extraction solvent chloroform, on the extraction yields of 2,4-D (●) and 2,4,5-T (○) from a 22 μM solution of PBC^+ in 2.5 mM sodium phosphate buffer (pH 8.0)—methanol (90:10, v/v), using the FIA system described under Experimental. Each data point represents the mean and standard deviation of at least four measurements.

result of the post-column mixing of the LC eluent with the aqueous reagent stream.

Unfortunately, solutions of the ADA^+ probe in 2.5 to 10 mM sodium phosphate buffer (pH 8.0) were unstable. Hence, ADA^+ could not be used in the ion-pair extraction system.

Separation of Phenoxyacid Herbicides and Coupling to the Reaction Detector

For the separation of phenoxyacid herbicides, several RP-LC systems have been

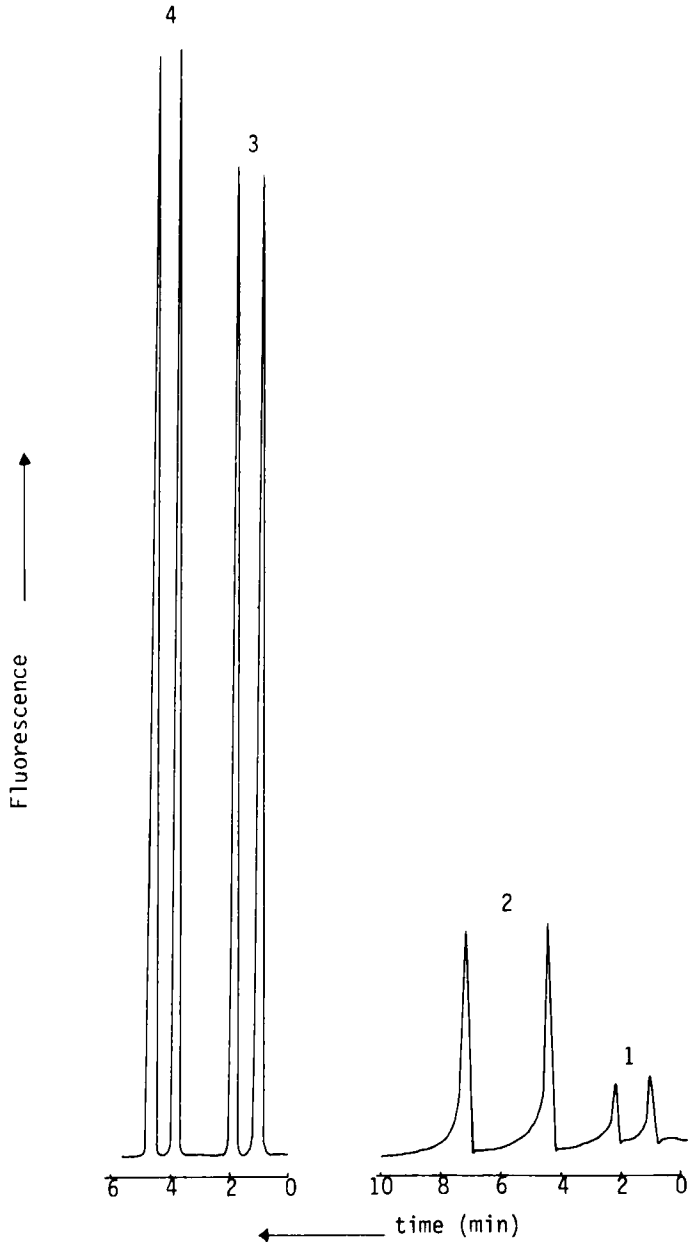

Figure 3 FIA recordings of 100 ng injections of 2,4-D, (1) without and (3) with 20% (v/v) 1-butanol added to the organic extractant chloroform, and of 2,4,5-T, (2) without and (4) with 20% (v/v) 1-butanol in the organic phase. Standard solutions of the herbicides in the aqueous phase were used. Injection volume, 10 μl.

Aqueous phase: 22 μM solution of PBC^+ in 10 mM sodium phosphate buffer (pH 8.0)—methanol (90:10, v/v), flow rate, 0.5 ml/min. Organic phase flow rate, 1.0 ml/min. Flow through detector, 0.3 ml/min. Detector settings: $\lambda_{ex} = 342$ nm (slit, 10 nm), $\lambda_{em} > 418$ nm (cut-off filter); photomultiplier voltage, 700 V; rise time, 1 s.

described.[23] If eluents are employed, which consist of mixtures of water and a modifier (e.g., methanol, acetonitrile), the herbicides usually elute near the column void volume and strongly distorted peaks are observed. This is mainly caused by ionic exclusion effects and can be avoided (i) by adding an ion-pairing agent to the mobile phase, (ii) by using mobile phases containing an inorganic salt, which causes salting out of the organic solutes, or (iii) by suppressing the ionization of the acids via the use of buffered mobile phases of low pH. Another possibility is the use of chemically bonded amino phases which, in acidic media, become protonated and can act as weak anion exchangers. In this case however, it is necessary to use a high concentration of, e.g. acetic acid in the mobile phase.

