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Trace analysis of fluorescein-derivatized phenoxy acid herbicides by micellar electrokinetic chromatography with laser-induced fluorescence detection

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Abstract

Micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection was used for the trace analysis of phenoxy acid herbicides. Capillary electrophoresis (CE) with LIF detection, which has not previously been used for pesticide analysis, overcomes the poor sensitivity of on-column UV detection. A novel derivatization procedure was developed which is suitable for nanogram amounts of organic acids. In this procedure, the acids are activated by hydroxybenzotriazol (HOBT) and diisopropylcarbodiimide (DIC) and reacted with 5-(aminoacetamido)fluorescein in dimethylformamide at ambient temperature. The fluorescent derivatives of all relevant phenoxy acid herbicides were separated in a single run by MEKC. A 488 nm Ar laser line was used for excitation. The reproducibility and reliability of the method were evaluated. The detection limit was 2 fg for a 4-nl injection, but for practical reasons, a minimum of 1 ng per compound should be subjected to the derivatization. The applicability of the described method to the extract of an aqueous sample was demonstrated.

1. Introduction

High-performance capillary electrophoresis (HPCE or CE) is a powerful, fast, and inexpensive analytical technique, yet in principle simple. In the past decade, numerous applications have been demonstrated in pharmaceutical and biochemical analysis [1–3]. There are, however, hardly more than a dozen reports on the use of CE for the analysis of pesticides [4–20]. This is principally due to the insufficient sensitivity of standard CE techniques with on-column UV detection. Consequently, most of these reports [4–13,15–19] deal with analytical concentrations in the ppm range in standard solutions or real extracts. Environmental trace analysis at sub-ppb levels (i.e., at concentrations in the ppb range after extraction), can become feasible only if sample preconcentration techniques or improved detection systems are used. The principal possibilities, as recently summarized by Nielen [14], include on-line isotachophoretic preconcentration, field-amplified injection techniques, or improved detection techniques such as laser-induced fluorescence (LIF) detection. Using CE with field-amplified injection after solid-phase extraction (SPE), Nielen [14] was able to determine phenoxy acid herbicides in 40-ml water samples at sub-ppb levels. Recently, Kaniansky et al. [20] achieved ultratrace determination of

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the herbicides paraquat and diquat in water by using CE with isotachophoretic sample pretreatment. Cai and El Rassi [6] accomplished a 10- to 35-fold preconcentration of triazine herbicides by using on-line coupling with capillaries having surface-bonded octadecyl functions. However, all these preconcentration techniques are hardly suitable for automated analysis with commercially available equipment and software.

Phenoxy acids, such as 2,4-D, constitute an important class of herbicides and are used worldwide in agriculture. A common method for their trace analysis in water samples is GC with electron-capture detection (ECD) after liquid–liquid extraction and derivatization with diazomethane [21]. Detection limits are in the upper ppt or low ppb range, but the hazards associated with diazomethane are a major disadvantage for this method. Butz et al. [22] recently described the analysis of 34 acidic pollutants, including all phenoxy acid herbicides, at low ppt levels in water. They used GC–MS with single-ion monitoring (SIM) after solid-phase extraction of the pollutants and derivatization with pentafluorobenzyl bromide. HPLC with UV detection can also be used, but detection limits for water samples are only in the ppb range, and the limited resolution provided by HPLC does not allow the analysis of a large number of herbicides in one run [23]. Sensitivity for HPLC can be improved by using LIF detection after derivatization of the phenoxy acids with 9-anthryldiazomethane (ADAM) [24]. Thus, detection limits in the upper ppt range could be achieved for spiked water samples. CE has also been applied to the analysis of phenoxy acids [9,12–14,16]. However, as discussed above, sensitivity is poor, and thus far only Nielen [14] has accomplished their trace analysis at sub-ppb levels in water samples.

A major goal of the analytical chemistry research program at the U.S. EPA (Las Vegas) is to ensure that novel analytical techniques become incorporated into the tools available for routine monitoring of pesticides and other pollutants in the environment. CE with LIF detection has not previously been used for pesticide analysis. It is a relatively simple, though highly sensitive technique. Of course, the need for fluorescent labelling of the analytes prior to analysis is an inherent disadvantage. In this paper we report on a novel procedure that allows the derivatization and subsequent analysis of nanogram amounts of all relevant phenoxy acid herbicides in a single run by micellar elektrokinetic chromatography (MEKC or MECC). The emphasis of this work was on developing conditions for derivatization and separation rather than on solid-phase extraction of real samples.

