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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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Use of immobilized copper ion chromatography and on-line mass spectrometry with atmospheric pressure chemical ionization for the profiling of complex mixtures of polycyclic aromatic compounds☆

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Abstract

Immobilized copper ion chromatography was combined with either diode array detection or atmospheric pressure chemical ionization mass spectrometry for the profiling of complex mixtures of polycyclic aromatic compounds (PACs). A heated pneumatic nebulizer interface was used to convey the eluent from the column into the ionization region of the mass spectrometer. Several modifications were made to the heated pneumatic nebulizer interface originally designed for liquid chromatography–mass spectrometry so that the mass spectrometer could be safely operated with large quantities of organic solvent passing into the ionization region of the instrument. A copper-coated Zorbax 300 SCX column was used in conjunction with a hexane, chloroform and acetonitrile gradient to accomplish fractionation of three extracts containing PACs. The column was able to separate portions of the extracts into a number of fractions, each of which contained homologous series of different PAC classes. Using this technique, it is possible to gain considerable insight into the types and relative amounts of minor components present in the PAC mixtures.

1. Introduction

In the analysis of complex mixtures of polycyclic aromatic compounds (PACs), a continuing problem is that of identification of many of the heterocyclic compounds present in the mixture. Generally these compounds are present at levels of 1% or less of their polycyclic aromatic hydrocarbon (PAH) analogues; PAHs are almost always the dominant PACs in the mixture. The principal reason for the determination of the PAC content of any given sample is the carcinogenic and mutagenic nature of many of these compounds [1]. For this reason substantial effort has been expended in the development of methods for their reliable determination in a variety of matrices [1]. Almost all such methods depend

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on some form of chromatography in the final analytical step to separate the mixture into its various components. In previous work, we have developed the thesis that such analyses are most reliably performed by using a mass spectrometer as the chromatographic detector [2,3]. Despite the level of effort, there has been relatively little work on the development of methods for the determination of compounds other than the dominant PAHs.

Interest in methods for the separation of the heterocyclic PACs, particularly those containing sulfur or nitrogen, from their hydrocarbon analogues has been spurred by the fact that many of these compounds are potent carcinogens [4]. Thus, assessment of potential exposure hazards requires methods of analysis for these compounds in environmental samples. In general, these methods involve some step, where the compounds of interest are separated and concentrated from the PAHs. The reasons for this selective concentration step are twofold. First, unambiguous compound identification in any chromatographic–mass spectrometric analysis depends upon the chromatographic technique having sufficient resolving power to deliver the individual analytes to the mass spectrometer at a slow enough rate to permit the acquisition of mass spectra of sufficient quality for positive identification. Second, the overall column loading must be kept low enough to prevent peak shape distortion and thus losses in resolving power, which could lead to co-elution of compounds and thus unreliable identification, or to poor peak area integration and thus unreliable quantification. In other words, a compound class-selective preconcentration step provides a wider margin for compromise in the choice of chromatographic conditions for the final analysis.

A variety of techniques have been developed which permit the analyst to isolate one particular group of heterocyclic PACs, usually the sulfur compounds, from the other components of the mixture. Thus a separation on a column of silica impregnated with palladium chloride has been developed for the isolation of sulfur heterocycles from a PAC mixture [5], and this technique has been applied to the characterization of several coal- and petroleum-derived products [6]. Other techniques have been developed using silver nitrate-impregnated silica [7–9], or other stationary phases containing mercury [10,11], to achieve the same end. We have recently developed a technique, based on the use of copper immobilized on silica, which permits the separation of complex mixtures of PACs from environmental samples into a number of less complex fractions, containing members of different compound classes [12]. In that work we were able to obtain a satisfactory separation of compounds containing nitrogen and oxygen from other classes of PACs. The sulfur heterocycles were not separated from the PAH fraction, so that the technique may be considered to be complementary to those methods focused on isolating the sulfur heterocycles from all other components. The copper–silica fractionation [12] permitted us to separate the carbazoles from other nitrogen species, allowing unambiguous chromatographic–mass spectrometric identification of many minor components in the mixture. Despite their success in separating some components of the mixture from others, such techniques mostly share a common weakness; viz., they all rely on lengthy open-column chromatographic steps to isolate the fraction(s) of interest before the analytical step, and all such manipulations must be performed before the analyst can determine whether or not the target compounds sought are actually present in the sample.

In the present work we describe the development of an on-line chromatographic–mass spectrometric technique for the rapid profiling of PAC mixtures. The technique applies the concept of immobilized metal ion chromatography to the problem [13]. A similar technique, using a carboxylic cationic exchange resin loaded with copper for the isolation of sulfides from petroleum-derived products, has already been described in the literature [7]. We have drawn on that work and developed a stationary phase in which the copper is immobilized on a strong cation exchange resin, for use as part of an on-line HPLC–MS profiling technique.

2. Experimental

2.1. Column preparation and conditioning

The column used for these experiments was a 25 cm × 4.6 mm Zorbax 300 SCX with 6 μm particle size (Chromatographic Specialities, Brockville, Canada) in which the stationary phase had been modified with copper. The stationary phase modification (akin to the silver loading commonly used in lipid chemistry [14]) was achieved by sequentially pumping the following solutions through the column (flow-rate of 1 ml/min, unless otherwise stated): deionized water (250 ml); 1 *M* $CuSO_4$ (150 ml at a flow-rate of 0.1 ml/min); deionized water (250 ml); methanol (250 ml); acetone (150 ml); ethyl acetate (150 ml); chloroform (150 ml); and hexane–chloroform (5:1) (150 ml). This sequence is identical to that used in conditioning silver–SCX columns for lipid class separations, except for the substitution of 1 *M* $CuSO_4$ in place of 1 *M* $AgNO_3$, and for the final rinses with the chloroform-containing solvents. When not in use, the column was stored in hexane. It was found that when the column had been in use for some time (ca. 1 month), its ability to successfully fractionate the PAC mixtures diminished. However, this problem could be overcome by reconditioning the column with the following solvents (at 1 ml/min, unless stated otherwise): hexane (75 ml); dichloromethane (75 ml); methanol (75 ml); deionized water (75 ml); 0.1 *M* HNO_3 (150 ml); deionized water (500 ml); 1 *M* $CuSO_4$ (150 ml at a flow-rate of 0.1 ml/min); deionized water (700 ml); methanol (150 ml); acetone (75 ml); ethyl acetate (75 ml); chloroform (75 ml); and hexane (75 ml).

2.2. Sample preparation and clean-up

Tar Pond extract

The Tar Pond extract originated from the Sydney Tar Ponds, Sydney, Canada, resulting from 80 years of uncontrolled discharge of the byproducts of a metallurgical coking operation [15]. The sample was isolated using the following procedure:

(1) A 5-g amount of Tar Pond sediment was Soxhlet extracted in dichloromethane (175 ml) for 24 h. After this time, the dichloromethane was evaporated to a final volume of 1 ml using a rotary evaporator.

(2) A 7-g amount of 100–200 mesh silica gel (Fisher Scientific, Nepean, Canada), dried at 160°C, was placed into a 25 cm × 2 cm fractionating column, and an additional 1 cm of copper metal powder (Fisher Scientific) was added. The copper had been previously cleaned with 0.1 *M* HNO_3 (100 ml) followed by deionized water (3 × 100 ml), acetone (3 × 100 ml), hexane (3 × 100 ml), and subsequently vacuum filtered to dryness. A 20-ml volume of diethyl ether was passed through the column and discarded. The dichloromethane extract was conveyed to the column and eluted with 20% dichloromethane in diethyl ether (3 ml) followed by 40% dichloromethane in diethyl ether (20 ml). The extracts were combined and rotary evaporated to approximately 1 ml. The purpose of this step was to remove elemental sulfur by reaction with the copper, and to trap compounds that may compromise subsequent chromatographic separations on the silica [2].

(3) A 20-g amount of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), pre-swollen in cyclohexane–methanol–dichloromethane (6:4:3) for 12 h, was placed into a 42 cm × 2 cm glass column. A 20-ml volume of the cyclohexane–methanol–dichloromethane solution was passed through the column and discarded. The extract from step 2 was added and eluted with 100 ml of cyclohexane–methanol–dichloromethane (6:4:3). The first 40 ml, which contained the aliphatic fraction, were discarded [16], the remaining 60 ml, which contained the PAC fraction [17], were collected. This fraction was rotary evaporated and dissolved in chloroform (10 ml) for analysis by immobilized copper ion chromatography (ICIC). The resulting solution contained approximately 75 mg of the original (5 g) mass of Tar Pond extract.

Harbour sediment, HS-3

HS-3 is a harbour sediment reference material that contains PAHs (Marine Analytical Chemistry Standards Programme, NRC, Halifax, Canada). A 35-g amount of this material was Soxhlet extracted and subjected to the first and second steps as described for the Tar Pond extract. For the third step, 6 g of 70–230 mesh neutral alumina (Merck, Darmstadt, Germany), which had been stored in an oven at 160°C were placed into a 25 cm × 2 cm fractionating column. To this were added 4 g of 60–120 mesh silica gel (BDH, Toronto, Canada). A 20-ml volume of pentane was passed through the column and discarded. The sample was added and eluted with pentane (35 ml), 5% benzene in pentane (40 ml), 15% benzene in pentane (40 ml) and methanol–benzene–diethyl ether (3:1:1) (25 ml). The first three fractions, which contained the aliphatic, monocyclic aromatic and bicyclic aromatic fractions, respectively [17], were discarded. The final fraction containing the PACs [18] was rotary evaporated and dissolved in 10 ml of chloroform for subsequent analysis. The resulting chloroform solution contained 50 mg of the original (35 g) HS-3 extract.