On the other hand, in order to obtain a satisfactory performance of the reaction detector system there are several restrictions with respect to the LC eluent. Firstly, the use of ion-pairing reagents or relatively high salt concentrations should be avoided, since all processes that influence the equilibrium of the ion-pair formation in the post-column reactor, will reduce the extraction yields of the herbicides. Secondly, high concentrations of acetic acid should not be used because after the separation, the carboxylic acid function of the analytes must be deprotonated, before ion-pair extraction can take place. Finally, as mentioned before, very high modifier contents are not allowed. As a result, C-8-bonded silica columns can not be used for the separation of herbicides. Therefore, a less polar stationary phase was chosen, i.e., C-8-bonded silica. The separation of a number of test compounds is shown in Figure 4. The repeatability of the retention times was less than 0.5%, for at least one month. Using an aqueous reagent stream containing NDA^+, dissolved in a 10 mM sodium phosphate buffer of pH 8.0, the analytes were on-line deprotonated and subsequently extracted as an ion-pair with NDA^+. The NDA^+ concentration of approx. 12.5 μM was found optimal in terms of the fluorescence background and the signal-to-noise (S/N) ratios of the extracted ion-pairs. The use of a chloroform-1-butanol (80:20, v/v) organic extractant again was found optimal in terms of the extraction recoveries and peak shapes of the herbicides.

Analytical Variables

The linearity of the total system as described in Figure 1, was good. Calibration curves of 2,4-D and 2,4,5-T were linear in the range of 1–100 ng with correlation coefficients of 0.9992 ($n=6$) and 0.9990 ($n=6$), respectively. The repeatability, based on peak height measurements, was determined performing repetitive injections of 100 ng 2,4-D and 2,4,5-T; relative standard deviations of 0.4% ($n=7$) and 0.5% ($n=7$) were obtained, respectively. The detection limits of 2,4-D and 2,4,5-T were about 400 pg (S/N = 3), as can be seen in Figure 5.

Application to Drinking Water

Breakthrough volumes on a 10 mm × 2 mm I.D. precolumn obtained for 2,3,6-TBA (the most polar representative), 2,4-D and 2,4,5-T, using aqueous samples acidified to pH 2.3 with phosphoric acid, were about 25, 30 and 50 ml, respectively.

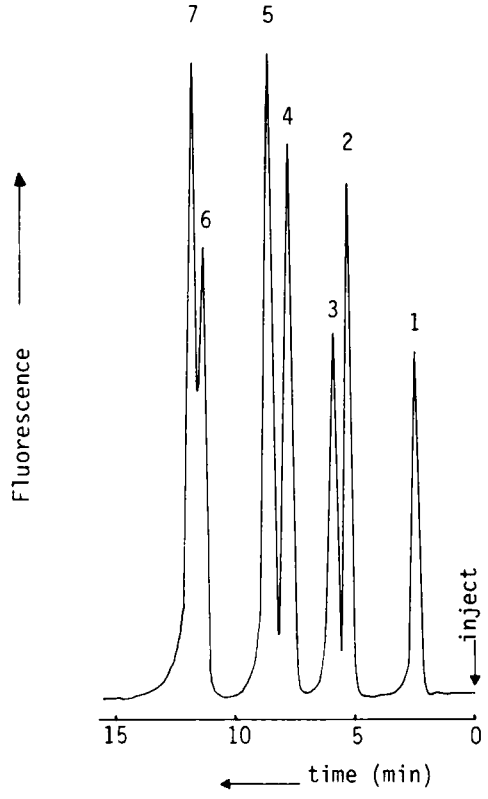

Figure 4 LC chromatogram of herbicides, using the system described in Figure 1. Chromatographic conditions: column, 150 mm × 3.1 mm I.D. 3 μm Hypersil MOS C-8; eluent, methanol-water (acidified to pH 2.5 with phosphoric acid) (55:45, v/v), flow rate, 0.6 ml/min; aqueous phase, approx. 15.5 μM NDA^+ in a 10 mM sodium phosphate buffer of pH 8.0 (containing 2.5% v/v methanol), flow rate, 0.6 ml/min; organic phase, chloroform—1-butanol (80:20, v/v), flow rate 1.0 ml/min; flow through detector, 0.3 ml/min; detector settings, $\lambda_{ex} = 260$ nm (slit, 10 nm), $\lambda_{em} > 470$ nm (cut-off filter); photomultiplier voltage, 935 V; rise time, 5 s.