2. Experimental

2.1. Chemicals

Phenoxy acid standards were obtained from Aldrich (Milwaukee, WI, USA) or Chem Service (West Chester, PA, USA). 5-(Aminoacetamido)fluorescein was obtained from Molecular Probes (Eugene, OR, USA). Other chemicals were purchased from Aldrich (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA). High purity solvents were used throughout (Baxter, McGaw Park, IL, USA). Deionized water was used (Barnstead/Thermolyne, Dubuque, IA, USA) for all aqueous solutions.

2.2. Instrumentation

All CE experiments used a P/ACE Model 5000 instrument (Beckman, Fullerton, CA, USA). It was equipped with a commercial Beckman LIF detector. In this setup, the exciting light (488 nm) was provided by an argon laser source (3 mW), and a 520-nm band pass emission filter was used. The Beckman System Gold software was used for system control, data collection, and processing.

2.3. Capillary electrophoresis

A 47 cm (40 cm to detector) × 50 μm I.D. fused-silica capillary (Beckman) was installed in a temperature-controlled (25°C) cartridge spe-

cially designed for the LIF detector. The voltage was 30 kV, and the current was ca. 60 μA with the running buffer described below. Injections were by pressure (35 mbar) for 5 s. The capillary was conditioned every morning before starting a sequence of runs by rinsing in the high-pressure mode for 5 min with 0.1 *M* sodium hydroxide, 5 min with water, and 5 min with the running buffer. Pre-run rinsing was performed for 4 min with the running buffer. After every run it was rinsed for 8–15 min (ca. 1/3 of the run time) with 0.1 *M* sodium hydroxide and for 4 min with water. When not in use, the capillary was kept filled with 0.01 *M* sodium hydroxide.

A 50 m*M* phosphate buffer (pH 7.0) was prepared by mixing appropriate amounts of 50 m*M* solutions of monobasic and dibasic sodium phosphate. Running buffer (100 ml) was prepared by dissolving 1.327 g of sodium dodecyl sulfate (SDS), 10.030 g of urea, and 22.2 ml of methanol in ca. 60 ml of 50 m*M* phosphate buffer (pH 7.0). After complete dissolution, more phosphate buffer was added to give a total volume of 100 ml. The resulting solution was ca. 39 m*M* in phosphate, 46 m*M* in SDS, 1670 m*M* in urea, and contained 22.2% (v/v) methanol. The buffer was filled into 5-ml minivials and stored in a freezer for up to a week. For optimal reproducibility of migration times and selectivity, the inlet buffer vial was replaced after every CE run.

2.4. Derivatization of phenoxy acids

The following individual solutions were prepared in dimethylformamide (DMF): 40 m*M* diisopropylethylamine (DIPEA), 20 m*M* hydroxybenzotriazol (HOBT) monohydrate, 750 m*M* diisopropylcarbodiimide (DIC), and 0.5 m*M* 5-(aminoacetamido)fluorescein. The solutions of HOBT and DIC were freshly prepared directly before use. The DIPEA solution was stable at room temperature, and the solution of the fluorescein reagent was stored for up to several weeks in a freezer. To a stock solution of the phenoxy acids in 250 μl DMF in a 5-ml minivial, 25 μl of each of the following reagent solutions were added: DIPEA, HOBT, DIC, and the fluorescein reagent. An electric 250-μl pipet (Rainin, Woburn, MA, USA) was used for this operation. After allowing the mixture to stand at ambient temperature overnight (18 h, or only 3 h if MCPB and 2,4-DB are not present; see Fig. 1), a 25-μl portion was taken and diluted with 150 μl of phosphate buffer (pH 7.0). A 40-μl portion of this solution was injected to the CE from a microvial. Because of slow deterioration, the analysis was performed within approx. 12 h. Excess reaction mixture was stored in a freezer for up to several days. Caution: all glassware must be rinsed with 50 % acetic acid to destroy remaining highly toxic carbodiimide, and excess DIC reagent must be destroyed in the same manner.