Heavy gas oil

Heavy gas oil (HGO) is one of the end-products of a refining process carried out by Syncrude Canada Ltd. for the production of naphtha and light and heavy gas oils from the Alberta Oil Sands. The PAC fraction was isolated using the following procedure:

(1) A 5-g amount of KOH was added to 400 ml of isopropanol and stirred for 1 h. A 75-ml volume of this solution was added to 5 g of 60–120 mesh silica gel (BDH), and the resulting slurry stirred for 24 h. The slurry was placed into a 42 cm × 2 cm glass column and the isopropanol solution was allowed to pass through the column, followed by 25 ml of chloroform which were discarded. A 250-mg amount of the HGO was added to the column and eluted with 70 ml of chloroform. The KOH-treated silica removed terpenoic acids which could interfere in subsequent chromatographic separations [18,19]. The chloroform was removed by rotary evaporation, and the resulting residue dissolved in 1 ml of pentane.

(2) The HGO extract was passed through a silica over alumina column as described for the harbour sediment. The methanol–benzene–diethyl ether (3:1:1) fraction was rotary evaporated, and the extract dissolved in chloroform (100 μl) in hexane (10 ml). The resulting solution contained 50 mg of the original (250 mg) HGO sample.

2.3. Instrumentation

A Hewlett-Packard (Palo Alto, CA, USA) 1090 Series II liquid chromatographic system was used for sample profiling. Hexane, chloroform and acetonitrile were used as the eluting solvents. A 25-μl volume of each extract was injected onto the copper-coated SCX column and eluted with the gradient described in Table 1 at a flow-rate of 1 ml/min. Detection in the first instance was performed using a Hewlett-Packard 1040 diode array detection (DAD) system set at a wavelength of 254 nm. For the HPLC–UV experiments the LC was controlled, and data collected and processed, by Hewlett-Packard 3365 Chemstation software installed on a Hewlett-Packard Vectra 486/33U computer.

The on-line ICIC–MS fractionation experiments were conducted using a Sciex API III triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmos-

Table 1
Mobile phase gradient employed for the separation of extracts containing PACs by ICIC–DAD and ICIC–APCI-MS using a copper-coated SCX column

Time (min)	Hexane (%)	Chloroform (%)	Acetonitrile (%)
0.00	100	0	0
8.00	85	15	0
15.00	0	100	0
17.00	0	100	0
22.00	0	50	50
27.00	0	100	0
29.00	0	100	0
31.00	100	0	0

pheric pressure chemical ionization (APCI) source. A 5-μl volume of each sample was injected onto the column and the eluent from the LC was conveyed into the ion source of the mass spectrometer through a heated pneumatic nebulizer interface. The interface has been described elsewhere [20]. However, due to the inherent danger of using hexane with the heated nebulizer interface [21], two modifications to the original design of the interface and source housing were used. First, a 200 MΩ high-voltage resistor was installed to limit the current through the corona discharge needle, and second, an over-pressure relief vent was added to the front end of the interface. Ultra-high-purity nitrogen was used as both nebulizer gas and make-up gas. The interface was operated at 400°C, with a nebulizer pressure of 60 p.s.i. (1 p.s.i. = 6894.76 Pa) and a make-up flow-rate of 1 l/min. The corona discharge needle was maintained at a current-controlled 3 μA discharge. Mass spectrometer control, data acquisition and data processing were accomplished using a Macintosh Quadra 950 microcomputer. The quadrupole mass spectrometer was operated using a dwell time of 2 ms per 1 u step for the mass range m/z 150–1000.

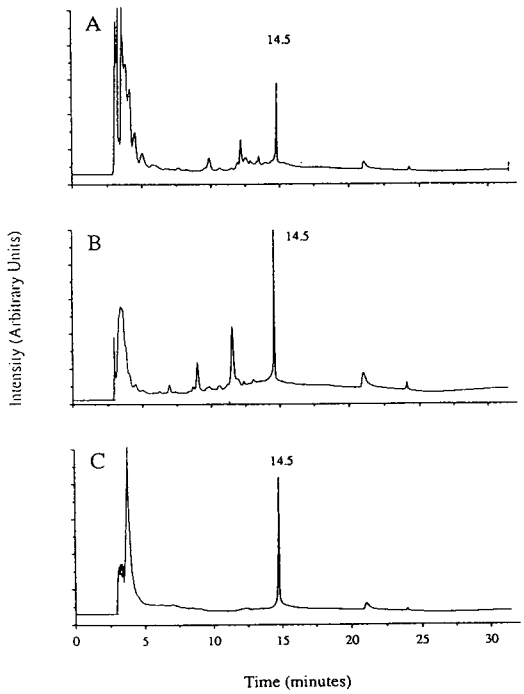

Fig. 1. Chromatograms obtained by the separation of (A) Tar Pond extract, (B) HS -3 and (C) HGO by ICIC–DAD (254 nm fixed) using a copper-coated SCX column.

3. Results and discussion

The extracts were initially passed through the copper-coated SCX column using the mobile phase gradient shown in Table 1, and were profiled using DAD. The resulting ICIC–DAD chromatograms for the extracts of the Tar Pond sediment, HS-3 and HGO, are shown in Fig. 1. It can be seen from the chromatograms that each extract has been separated into several distinct fractions, with some peaks common to all three extracts. Most of the fractionation takes place in the early part of the mobile phase gradient (100% hexane going to 100% chloroform). The use of hexane as a fractionating solvent was critical to the success of the technique. Other potential fractionating solvents were used [e.g., methanol, hexane–chloroform (1:1)], but it was found that all of the components of the extract eluted in one unresolved peak. To evaluate the reproducibility of the technique each fraction was passed through the column eight times. It was found that the retention time for the peak labelled at 14.5 min in Fig. 1 had an average retention time of 14.54 min with a relative standard deviation (R.S.D.) of 1.1%. However, when the column had been in use for a long time (1 month), its ability to resolve the components of the extracts diminished somewhat. Reconditioning of the column with solvent alone was not found to sufficiently improve resolution. However, when the column was reconditioned with the solvents and $CuSO_4$ solution, as described in the Experimental section, its performance returned to the original without any change in peak retention times.

In order to be able to identify the compounds present in the chromatographic peaks, either as impurities generated from the column or as components of the extracts, the extracts were injected into the HPLC system and detected by

APCI-MS. The total ion chromatogram (TIC) obtained by ICIC–APCI-MS for the Tar Pond extract is shown in Fig. 2A. It can be seen that the introduction of the HPLC eluent into the ion source of the mass spectrometer has been achieved without any loss of chromatographic resolution. It was found that the copper-coated SCX column was able to separate the Tar Pond extract into fractions containing components of various homologous series. This is exemplified in Fig. 2B which shows the mass spectrum obtained for the peak indicated in the inset of Fig. 2A. The mass spectrum indicates the presence of protonated molecules $[M+H]^+$ of pyrene, M_r 202, and alkylated pyrene derivatives. The peak marked "1" in each fraction (see Fig. 2, and Figs. 3 and 4 later) arises from copper compounds which elute from the column at the onset of the acetonitrile portion of the gradient. It is unclear whether these compounds arise from previous clean-up steps or are formed by a reaction between the copper on the column and components of the sample. The mass spectrum (not shown) of this peak shows two major sets of double peaks, at 2 u intervals as a result of the copper isotopes (^{63}Cu and ^{65}Cu) forming oxides (m/z 158 $[^{63}Cu_2O_2]^{+\cdot}$ and m/z 160 $[^{63}Cu^{65}CuO_2]^{+\cdot}$) as well as complexes with acetonitrile {m/z 170 $[^{63}Cu^{65}Cu(CH_3CN)+H]^+$ and m/z 172 $[^{65}Cu^{65}Cu(CH_3CN)+H]^+$}. For both the oxide and the acetonitrile cluster ion, peaks attributable to the remaining possible isotopic combinations {$[^{65}Cu_2O_2]^{+\cdot}$ and $[^{63}Cu_2(CH_3CN)+H]^+$} were not detected. None of the other peaks in the chromatograms exhibited the presence of copper complexes, indicated by the absence of regular series of mass spectral peaks separated by 2 u.

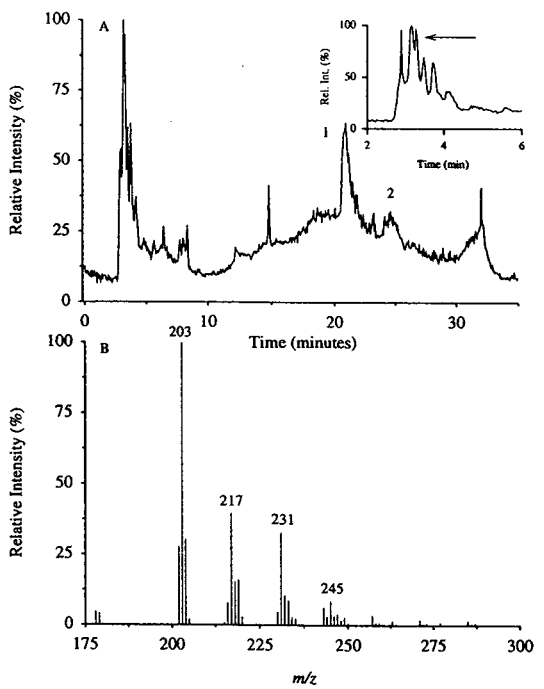

Fig. 2. (A) Total ion chromatogram (m/z 150–1000) obtained by the separation of the Tar Pond extract by ICIC–APCI-MS using a copper-coated SCX column. (B) Mass spectrum, obtained from the peak marked in the inset of (A), showing the components of the peak to be an homologous series of pyrene (M_r 202) and alkylated pyrene compounds.

It has been found previously that the relative responses of low- and high-molecular-mass (>200) PACs in APCI-MS are qualitatively different, when the sample is introduced under supercritical fluid chromatography conditions [22,23], and the analysis of PACs by the present ICIC–APCI-MS technique yielded similar results. The mass spectra of the lower-molecular-mass compounds are characterized by the formation of both molecular ions $[M]^{+\cdot}$ and protonated molecules $[M+H]^+$. Typically, the higher-molecular-mass PACs form only protonated molecules in an APCI environment (see Fig. 2B). The reasons for these phenomena have been discussed in previous publications [22,23].