A standard solution in the mobile phase, containing 70 ng of each herbicide was injected; injection volume, 10 μl. Peaks: 1. 2,3,6-TBA; 2. 2,4-D; 3. MCPA; 4. 2,4,5-T; 5. MCPP; 6. 2,4-DB; 7. MCPB.

Therefore, 20 ml drinking water samples were analyzed in order to prevent losses of the analytes. In Figure 6, a chromatogram of a blank drinking water sample, as well as of a sample spiked with 5 ng/ml of each 2,4-D and 2,4,5-T, is shown. From this figure, it is clear that sensitive and selective detection of phenoxyacid herbicides can be obtained by applying on-line post-column ion-pair extraction with a fluorescent counter ion, after trace enrichment by solid-phase extraction. Detection limits, in units of concentration, can easily be as low as 50–100 ppt.

CONCLUSIONS

The described post-column ion-pair extraction detector can be used in the LC

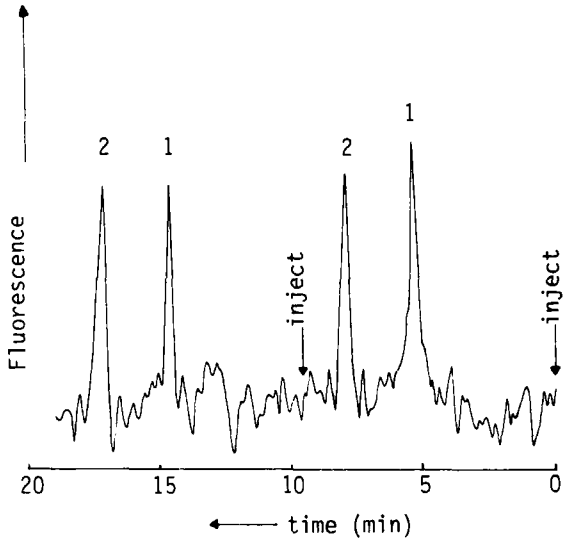

Figure 5 Duplicate determination of the detection limit of (1) 2,4-D and (2) 2,4,5-T, using the system described in Figure 4. A standard solution in the mobile phase, containing 1 ng of the phenoxyacid herbicides was injected. The injection volume was 10 μl.

determination of phenoxyacid herbicides. Low detection limits are obtained and the selectivity of the system appears to be good.

The introduction of a cationic function into the fluorophore NDA, by means of the reaction with 2-aminoethyltrimethylammoniumchloride hydrochloride, is only one example of the synthesis of a quaternary ammonium fluorophore suitable for ion-pair extraction of carboxylic acids. It should be realized that many fluorescent labels for the derivatization of compounds with a primary amine function[24] can be evaluated for this purpose. Because the derivatization of carboxylic acids prior to LC is still difficult, especially with respect to the required reaction conditions—i.e., elevated temperature, long reaction time, the necessity of using non-aqueous solvents and off-line time-consuming procedures—, the use of on-line post-column ion-pair extraction can well be a useful alternative.

Preliminary experiments with fatty acids and prostaglandins show that sensitive fluorescence responses can also be obtained in the ion-pair extraction detector for other compounds with a carboxylic acid functional group. Therefore, the application area of this system will be further evaluated in the near future, testing various fluorescent cations and different compounds with a carboxylic acid functional group.

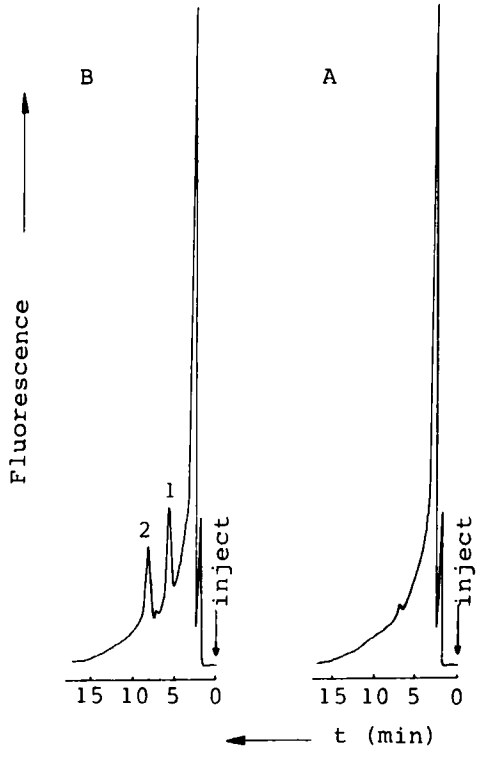

Figure 6 Analysis of drinking water, using 20 ml samples, A, non-spiked sample; B, sample spiked with 5 ng/ml of each 2,4-D and 2,4,5-T. Preconcentration was carried out as described under Experimental. Other conditions, see Figure 4.

Acknowledgements

We thank G. Gübitz (Graz, Austria) and P. J. M. Kwakman (Free University) for stimulating discussions.

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