2.5. Extraction of phenoxy acids from water

Distilled water (250 ml) was spiked with 1 nmol/l of every phenoxy acid, fortified with 1.5 ml of methanol and adjusted to pH 5.5 with monobasic and dibasic phosphate. As specified in the manufacturer's instructions, a C_{18} membrane disk (Empore 3M, distributed by Varian, Harbor City, CA, USA) was washed and conditioned with methanol. The sample was passed through the disk at a flow-rate of approx. 0.2 l/min to remove non-acidic contaminants. After washing the disk with 100 ml of water (adjusted to pH 5.5 and containing 0.5% (v/v) methanol), the purified eluate was acidified to pH 1.5 with 10 ml of 10 *M* hydrochloric acid and again subjected to solid-phase extraction with a clean disk. After passing the sample through, the disk was dried by applying a vacuum for 1 h. The herbicides were then eluted with two 8-ml portions of methanol. A boiling chip was added to the combined eluate, which was then taken to dryness using an oil bath at 110°C. The residue was dried in an oven at 110°C for 20 min. The derivatization was carried out as described in section 2.4, except that 40 m*M* HOBT, 900 m*M* DIC, and 2.5 m*M* fluorescein reagent solutions were used, and that the reaction time was only 2 h.

3. Results and discussion

3.1. Choice of the derivatizing reagent

Commonly used fluorescent labelling reagents for organic acids are bromomethyl coumarin derivatives and 9-anthryl- or 1-pyrenyl-diazomethane (ADAM and PDAM) [25,26]. PDAM was recently used for the determination of short-chain dicarboxylic acids in blood serum in the μmol/l concentration range by CE with LIF detection [27]. Compared with those reagents, derivatization with a suitable fluorescein reagent offers several advantages:

- sensitivity is higher because fluorescein derivatives are among the most intensely fluorescent molecules;
- the excitation wavelength exactly matches the 488 nm light provided by an argon laser that is used in at least one commercially available CE system with LIF detection;
- the use of long-wavelength excitation light reduces problems with light scattering and thus enhances sensitivity;
- the carboxy group on fluorescein may make it easier to separate the derivatives as anions with CE techniques.

No analytical derivatization procedure for low-molecular-mass organic acids with a fluorescein reagent has been described in the literature. In initial experiments we attempted the esterification of phenoxy acids with 5-bromomethyl fluorescein. However, this procedure proved to be more complex than the well-established analogous reaction with 4-bromomethyl-7-methoxycoumarin in acetone with potassium carbonate as a base [28,29]. The derivatization was only partly successful for microgram amounts of phenoxy acids in DMF after deprotonation with a very small amount of the hindered base 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU). Nevertheless, the reagent tended to decompose and this approach was finally abandoned.

A more suitable derivatization procedure for the phenoxy acids pictured in Fig. 1a was found in the formation of an amide linkage by employing a fluorescein reagent with a reactive amino group. Several fluorescein reagents with amino functions have been used for the derivatization of certain reactive carboxy groups in proteins and other biomolecules, employing either carbodiimide-mediated or enzyme-catalyzed coupling reactions [25]. The carboxy function found in fluorescein itself is not reactive to carbodiimides [25]. This is also verified in this work because the reagent does not readily react with itself, even after overnight contact at large excess (cf. below). Thus far, amine-substituted fluorescein reagents have only been used in biochemical applications, and no generally applicable analytical derivatization procedure has been reported for the labelling of small organic acids with these reagents.

We did not use the readily available aminofluoresceins (fluorescein amines) as fluorescent labelling reagents for phenoxy acids because aromatic amino groups are not reactive enough with carboxylic acids such as those of proteins [25]. The phenoxy acid substrates are resistant to amide formation and are unreactive without an activating agent (cf. below). Shipchandler et al. [30] reported on the usefulness of 4′-(aminomethyl)fluorescein as a reagent in immunodiagnostic techniques. However, that reagent is chiral and exists as a mixture of two rotamers [30], and as a consequence the derivatization of chiral phenoxy acids would lead to a mixture of diastereomers. Therefore, we chose another commercially available reagent, 5-(aminoacetamido)fluorescein (fluorescein glycine amide, Fig. 1b), previously used for the derivatization of milligram amounts of the undecapeptide O-acetyl-cyclosporin A [31]. The reagent's excitation wavelength at 489 nm best matches the argon laser line at 488 nm.