The TIC obtained by ICIC–APCI-MS for the harbour sediment HS-3 is shown in Fig. 3A. Comparison of the ICIC–DAD chromatogram (Fig. 1B) and the TIC shows that the ICIC–APCI-MS system has only been partially successful in matching the HPLC–DAD chromatogram. However, the components of each peak in the profile could be detected by extracting reconstructed ion chromatograms for selected masses. Fig. 3B shows the mass spectrum obtained from the major peak indicated in the TIC. This mass spectrum is rather uninformative, consisting of a peak at every mass over the range scanned. However, the intensity profile of the envelope of

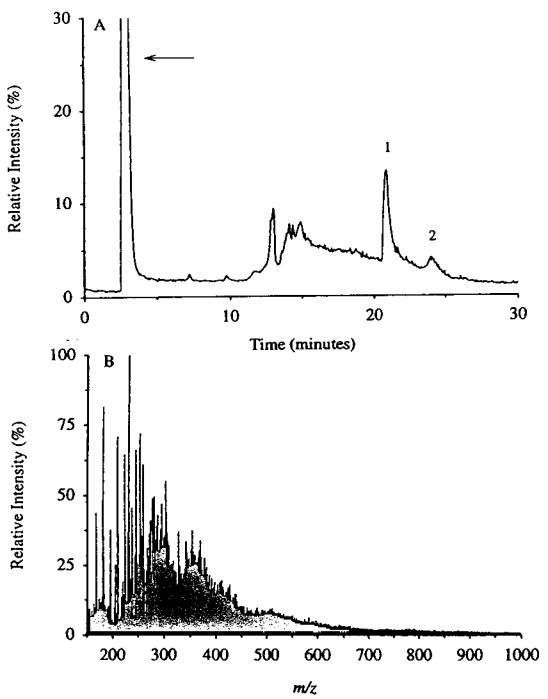

Fig. 3. (A) Total ion chromatogram (m/z 150–1000) obtained by the separation of the HS-3 extract by ICIC–APCI-MS using a copper-coated SCX column. (B) Mass spectrum obtained from the peak marked in the TIC above showing the components of the peak to include PACs of molecular masses that encompass the whole mass range scanned by the mass spectrometer.

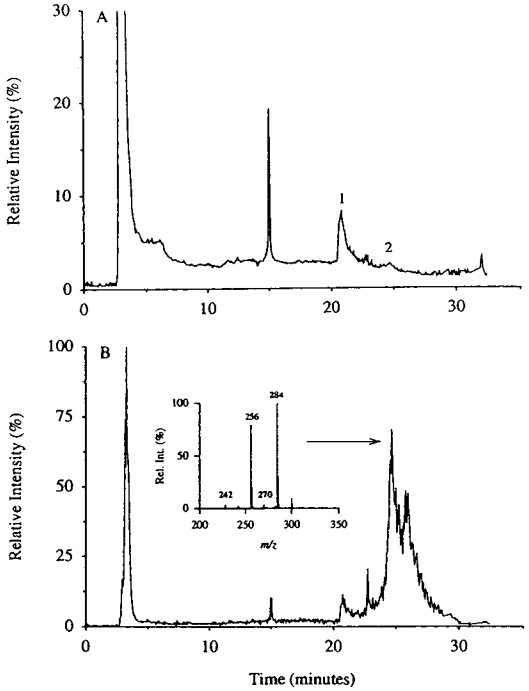

Fig. 4. (A) Total ion chromatogram (m/z 150–1000) obtained by the separation of the HGO extract by ICIC–APCI-MS using a copper-coated SCX column. (B) Reconstructed ion chromatogram of m/z 284 with the inset showing the mass spectrum of the indicated peak. The mass spectrum of this peak shows the possible presence of an homologous series of acridinones.

mass spectral peaks arising from this chromatographic peak is qualitatively very different for each of the three extracts.

Fig. 4A shows the TIC obtained by ICIC–APCI-MS of the HGO extract. This extract showed good correlation between the ICIC–DAD chromatogram (Fig. 1C) and the TIC. The reconstructed ion chromatogram of m/z 284 (Fig. 4B) shows a peak corresponding to that indicated by "2" in all of the TICs shown (see Figs. 2–4). The inset of Fig. 4B shows the mass spectrum obtained from this peak, which is dominated by four peaks at m/z values each separated by 14 u. This sequence of mass spectral peaks could possibly be explained as arising from an homologous series of compounds of the general formula ($C_{14+a}H_{11+2a}NO_3$), for $a = 0$–3, consistent with the acridinones, although their identities could not be confirmed due to a lack of authentic standard compounds.

The column, like the copper-coated silica open column described previously [12], effects a partial separation of the PAC fraction into distinct compound classes. This may be demonstrated by considering the mass chromatogram for m/z 209, shown in Fig. 5A. This mass is typical of a family of alkylated fluorenones with three substituent carbons. The mass spectrum acquired at the crest of the peak at a retention time of 7.9 min, shown in Fig. 5B, indicates that there are several members of a homologous series of (possibly) alkylated fluorenones present in this peak (peaks at m/z 209, 223, 237) as well as some other unidentified compounds. This group of compounds has been well resolved from the PAH peak, eluting at 3 min.

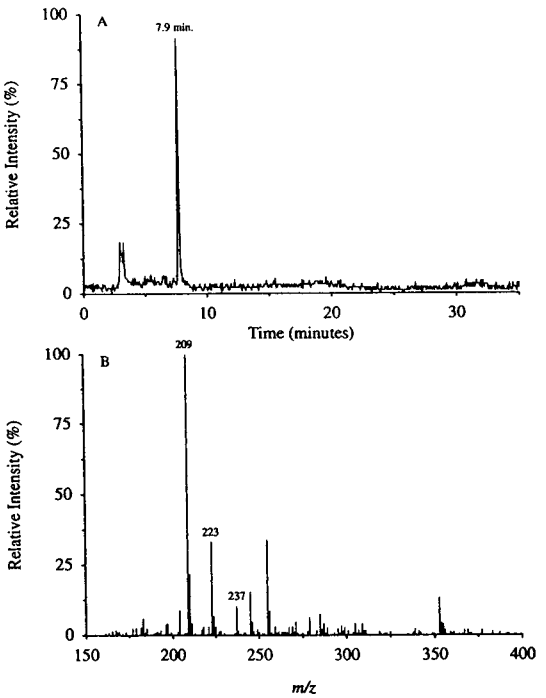

Fig. 5. (A) Mass chromatogram for m/z 209, extracted from the total ion chromatogram of Fig. 2A. (B) The background subtracted mass spectrum obtained at the crest of the peak at 7.9 min in (A).

4. Conclusions

The ICIC profiling technique appears to be a useful rapid method for revealing the relative amounts of some different classes of PACs, in complex mixtures of PACs from environmental and petroleum-derived sources. Using this method it is possible to demonstrate the presence or absence of several different classes of PACs in a mixture. The HPLC–ICIC–DAD profiles may be used to gain insight into the overall complexity of the PAC fraction, and thus to act as a guide to subsequent analytical procedures. Depending upon the needs of the analyst, it is possible to collect the fraction(s) of interest and submit them to further steps. As in the earlier work [12], the separation of other classes of PACs from the PAHs allows collection of high-quality mass spectra, sometimes permitting compound identification in suitable cases, in subsequent chromatographic–MS analyses. Work in progress in our laboratory has shown that the combination of ICIC fractionation with subsequent HPLC–MS–MS analysis can provide unambiguous identification of sulfur [24] and nitrogen [25] heterocycles in different fractions of the Tar Pond extract.

The three samples studied in this work have very different HPLC–UV profiles, which indicated significant differences in the types and amounts of various classes of PAC present. These samples were further examined using HPLC–ICIC–APCI-MS, indicating that the differences indeed derive from differences in the amounts of compounds of various PAC classes present in the mixtures of PACs present in the extracts. Using this technique, it is possible to obtain considerable information about the types and relative amounts of these minor components.

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JOURNAL OF CHROMATOGRAPHY A

Determination of aldehydes by high-performance liquid chromatography with fluorescence detection after labelling with 4-(2-carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole

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Abstract

The utility of the fluorescent labelling reagent 4-(2-carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) for the determination of aldehydes was evaluated. The labelling conditions were optimized with model aldehydes (benzaldehyde and *n*-butanal). Under the relatively mild reaction conditions of 65°C for 10 min in acidic medium, all the aldehydes tested were quantitatively derivatized with the reagent to yield stable and highly fluorescent hydrazone derivatives. The maximum excitation (ca. 450 nm) and emission (ca. 540 nm) wavelengths were essentially the same for all the aldehydes. The fluorescence intensity was substantially affected by the solvents in the medium, being higher in organic than protic solvents. However, pH in the range 2.7–11.5 had little effect on the fluorescence properties. The derivatives obtained from six aliphatic aldehydes with DBD-ProCZ were completely separated by reversed-phase liquid chromatography with aqueous acetonitrile. The on-column detection limit (signal-to-noise ratio = 3) with fluorescence detection is at the sub-picomole level.

1. Introduction

Fluorescence detection is the method of choice in numerous determinations of target materials at their real-life levels. The recent upsurge of interest in this area has resulted in many new fluorescence labelling reagents, as documented by the review literature [1,2]. A number of such reagents, e.g., 6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl-propionohydrazide (DMEQ-hydrazide) [3], fluoren-9-yl-methoxycarbonylhydrazine (Fmoc-hydrazine) [4], anthracenecarboxylic acid hydrazides [5], O-(anthrylmethyl)hydroxylamines [5], 4-hydrazino-7-substituted-benzoxadiazoles [6,7] and 5-hydrazino-N,N-dimethylnaphthalene-1-sulfonamide (Dns-hydrazine) [8–11], have been developed for the determination by high-performance liquid chromatography (HPLC) of carbonyl compounds such as aldehydes and ketones. Many of the reagents for labelling of aldehydes and ketones possess a hydrazino group ($-NHNH_2$) as

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the reactive site [3–13]. These reagents have been applied to the determination of carbonyl compounds at trace levels. However, reliable derivatization procedures based on novel labelling reagents that offer high sensitivity, high selectivity and good fluorescence properties in addition to good stabilities of the reagents and the derivatives are still needed.