3.2. Optimization of derivatization conditions

We adopted a method commonly used in peptide synthesis [32,33], i.e., a one-step procedure consisting of the activation of the analytes with HOBT [33] and carbodiimide and reaction with the amine, using DIPEA as a base and DMF as the solvent. DIC [34] was found more suitable than dicyclohexylcarbodiimide (DCC)

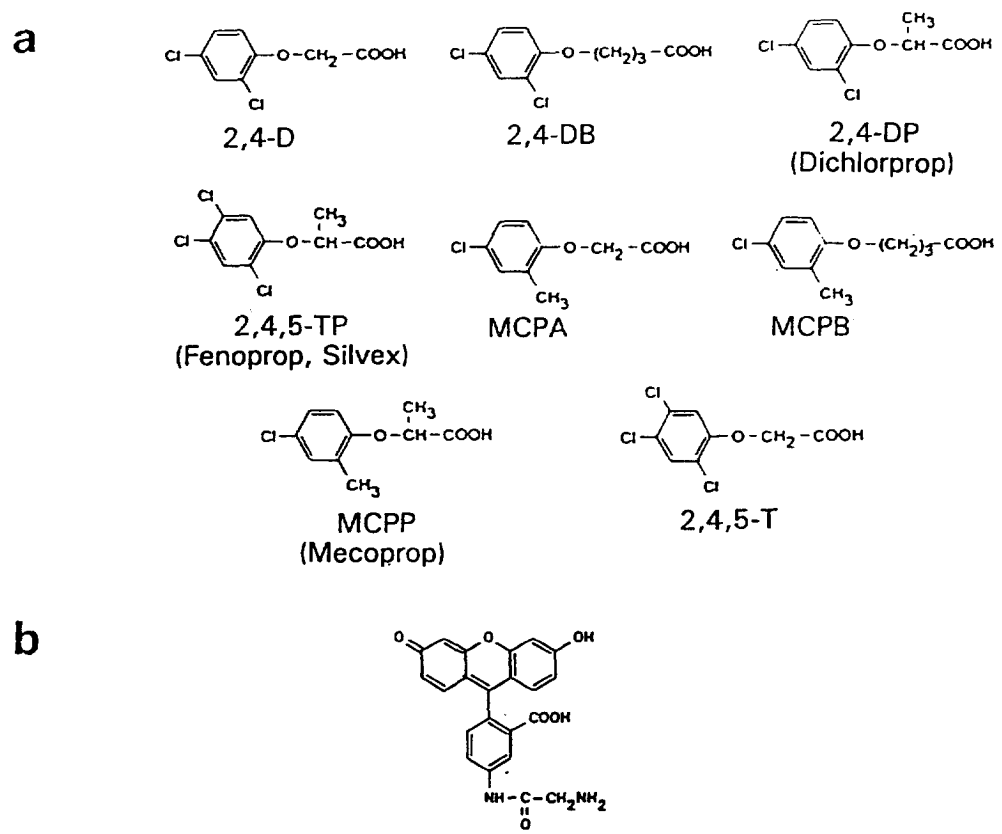

Fig. 1. Structures of (a) the phenoxy acid herbicides and (b) the derivatizing reagent 5-(aminoacetamido)fluorescein (fluorescein glycine amide).

because no precipitate formed upon dilution of the reaction mixtures with an aqueous buffer. In initial experiments we used a slight excess of the phenoxy acid to test the completeness of the procedure by MEKC with LIF detection. When the concentrations were lowered to analytically useful levels, the reaction became considerably slower. Part of the labelling reagent, which was now used in a large excess, was lost to side reactions. By carefully optimizing the concentrations of all reagents, we maximized the rate of reaction, while suppressing decomposition reactions of the labelling reagent to a reasonably small extent. The reaction was unsuccessful without any HOBT present, although only a small amount was needed. The concentration of DIPEA did not seem to influence rates and extent of main and side reactions. Our conditions may be compared to those of Mechref and El Rassi [35] who first derivatized acidic carbohydrates with aminoaromatic sulfonic acids for sensitive detection in CE.

The optimized derivatization conditions are described in the Experimental section. Under these conditions, the reaction is complete for most analytes after 3 h at ambient temperature. Only the butyric acid derivatives, MCPB and 2,4-DB (Fig. 1a), react more slowly (ca. 1/8), as can be seen by following the reaction kinetics using MEKC with LIF detection. For MCPB and 2,4-DB, the derivatization is complete only after one night (18 h) at room temperature. Heating to 60°C was not found useful since it enhances side reactions. The reaction can be considered complete for all analytes when MEKC analysis yields the same peak areas for the MCPB and

2,4-DB derivatives as it does for the other acid derivatives. After 18 h, only approx. 30% of the reagent are lost to side reactions.

3.3. Optimization of analytical conditions

After optimization of the running buffer, the separation of all investigated phenoxy acid adducts, as well as the excess reagent and its decomposition products, could be achieved by MEKC in a single run. Although they are not herbicides, 3,4-D and 2,3-D were also studied since these compounds could serve as internal standards or herbicide surrogates. Typical chromatograms for all the phenoxy acids are shown in Fig. 2. For the selected capillary dimensions, a separation efficiency corresponding to 170 000–230 000 theoretical plates was observed.

Capillary zone electrophoresis (CZE) was not successful, and both methanol and urea were necessary as buffer additives to provide the required selectivity. Comparable results were not obtained when a phosphate buffer at pH 7.5 or borate buffer at pH 8.4 was used instead of the phosphate buffer at pH 7.0. As often observed in CE, the separation was best when the pH of the running buffer is close to the pK_a of the analytes, which is 6.5. A lower pH cannot be chosen because only the anions of the fluorescein derivatives are fluorescent [25]. The peak of 3,4-D often tended to be broadened. Surprisingly, the electric field strength also affected the selectivity to a certain extent; the separation of 2,3-D and MCPP deteriorated upon changing the voltage from 30 to 25 kV when the capillary length was kept the same.

It should be noted that, as a consequence of the large excess of the reagent, even minor decomposition products can give additional peaks, which can easily be larger than those of the phenoxy acid derivatives. Fortunately these peaks are concentrated in the first part of the chromatogram and therefore do not interfere. A very small peak appeared after 2,4-DB and another one after 2,4-DP. These small peaks were fully separated from the phenoxy acid peaks and were also found in a blank derivatization run (i.e., without any phenoxy acids present) (Fig. 2g). Prior to analysis, a 25-μl portion of the reaction mixture was diluted with 150 μl phosphate buffer (pH 7.0). Considerable peak broadening resulted if less than 150 μl of buffer were added. The concentration of the buffer used for dilution had no obvious influence on analysis. A borate buffer at pH 8.4 could not be used for dilution of the sample because peak splitting was then observed for 2,4,5-T and 2,4,5-TP when methanol concentrations below 22% (v/v) in the running electrolyte were employed.

3.4. Reliability and reproducibility

To evaluate the described procedure, six individually prepared mixtures, each containing 100 pmol of every investigated phenoxy acid, were derivatized and analyzed in consecutive runs. The results are listed in Table 1. Relatively stable migration times could be obtained when the column was thoroughly rinsed with sodium hydroxide after every run, as described in the Experimental section. The replacement of the running buffer after every run further enhanced reproducibility. The day-to-day reproducibility is affected by any changes of the capillary surface that affect the electroosmotic flow (EOF). Therefore, it is better to rely on relative migration times, whose reproducibility was found to be much better. As far as the peak areas are concerned, the reproducibilities were good, except for MCPB and 2,4-DB. We attribute the somewhat larger standard deviation for those herbicides to their much slower rate of derivatization. They may not always be quantitatively derivatized, sometimes only to ca. 95%. A complete derivatization of MCPB and 2,4-DB in real extracts may become impossible in cases where the extracts contain substances that accelerate the decomposition of the derivatizing reagent.

3.5. Limit of detection

For the derivatization and injection conditions described in the Experimental section, the injection volume according to Hagen–Poiseuille's law is approx. 4 nl for a 175 mbar s injection [18].