Recently, we have developed the benzoxadiazole-based fluorescence labelling reagents 4 - (2 - carbazoylpyrrolidin - 1 - yl) - 7 - (N,N - dimethylaminosulfonyl)-2,1,3-benzoxadiazole [(R)-(+)- and (S)-(−)-DBD-ProCZ] and 4-(2-carbazoylpyrrolidin - 1 - yl) - 7 - nitro - 2,1,3 - benzoxadiazole [(R)-(+)- and (S)-(−)-NBD-ProCZ] for the precolumn derivatization of carbonyl compounds for HPLC analysis [14]. These reagents have been successfully applied to the stereoselective resolution of several racemic ketones by reversed-phase and/or normal-phase chromatography. This paper deals with the precolumn derivatization of achiral aldehydes with DBD-ProCZ and the separation and fluorescence detection of the derivatives by reversed-phase HPLC.

2. Experimental

2.1. Materials and reagents

4-(2-Carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) was synthesized as described previously [14]. Benzaldehyde, *n*-butanal, *n*-pentanal, *n*-hexanal, *n*-heptanal, *n*-octanal and *n*-nonanal were purchased from Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), acetonitrile, methanol, ethanol and water were of HPLC grade (Wako, Osaka, Japan). All other chemicals were of analytical-reagent grade and were used as received.

2.2. Apparatus

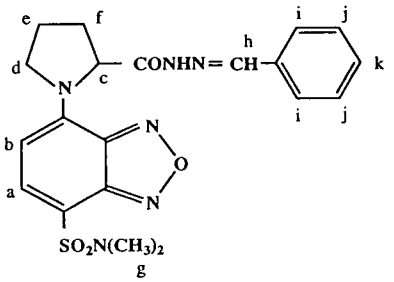
Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian (Palo Alto, CA, USA) Jemini-300 instrument at 300 MHz using tetramethylsilane (0.00 ppm) as the internal standard. For describing NMR characteristics, the following abbreviations are used: s = singlet, bs = broad singlet, d = doublet and m = multiplet. Mass spectrometry (MS) was carried out on a JEOL (Tokyo, Japan) DX-300 instrument [70 eV, electron-impact (EI) ionization]. For measurement of excitation and emission spectra, a Shimadzu (Kyoto, Japan) RF-5000 spectrofluorimeter with a 1-cm quartz cell was employed without spectral correction. Melting points (m.p.) were measured with a Yanagimoto (Tokyo, Japan) micro melting point apparatus.

2.3. HPLC

The high-performance liquid chromatograph consisted of two LC-10AD pumps (Shimadzu) and an SCL-10A system controller (Shimadzu). Sample solutions were injected with a SIL-10A autoinjector (Shimadzu). The analytical column was an Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) (GL Sciences, Tokyo, Japan). The column was maintained at 40°C with a CTO-10AC column oven (Shimadzu). A Shimadzu RF-550 fluorescence monitor equipped with a 12-μl flow cell was used for the detection of the derivatives. The excitation and emission wavelengths were fixed at 450 and 540 nm, respectively. The peak areas obtained from the fluorescence monitor were computed with a C-R7A Chromatopac (Shimadzu). All mobile phases were degassed with a DGU-3A on-line degasser (Shimadzu). The flow-rate of the eluent was 1.0 ml/min.

2.4. Synthesis of benzaldehyde derivative with DBD-ProCZ

A 2% solution of TCA in acetonitrile (0.5 ml) was added to a mixture of benzaldehyde (21 mg, 0.2 mmol) and DBD-ProCZ (14 mg, 0.04 mmol) dissolved in 9 ml of acetonitrile. After stirring at room temperature for 30 min, the solution was heated at 50°C for 15 min. The solvent in the reaction mixture was evaporated under reduced pressure. Acetonitrile (1 ml) and water (30 ml) were added to the remaining residues, then the



Benzaldehyde derivative

excess amount of benzaldehyde was extracted with light petroleum (b.p. 30–60°C) (20 ml). Benzaldehyde labelled with DBD-ProCZ in the aqueous layer was extracted three times with ethyl acetate (25 ml). The combined ethyl acetate extract was evaporated in vacuo and the residue was recrystallized from acetonitrile as orange crystals, m.p. ca. 130°C, yield 16 mg (90%). NMR (ppm) in $CDCl_3$, 9.52 (1H, bs, h), 7.88 (2H, m, i). 7.74 (1H, d, a + k), 7.46 (2H, m, j), 6.07 (1H, m, b + c), 3.7–4.1 (2H, bs, d), 2.44 (2H, m, f), 2.23 (2H, m, e), 2.81 (6H, s, g); EI-MS, m/z 442 (M^+).

2.5. Labelling procedure for aldehydes and HPLC separation

A 50-μl volume of 0.5% TCA in acetonitrile was added to a mixture of 50 μl of DBD-ProCZ (1 mM) in acetonitrile and 50 μl of aldehydes (5–50 μM) in water–acetonitrile (7:3). After heating at 65°C for 10 min, the reaction solution was cooled in ice–water to stop the reaction. An aliquot (5 μl) of the derivatization solution was subjected to HPLC. The reagent blanks without aldehydes were also treated in the same manner.

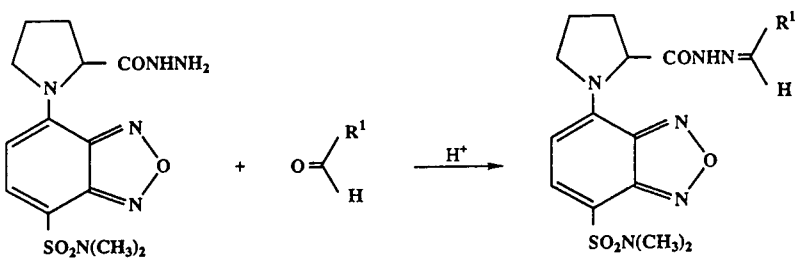
2.6. Fluorescence properties

Stock standard solutions (1 mM each) of the reagent (DBD-ProCZ) and its benzaldehyde derivative were prepared in acetonitrile. For studies of the effects of organic solvents or pH on the fluorescence properties, 50-μl portions of the stock standard solutions were added to 2 ml of the organic solvents or aqueous solutions at various pHs (2.7–11.5). The fluorescence spectra and fluorescence intensities were measured with a spectrofluorimeter within 5 min after preparation of the solutions.

3. Results and discussion

3.1. Fluorescence properties of the reagent and the derivatives

Fig. 1 shows the labelling reaction of aldehydes with DBD-ProCZ. The fluorescence properties of the reagent and its benzaldehyde derivative in various solvents are shown in Table 1. The excitation maxima of these compounds were essentially the same in protic, aprotic and organic solvents, whereas the emission maxima were shifted towards the short-wavelength region



DBD-ProCZ Hydrazone Derivative

Fig. 1. Reaction of DBD-ProCZ with aldehydes.

Table 1
Effect of solvent on the fluorescence properties of the reagent and benzaldehyde derivative

Solvent	λ_{max} (nm)		FI[a]	
	Excitation	Emission	DBD-ProCZ	Derivative
Water	450	550	5.5	4.7
Dimethyl sulfoxide	451	541	49	145
Methanol	451	541	92	90
Acetonitrile	450	535	195	220
Acetone	451	531	390	290
Benzene	450	522	260	310
Ethyl acetate	450	525	250	350

[a] FI = Fluorescence intensity.

with increasing hydrophobicity of the medium. The fluorescence intensities (FI) of the reagent and the derivative were higher in organic solvents such as benzene and ethyl acetate than protic solvents. Although the FI of the reagent in acetone was higher than that of the derivative (390 versus 290), the reason is not obvious. The excitation and emission wavelengths were stable over a wide pH range (2.7–11.5) (λ_{max}: excitation 450 nm and emission 550 nm). Table 2 shows the FI at various pHs. The FI was relatively stronger in neutral and slightly alkaline solutions. The lower intensity at pH 11.5 does not appear to be due to decomposition, but to changes in ionization, because the intensity recovered on changing the pH from 11.5 to 4.0. This suggests that the derivative exhibits good stability over a wide pH range. As essentially the same fluorescence properties were observed for the reagent and its derivatives, it is likely that the fluorescence is due to the fluorophore of the benzofurazan moiety.

Table 2
Effect of pH on the fluorescence properties of the reagent and benzaldehyde derivative

pH	FI[a]	
	DBD-ProCZ	Derivative[b]
2.7	280	280
3.5	290	290
4.7	280	310
5.5	290	330
6.7	290	350
7.7	310	400
8.7	305	405
9.5	330	400
10.4	330	360
11.5	290 (290)[c]	200 (290)[c]

[a] FI = Fluorescence intensity.
[b] Maximum wavelengths of the derivative at all pHs tested are ca. 450 nm (excitation) and ca. 550 nm (emission).
[c] FI data in parentheses are the values after addition of dilute HCl to change the pH from 11.5 to 4.0.

3.2. Optimization of the derivatization

The labelling reaction is affected by various parameters, such as the concentration of the reagent, the pH of the reaction solution and the reaction temperature. Therefore, the derivatization conditions were optimized using benzaldehyde and *n*-butanal, which were selected as the representative aromatic and aliphatic aldehydes, respectively. The labelling reactions of carbonyl compounds with hydrazines such as DBD-ProCZ are usually accelerated by an acid in the medium to yield the corresponding hydrazone derivatives via addition to the carbonyl group followed by dehydration.

Initially, the relative yields of hydrazone derivatives with various acid catalysts were studied by reversed-phase HPLC. Essentially the same yields of the derivatives were obtained with the four acids tested (acetic acid, hydrochloric acid, TFA and TCA). No reaction was observed in the

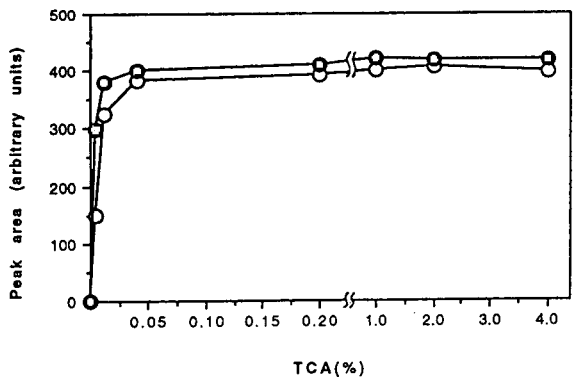


Fig. 2. Effect of TCA concentration on the tagging reaction. ○ = *n*-Butanal; ● = benzaldehyde.

absence of the catalyst. TCA was selected as the derivatization catalyst for subsequent experiments. With respect to the concentration, a small amount of TCA provided quantitative yields of the derivatives, as shown in Fig. 2. The yields were almost the same in the concentration range 0.05–4%. Some unknown peaks appeared with TCA concentrations higher than 5%.

The solvent in the reaction mixture also affected the labelling reaction. As illustrated in Fig. 3, the yields of the derivatives were highest in acetonitrile and lowest in DMF. The labelling reactions in benzene and tetrahydrofuran were not investigated because the reagent has poor solubility in these solvents.

The concentration of DBD-ProCZ is critical for the labelling reaction; higher concentrations gave higher reaction yields. Quantitative labelling was achieved with the use of a 20 molar excess of the reagent.

Reaction temperature is also a critical parameter for the derivatization. The effect was tested in acetonitrile–water (7:3) containing 0.05% TCA. As depicted in Fig. 4A, the labelling reaction proceeds under the mild condition of room temperature (ca. 25°C), and the reactions of both aldehydes were complete after 20 min. Judging from the curves in Fig. 4A, the reaction of the aliphatic aldehyde (*n*-butanal) appears to be faster than that of the aromatic aldehyde (benzaldehyde). The derivatization reaction proceeds faster at elevated temperature (65°C). As shown in Fig. 4B, the reaction of benzaldehyde was completed after 5 min with little additional change over 15 min. The same effect was noted with *n*-butanal. The labelling of the

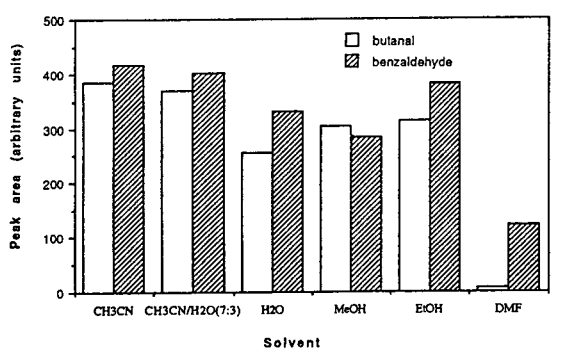


Fig. 3. Effect of solvent on the derivatization.

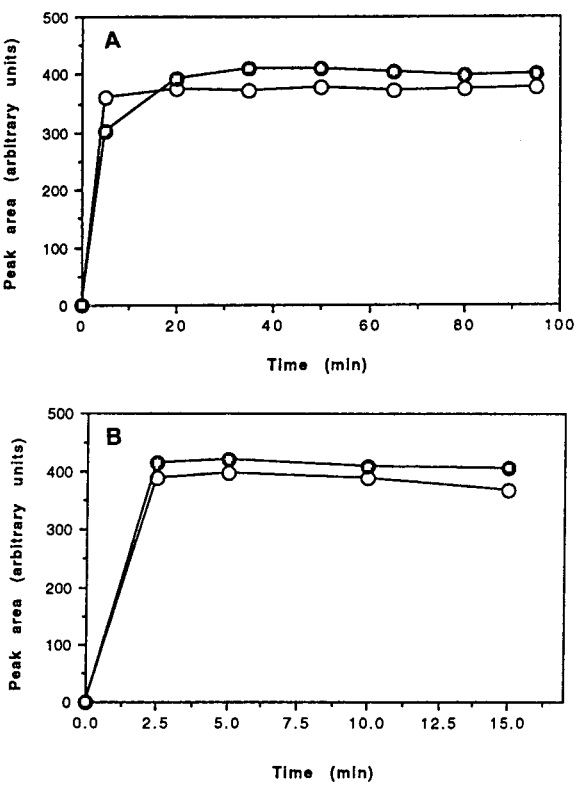


Fig. 4. Derivatization reaction as a function of time: (A) at room temperature; (B) at 65°C. ○ = *n*-Butanal; ● = benzaldehyde.

aldehyde proceeds to greater than 95% as determined by comparison of the peak areas obtained from the authentic derivative and the reaction mixture. Further, no degradation of the derivatives was observed after storage for 90 min at room temperature. The reagent and its derivative in acetonitrile are also stable for at least 3 weeks in a refrigerator at 5°C. Based on these observations, a reaction period of 10 min with a 100 molar excess of DBD-ProCZ at 65°C in water–acetonitrile (7:3) containing 0.05% TCA was selected for the reaction. Under these conditions, a linear relationship was obtained between the peak area of the derivative and the concentration of the aldehyde (1–12 nmol/ml of benzaldehyde or *n*-butanal) in the reaction medium. The slope, intercept and correlation coefficient (γ) were 52.09, 4.35 and 0.999 (benzaldehyde) and 48.38, −4.68 and 0.999 (*n*-butanal), respectively. The relative standard deviations (R.S.D.) for benzaldehyde and *n*-butanal (10 nmol/ml each) with the proposed procedure were 1.0% and 3.1% ($n = 5$), respectively.

3.3. HPLC separation of labelled aliphatic aldehydes

Fig. 5 shows the chromatographic separation of six aliphatic aldehydes (C_4–C_9) after derivatization with DBD-ProCZ. The resulting hydrazone derivatives were completely separated by a reversed-phase ODS column with simple linear gradient elution with aqueous acetonitrile (50–70% for 20 min) with a mobile phase containing 10 m*M* sodium phosphate buffer (pH 7.1). The large peak at ca. 2.5 min is unreacted DBD-ProCZ. The earlier peak is a system peak. The unnumbered peaks appear to be due to impurities in the analytes. The detection limit (signal-to-noise ratio = 3) for both the aliphatic and aromatic aldehydes is in the range 100–150 fmol per 5-μl injection volume (corresponding to 1 pmol of aldehyde). The detection limits were calculated from the peak heights of the derivatives, obtained from ten-fold dilution of the derivatized solution (2 nmol/ml of each aldehyde). The sensitivity is comparable to those of methods using labelling reagents reported by other research groups [3,4,6].

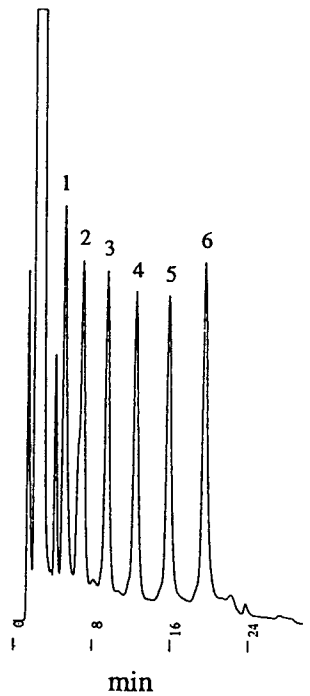


Fig. 5. Chromatographic separation of aliphatic aldehydes labelled with DBD-ProCZ. Peaks: 1 = *n*-butanal; 2 = *n*-pentanal; 3 = *n*-hexanal; 4 = *n*-heptanal; 5 = *n*-octanal; 6 = *n*-nonanal. Each peak except the reagent corresponds to 15 pmol. Eluent A, 10 m*M* phosphate buffer (pH 7.1); eluent B, acetonitrile; linear gradient elution from A–B (50:50) to A–B (30:70) in 20 min. For other HPLC conditions, see Experimental.

3.4. Preliminary application

As an application of the proposed method, the determination of carbonyl compounds in French lotion was tried. The chromatograms shown in Fig. 6 were obtained from derivatized samples of (A) the lotion and, (B) the reagent blank without lotion, and from the underivatized lotion samples with (C) FL detection at 540 nm (excitation at 450 nm) and (D) UV detection at 200 nm. Four main carbonyl components (aldehydes and/or ketones) seem to be present in the lotion (Fig. 6A). No attempt was made to characterize the four carbonyl-containing compounds because