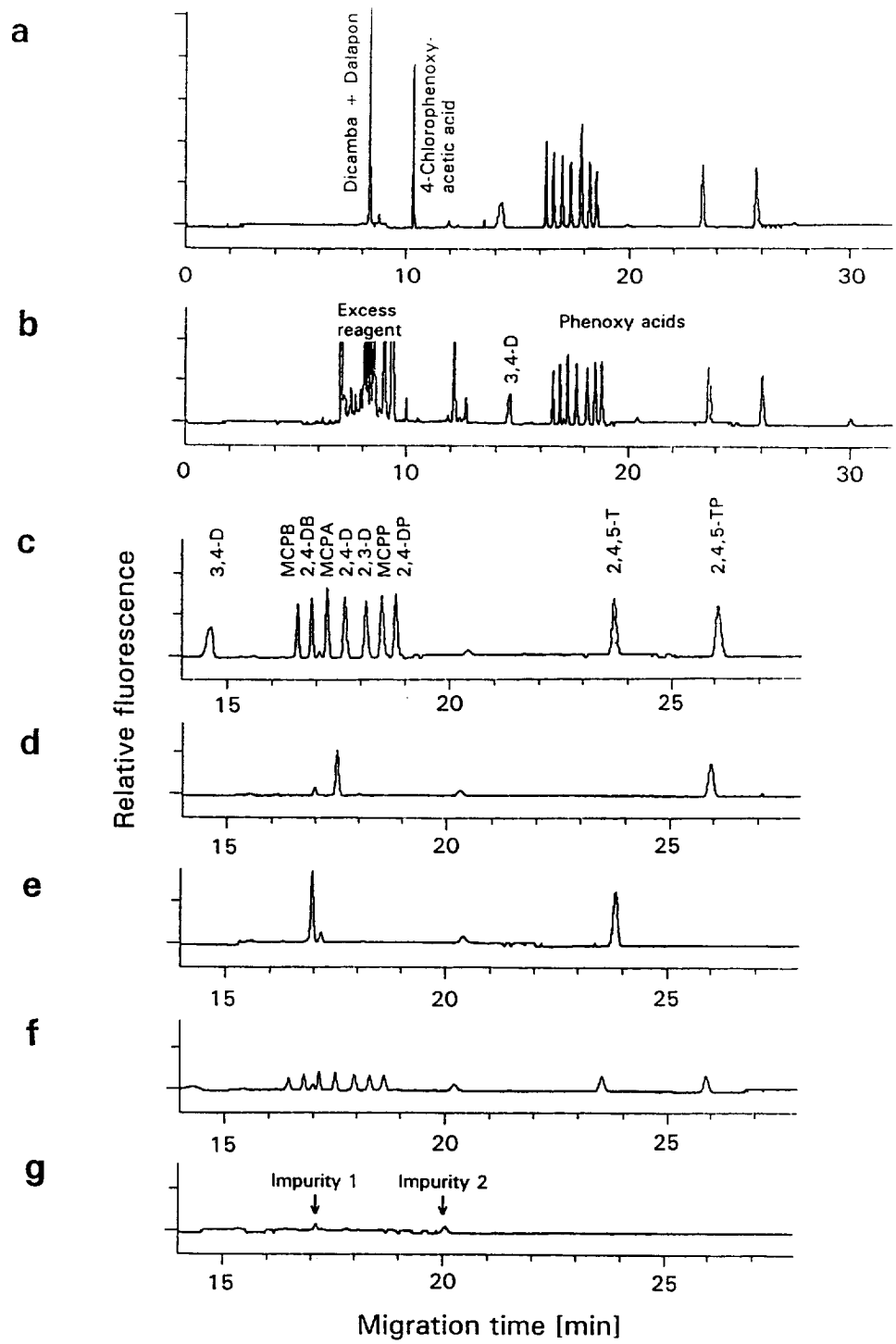

Fig. 2. Chromatograms from trace analysis of phenoxy acid herbicides by MEKC with LIF detection after derivatization of stock solutions as described in the Experimental section. Analytical conditions: voltage, 30 kV; capillary, 47 cm (40 cm to detector) × 50 μm I.D.; buffer, 39 m*M* phosphate pH 7.0, 46 m*M* sodium dodecyl sulfate, 1670 m*M* urea, 22.2% (v/v) methanol. (a) Artificial mixture of acid derivatives prepared at a semi-preparative scale; (b) derivatization of a mixture containing 100 pmol (approx. 20 ng) per phenoxy acid; (c) enlargement of the relevant part of chromatogram b; (d) derivatization of 2,4-D and 2,4,5-TP, 100 pmol each; (e) derivatization of 2,4-DB and 2,4,5-T, 100 pmol each; (f) derivatization of a mixture containing 20 pmol per phenoxy acid; (g) blank derivatization without phenoxy acids.