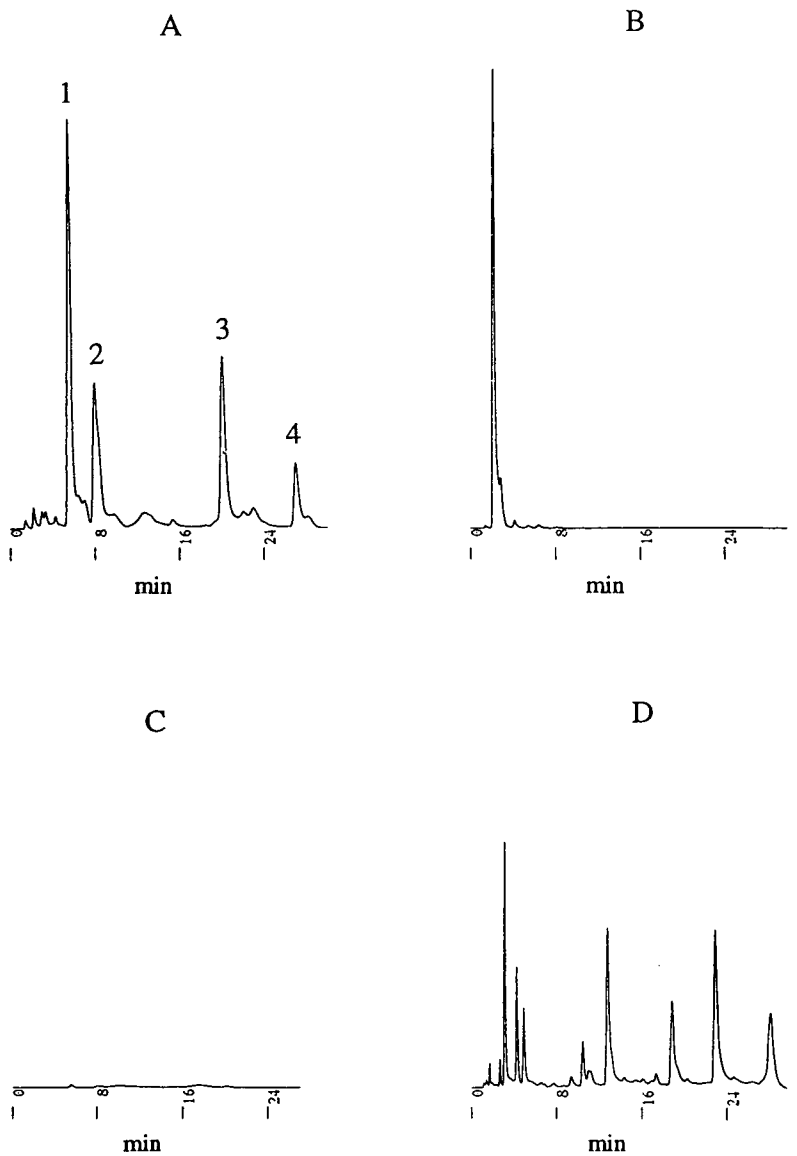


Fig. 6. Chromatogram obtained from a French lotion sample before and after derivatization with DBD-ProCZ. Peaks 1–4 = derivatives of carbonyl compounds. (A) Derivatized solution of the lotion sample; (B) derivatized solution without lotion; (C) underivatized solution with FL detection (excitation at 450 nm and emission at 540 nm); (D) underivatized solution with UV detection at 220 nm. HPLC conditions as in Fig. 5.

this was outside the scope of the present research. Highly sensitive fluorescence detection seems not be necessary in this instance, as the contents in the sample are very high. However, it should be noted that the fluorescence method is much superior to those with UV detection in terms of selectivity, as can be seen by comparing chromatograms C and D in Fig. 6. many interfering peaks arise from the UV-absorbing compounds present in the sample when UV detection is used. However, these compounds usually do not fluoresce at relatively long excitation and

emission wavelengths such as those used is the proposed method. Hence fluorescence detection at long wavelengths is preferable even if highly sensitive detection is not critical for the analysis.

4. Conclusions

The proposed HPLC–fluorescence detection procedure for the trace determination of aliphatic and/or aromatic aldehydes offers advantages in terms of the ease of reaction, the sensitivity of the fluorescence properties of the resulting hydrazone and the stabilities of the reagent and the derivatives. The detection limits are similar to the levels achieved with the methods reported previously [3,4,6]. However, the selectivity seems to be much superior to those of the other methods, because the fluorescence maxima of the derivatives resulting from DBD-ProCZ are longer than those for the derivatives obtained from Fmoc-hydrazine ($\lambda_{ex.}$ 270 nm and $\lambda_{em.}$ 320 nm) [4], DMEQ-hydrazide ($\lambda_{ex.}$ 362 nm and $\lambda_{em.}$ 442 nm) [3] and Dns-hydrazine ($\lambda_{ex.}$ 350 nm and $\lambda_{em.}$ 500 nm) [9]. In addition, the sensitivity may be improved with peroxyoxalate chemiluminescence (CL) detection, as the determination of trace amounts of carboxylic acids after labelling with similar tagging reagents having a DBD moiety was successfully achieved with the CL method [15]. Studies of the application of the method to environmental pollutants and the quality control of drugs are in progress.

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JOURNAL OF CHROMATOGRAPHY A

Applicability of coupled-column liquid chromatography to the analysis of β-agonists in urine by direct sample injection I. Development of a single-residue reversed-phase liquid chromatography–UV method for clenbuterol and selection of chromatographic conditions suitable for multi-residue analysis

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Abstract

Optimisation procedures originally applied to coupled-column RPLC–UV for the residue analysis of polar pesticides were evaluated for the analysis of β-agonists in human and bovine urine using direct sample injection. Two approaches have been studied: (i) a multi-residue method (MRM) for the clean-up and separation of eight different β-agonists (isoprenaline, cimaterol, terbutaline, salbutamol, fenoterol, ractopamine, clenbuterol and mabuterol) and (ii) a single-residue method (SRM) focussed at the detection of clenbuterol residues in samples of urine.

Both approaches provided efficient procedures to process urine samples automatically with coupled-column LC. Particular attention was paid to selecting analytical conditions suitable for thermospray MS detection, which is to be investigated in the near future.

Though UV detection cannot offer enough selectivity for the simultaneous screening of a group of β-agonists, coupled-column RPLC–UV proved to be very powerful in SRM, allowing the detection of clenbuterol at the μg/l level in filtered (0.45 μm) human and bovine urine after direct sample injection.

1. Introduction

The illicit use of β-agonists in zootechnics and in sports is well documented [1–5]. In both cases the side effects of these drugs on protein synthesis and lipolysis are sought, although their stimulatory activity on respiration could also be exploited for doping purposes [6].

Many single-residue methods (SRMs) or multi-residue methods (MRMs) have been proposed for the analysis of β-agonists in biosamples based on immunoassay techniques [7–10], or involving high-performance liquid chromatographic (HPLC) [6,11–17] or gas chromatographic (GC)

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separation [5,18–31] after purification of the analytes by means of either liquid–liquid partition [6,11,12,15–18,20,24,28], solid-phase extraction [5,13,17,21,23,30,31] or immunoaffinity chromatography [27,29,31,32]. The need of a confirmation defensible in the court of justice, i.e. based on mass spectrometric (MS) identification, and the availability of low-priced bench top GC–MS instruments explains the development of a large number of methods based on GC separation. Nevertheless, the use of GC is significantly limited by the high polarity of this class of compounds, requiring a derivatisation step prior to injection. Furthermore, the derivatisation procedures proposed for β-agonists are not entirely satisfactory owing to the low specificity of the resulting electron impact (EI) mass spectra (e.g. silyl derivatives) or to the restricted range of applicability (e.g. derivatives with boronic acids) [5,24]. This problem can be alleviated by using a very selective sample pretreatment technique such as immunoaffinity chromatography.

HPLC has significant advantages over GC for the analysis of β-agonists, the main ones being the lack of need for derivatisation and a good solvent compatibility of sample with the chromatographic system. However, its application was found to be limited because of the insufficient selectivity and/or sensitivity of common LC detectors on one hand, and difficulties of its interfacing with MS on the other. The recent development of effective HPLC–MS interfaces, such as thermospray or electrospray, however, opens new possibilities in the bioanalysis of these drugs [12,15,33,34]. Furthermore, as has been demonstrated in numerous publications, HPLC column switching offers the possibility to automate sample processing and to enhance sensitivity and selectivity by means of large volume injections and clean-up on the first column.

Recently, at the RIVM, optimisation procedures and strategies in method development have been derived for the determination of polar pesticides in various matrices using coupled-column RPLC and UV detection [35–40]. Basically, two method development approaches are used: MRMs for the simultaneous determination of a group of polar pesticides of widely different polarity in various types of samples [35–37], and SRMs for the rapid, sensitive assay of single analytes in aqueous environmental samples [38,39]. Both approaches make use of formulated criteria for important parameters such as clean-up, resolution, time of chromatographic run and (gradient) elution profile [36–40].

In this study the applicability of the SRM and MRM approaches for pesticides was investigated for the automated processing of β-agonists in urine samples including particular care in selecting analytical conditions suitable for thermospray (TSP) HPLC–MS, the desired technique in the bioanalysis of β-agonists which will be a matter of future investigation.

2. Experimental

2.1. Reagents

The β-agonists isoprenaline · HCl, cimaterol free base, terbutaline · H_2SO_4, salbutamol · H_2SO_4, fenoterol · HBr, ractopamine · HCl, clenbuterol · HCl and mabuterol free base, all with purity >99%, were obtained from the Laboratory for Residue Analysis (RIVM). HPLC-grade methanol and formic acid were purchased from J.T. Baker (Deventer, Netherlands). Ammonium acetate and triethylamine were from Aldrich (Axel, Netherlands) and Merck (Darmstadt, Germany), respectively. HPLC-grade water was prepared by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA). For linear binary gradient elution water was used as solvent A and a mixture of methanol–water (95:5, v/v) as solvent B. Both solvents contained 0.1 *M* ammonium acetate, 0.17 *M* formic acid and 0.01 *M* triethylamine. Isocratic and step-gradient elutions were performed using mobile phases made of different proportions of methanol and water, containing 0.1 *M* ammonium acetate and 0.01 *M* triethylamine with (pH about 3.8) or without (pH about 7.5) 0.17 *M* formic acid.

Separate stock standard solutions (1 mg/ml expressed as free bases) were prepared in HPLC-

grade water containing 2.5% of methanol with the exception of isoprenaline and mabuterol which were dissolved in ethanol. For HPLC analyses dilutions were made in HPLC-grade water, with evaporation of ethanol under nitrogen flow when isoprenaline and/or mabuterol were added.

2.2. Equipment

LC instrumentation used for the experimental work consisted of the following components (see Fig. 1): an ASPI 232-401 autosampler, AS (Gilson, Villiers-le-Bel, France) equipped with a programmable high-pressure valve, HV (type 7010; Rheodyne, Cotati, CA, USA); a binary gradient pump, P-1, Model 250 from Perkin-Elmer (Norwalk, CT, USA) with a helium degassing system from Perkin-Elmer equipped with air-tight bottle connections from Omnifit (Cambridge, UK) to deliver mobile phases to the pumps under light pressure (60 p.s.i.; 1 p.s.i. = 6894.76 Pa); two isocratic Model 306 Gilson pumps, P-2 and P-3; one isocratic Model 305 Gilson pump, P-4; a two-valve switching device, MUST (Spark Holland, Emmen, Netherlands); a manometric module, MM (Model 805, Gilson). All flow-rates were set at 1 ml/min.

Detection (UV at 245 or 285 nm), and collection of UV spectra (190–370 nm) were performed on a photodiode array detector, D, Model 1000S from ABI (Foster City, CA, USA) equipped with a Kipp and Zonen (Delft, Netherlands) recorder.

Two 50 × 4.6 mm I.D. columns, packed with Microspher C_{18}, 3-μm particles, from Chrom-

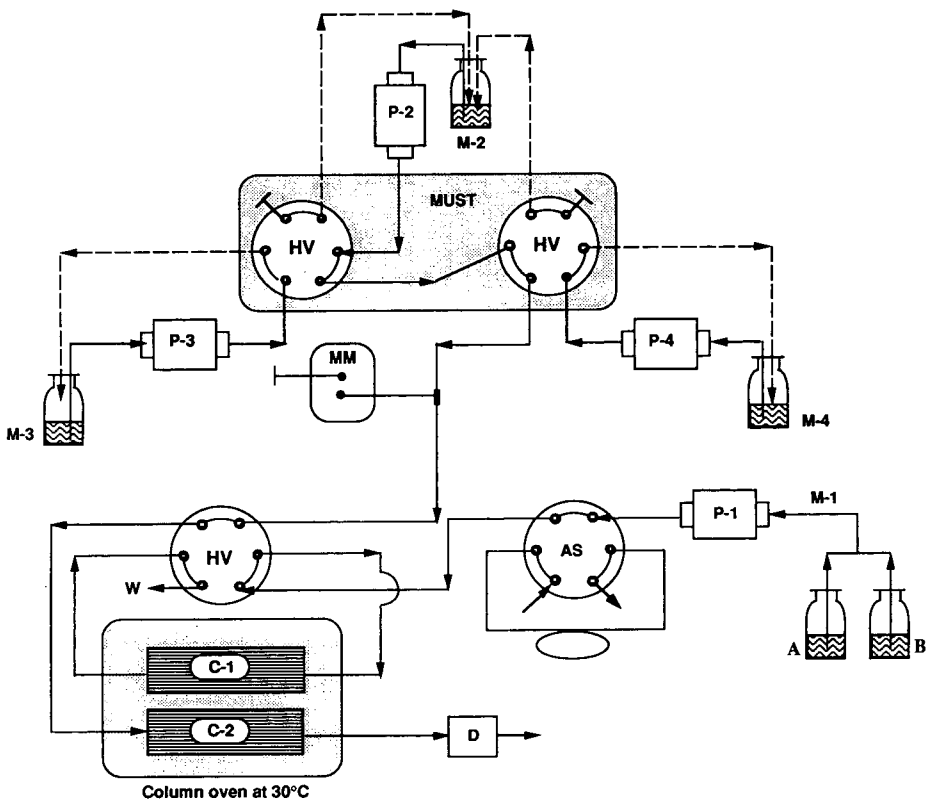


Fig. 1. Overview of the LC system used for experimental work. AS = Autosampler; HV = high-pressure valve; P-1 = binary LC pump; P-2, P-3, P-4 = isocratic LC pumps; MUST = two-valve switching device; MM = manometric module; C-1, C-2 = first and second separation columns; M-1, M-2, M-3, M-4 = mobile phases; D = UV photodiode array detector; W = waste.

pack (Middelburg, Netherlands) were used as first (C-1) and second column (C-2) for column switching experiments. A 10 × 3 mm I.D. guard column packed with 40-μm pellicular C_{18} material (Chrompack), was inserted before C-1. A 3-μm Microspher C_{18} column (100 × 4.6 mm) was used to establish gradient elution profiles without column switching. The columns were maintained at 30°C with a laboratory-made column oven connected to a circulating water system (Model 1441; Braun, Germany).

Calculations were performed with the program OPTIME Version 3.1 (RIVM) using a Macintosh MC II (Apple, Cupertino, CA, USA) personal computer.

2.3. Urine sample pretreatment

Human and bovine urine samples were filtrated on 0.45-μm Millex-HA (Millipore) filters prior to injection.

3. Results and discussion

3.1. General aspects

The β-agonists selected in the study for the MRM approach are listed in Table 1. The first step in the set-up of a coupled-column RPLC method is to select appropriate mobile and stationary phases [36–39]. In this case, special attention has to be paid to the physico-chemical properties of the analytes (basic or amphoteric, see Table 1) and also to the detection mode that will be applied to the presently developed procedure in a planned future study (TSP-MS). In fact, though there is good compatibility between RPLC and TSP-MS, a significant limitation is the necessity of a buffer in the mobile phase to ensure direct TSP ionisation ("buffer" ionisation). Furthermore, only volatile buffers such as ammonium acetate can be used to avoid interface clogging [41,42].

Taking into account this limitation, and based on preliminary results obtained earlier for the RPLC–TSP-MS analysis of β-agonists at the Institute of Legal Medicine of Pavia (data not published), mixtures of methanol and water containing ammonium acetate, formic acid and triethylamine (see Experimental) to reduce peak tailing of the analytes, were selected as mobile phase constituents.

Cartridge columns (50 × 4.6 mm I.D.) packed with 3-μm Microspher C_{18} were selected to achieve efficient separation, high sample load and on-line clean-up with column switching.

After the selection of the LC constituents, the UV spectra of the analytes were recorded and their retention behaviour was determined by constructing the second-order polynomial ln k vs. φ relations, where φ is the percentage of methanol in the mobile phase. The UV characteristics are listed in Table 1 and the retention behaviour is displayed in Fig. 2. As can be seen from this figure all individual analytes possess a proper RPLC retention ($1 < k < 10$) in various isocratic mobile phase composition. However, a satisfactory isocratic separation of all analytes is impossible and thus gradient elution is necessary. When linear gradient elution was applied, to both solvents A and B the same quantity of buffer constituents was added in order to avoid ions dilution in the mobile phase under gradient elution owing to the increment of the percentage of organic solvent, and thus to prevent possible disturbances on ionisation processes [42].

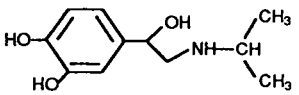
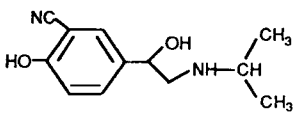
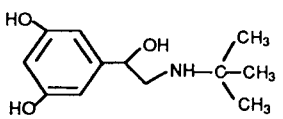
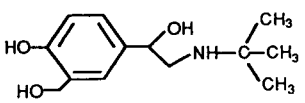
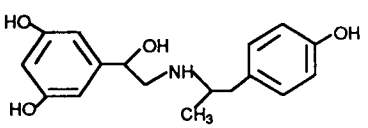
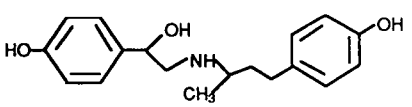
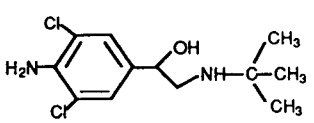
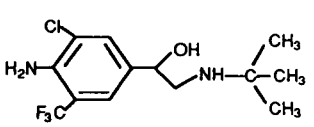
At this point our approach in method development is divided into SRM and MRM methodology, which will be discussed separately below.

3.2. SRM approach

One of the most favourable aspect of automated sample processing using LC–LC is the utilisation of the separation power of the first column (C-1). Beside the opportunity, in the case of aqueous samples, to enlarge sample injection volume for the improvement of the sensitivity, it offers the possibility to perform an efficient clean-up.

Another crucial feature of LC–LC is the transfer volume i.e. the time that C-1 is coupled on-line to the second column (C-2). In complex samples it is unavoidable that part of the interferences will be transferred together with the

Table 1
Names, structural formulae and UV characteristics of the β-agonists considered

Compound	Structural formula	λ_{max} (nm)	ϵ_{max} (l/mol · cm)
Isoprenaline		231 280	3200 2600
Cimaterol		249 325	8500 3700
Terbutaline		231 280	2900 3000
Salbutamol		231 280	3000 1500
Fenoterol		231 280	3100 2900
Ractopamine		231 280	2700 2000
Clenbuterol		245 300	7000 2000
Mabuterol		245 300	9700 2800