Table 1
Reliability and reproducibility data for the derivatization[a] of phenoxy acids with fluorescein glycine amide and analysis of the derivatives by MEKC[b]

Analytes	Relative standard deviation (%) of absolute migration times ($n = 6$)	Mean relative migration times (2,3-D = 1)	R.S.D. (%) of relative migration times ($n = 6$)	Mean corrected[c] relative peak areas (2,3-D = 100)	R.S.D. (%) of relative peak areas ($n = 6$)
3,4-D	1.9	0.784	1.1	140	1.9
MCPB	2.4	0.894	0.3	87	10.6
2,4-DB	2.5	0.917	0.2	95	9.7
MCPA	2.7	0.945	0.1	114	2.0
2,4-D	2.6	0.970	0.1	110	1.5
2,3-D	2.7	1	–[d]	100	–[d]
MCPP	3.0	1.021	0.1	109	2.9
2,4-DP	2.9	1.043	0.2	104	3.8
2,4,5-T	3.6	1.359	0.9	89	1.6
2,4,5-TP	3.9	1.519	1.3	86	3.2

[a] The derivatization was carried out six times using individually prepared mixtures, each containing 100 pmol per phenoxy acid.
[b] Analytical conditions as in Fig. 2; cf. Experimental section.
[c] With on-column detection, the peak areas directly depend on the migration times. To obtain comparable values, the data acquisition software automatically corrects the measured peak areas accordingly.
[d] Not applicable.

For stock solutions of phenoxy acids, the detection limit per injection is 2 fg for a signal-to-noise ratio of 8. However, handling and derivatization for practical reasons require a minimum of approx. 1 ng. For extracts of real samples, higher detection limits have to be expected, depending on the sample.

3.6. Extraction from water samples

The extraction of water samples spiked with phenoxy acids was performed by solid-phase extraction using C_{18} membrane disks under similar conditions as described previously [16,22]. Before extraction at pH 1.5, the sample was purified by means of another extraction step at pH 5.7, thus removing non-acidic organic compounds. However, we found that there were still side reactions occuring during our derivatization procedure, resulting in enhanced decomposition of the derivatizing reagent and in additional peaks in the chromatogram. For water samples spiked at 1 nmol/l (approx. 0.2 ppb) per phenoxy acid, recoveries in the 60–80% range were obtained for most analytes. The recovery of 2,4,5-TP was poor (approx. 30%). An example is shown in Fig. 3. MCPB and 2,4-DB are barely visible since the reaction time for derivatization was only 2 h. After longer reaction times, the background noise level resulting from side reactions was found to be higher. Further work towards optimization and development of suitable conditions for sample preparation and extraction is in progress.

4. Conclusion

MEKC with LIF detection is applicable to the determination of nanogram levels of phenoxy acid herbicides. The described procedure can be performed with commercial equipment, using an autosampler for automated analysis. The high efficiency of CE allows the separation of the fluorescein derivatives of all target phenoxy acids in a single run while still not crowding the chromatogram. However, the derivatization procedure is not as robust as some well-established

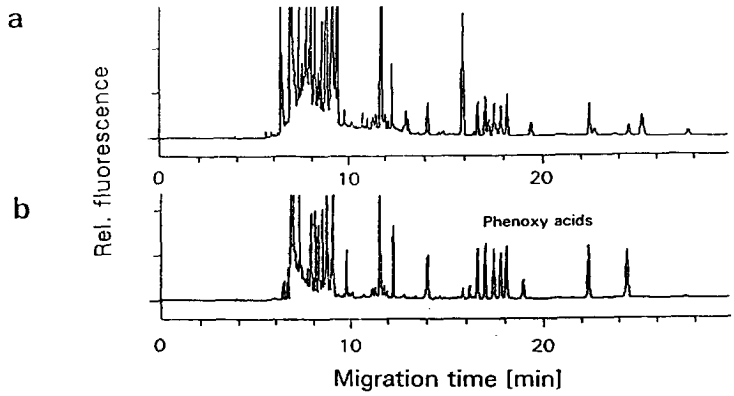

Fig. 3. (a) Trace analysis of phenoxy acid herbicides in an extract of a 250-ml water sample spiked to give 1 nmol/l per acid; (b) reference: direct derivatization of corresponding amounts of phenoxy acids from a stock solution. Peak assignment and other conditions as given in Fig. 2.

procedures. Extracts of real samples may contain compounds that enhance the decomposition of the derivatizing reagent, thus necessitating a modification of the procedure. It is expected that analytical conditions similar to those developed in this work can be applied not only to phenoxy acids, but also to other classes of compounds after derivatization with a suitable fluorescein reagent.

Notice

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