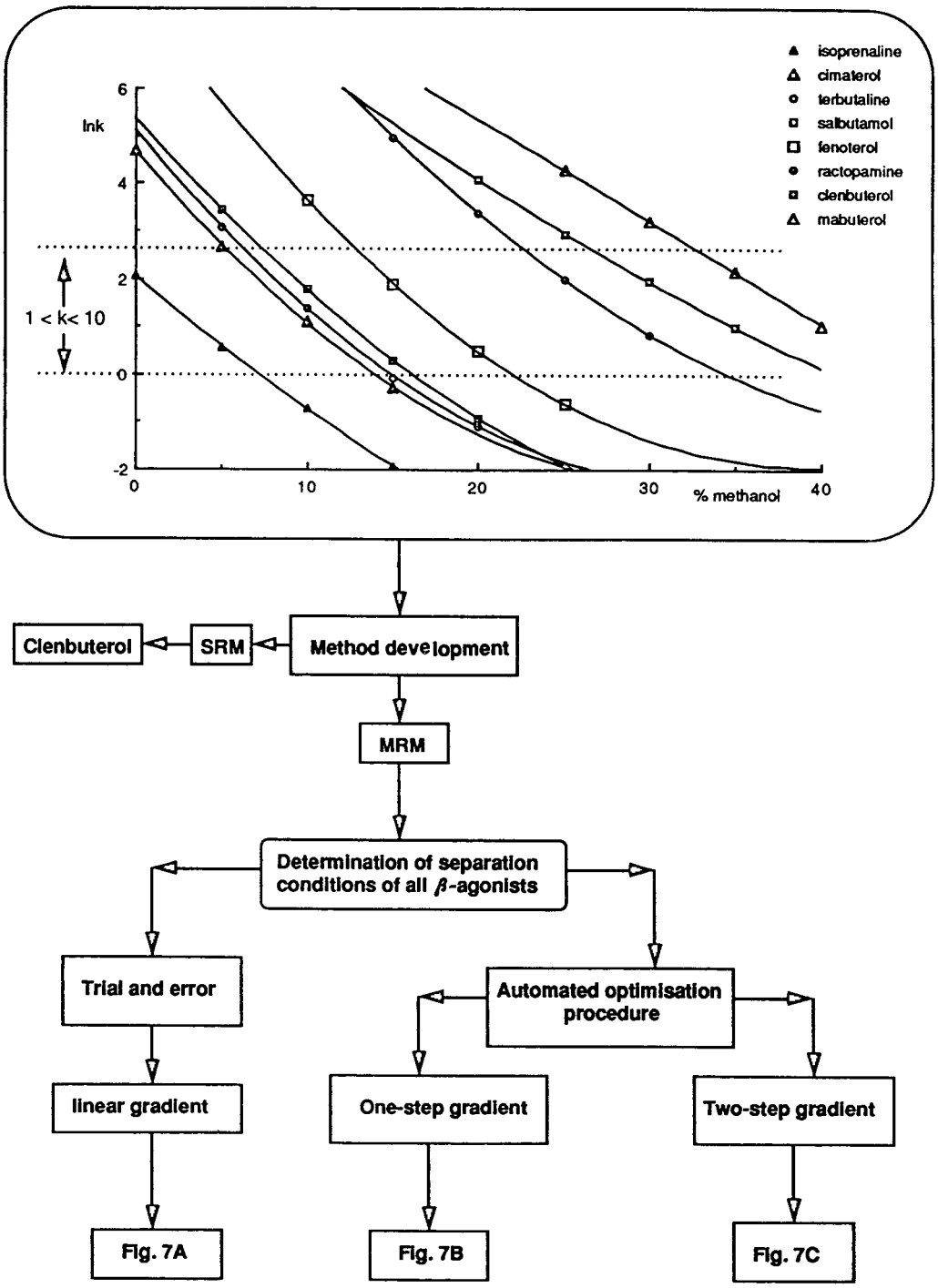


Fig. 2. Retention behaviour of the selected β-agonists on a 3-μm Microspher C_{18} column (50 × 4.6 mm I.D.) using methanol–buffer (0.1 *M* ammonium acetate, 0.17 *M* formic acid and 0.01 *M* triethylamine) as the mobile phase and flow path for the selection of adequate separation conditions (see Figs. 3 and 4).

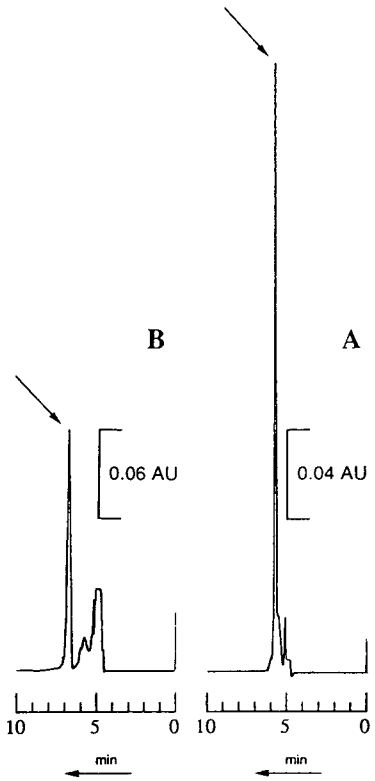


Fig. 3. Coupled-column RPLC–UV (245 nm) produced by the injection (150 μl) of blank human urine spiked with clenbuterol (10 μg/ml) with (A) and without (B) the addition of formic acid in the mobile phase, M-2 (40% of methanol in buffer, pH about 7.5) of C-2; M-1 = 25% methanol in buffer (pH about 3.8); clean-up volume, 3.90 ml; transfer volume, 0.50 ml. Clenbuterol is indicated by an arrow.

analytes. Hence, the attainable selectivity will be determined by (i) the effectiveness of clean-up on C-1 (clean-up volume) and (ii) the volume of the analyte fraction (transfer volume). In other words, optimal selectivity will be obtained in SRMs with the application of a minimal transfer volume. In pesticide residue analysis it has been clearly demonstrated [38,39] that coupled-column RPLC–UV provide sensitive, selective and moreover rapid and thus highly cost-effective SRMs. Hence it is very attractive to investigate the potential of this technique to this field of analysis.

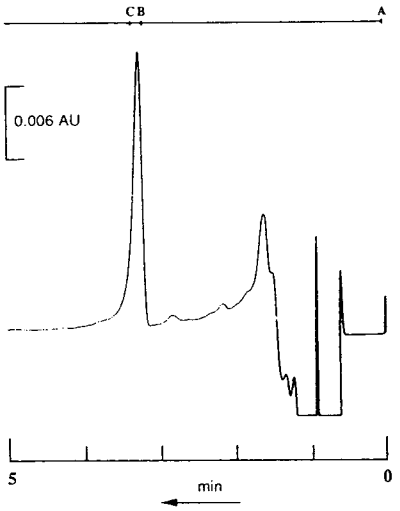


Fig. 4. RPLC–UV (245 nm) showing the elution of clenbuterol (150 ng in 1.5 ml of water) on C-1 for the establishment of column-switching conditions. Mobile phase M-1, 28.5% methanol in buffer (pH about 3.8). A = Injection of 1.5-ml sample; A–B = clean-up time; B = C-1 on-line C-2; B–C = transfer time; C = C-1 off-line C-2.

Sensitivity and selectivity depend largely on the RPLC–UV properties of the analyte. In UV detection both selectivity (wavelength, nm) and sensitivity (ϵ, l/mol · cm) play a role; the analyte's C_{18} retention is important, since it influences both the maximum tolerable sample injection volume (sensitivity) and the potential for separation between analytes and early eluting interferences (selectivity).

Based on our experience [37–39] some rules in the process of single-residue method development using coupled-column RPLC for environmental samples should also be applied to urine samples:

(1) The clean-up volume, which is the volume of mobile phase used on column C-1, should at least be twice the dead volume of that column; in practice this means that the capacity factor, k, should be greater than 1.

(2) The capacity factor of the analyte in the mobile phases should not exceed 10, in order to achieve short times of analysis and good sensitivity.

(3) The eluotropic strength of the first mobile phase (M-1) must never exceed that of the second mobile phase (M-2).

(4) The use of gradients based on pH changes in M-1 and M-2 in the case of acidic or basic analytes.

Being a compound for which the availability of a rapid screening method is of great importance, and having rather favourable RPLC–UV properties (see Fig. 2 and Table 1), clenbuterol was selected to test the SRM methodology for the set-up of an automated coupled-column RPLC–UV procedure for its determination in samples of urine.

Initially, 25% methanol in acidic buffer (see above) was selected as the mobile phase on C-1 (M-1). It provides a clean-up of about ten column volumes (V_0) and an elution volume of clenbuterol of about 500 μl. For the second mobile phase, M-2, 40% of methanol was chosen, which gives a considerable peak compression on C-2, favourable towards sensitivity. Fig. 3A, shows the chromatogram obtained with column switching for the direct injection of 150-μl urine samples. Although the heart-cutting procedure seems rather efficient, the analyte elutes on top of interferences, which certainly will make the detection of clenbuterol at low levels difficult. Based on the experiences that acidic interferences can be removed by liquid–liquid extraction with an organic solvent from the acidified sample [5], a mobile phase on C-2 was prepared, containing the same constituents as before but without formic acid (pH of about 7.5), which should remove acid interferences as unretained compounds. Therefore, a gradient based on a pH change was investigated by using, as M-2, a mobile phase without the addition of formic acid. The obtained considerable increase in selectivity is clearly demonstrated in Fig. 3B.

The next step was to increase the sample injection volume. Experiments indicated that volumes up to at least 1.5 ml did not affect the elution volume of clenbuterol on C-1. Selecting this urine sample volume to perform on-line injections, only a part of the analyte containing volume (150 μl, see Fig. 4) was chosen as a good compromise between sensitivity and selectivity. The percentage of modifier in both mobile phases was also slightly adjusted to decrease clean-up time on C-1 and to increase separation on C-2.

The obtained coupled-column RPLC–UV procedure was tested with the analysis of human urine spiked with clenbuterol at a level of 10 μg/l. Examples of RPLC–UV analyses of a blank and a spiked urine sample are given in Fig. 5A and B, respectively. The method showed a good repeatability at this level (R.S.D. = 9.1%, $n = 8$), and moreover the system appeared to be robust over a period of two weeks in which at least 50 samples of 1.5 ml of urine were processed. It must be mentioned that sometimes a small shift in retention (4–6 s) of clenbuterol in different spiked urine samples was observed, requiring a re-adjustment of column switching conditions. An additional buffering of the sample of urine prior to injection may solve this inconvenience, but has to be further investigated.

The availability of a real-life bovine urine

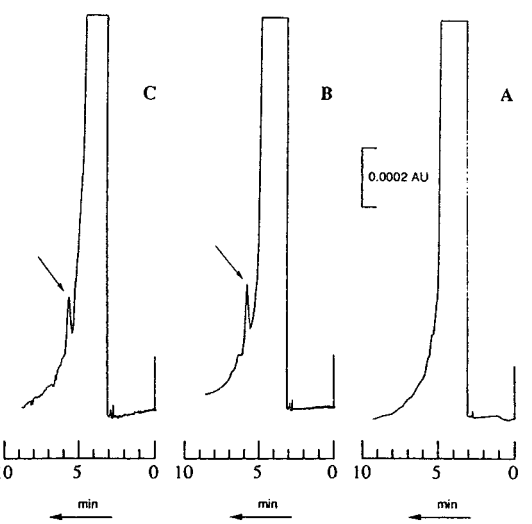


Fig. 5. Coupled-column RPLC–UV (245 nm) produced by the injection of 1.5 ml of (A) blank human urine, (B) blank human urine spiked with clenbuterol (10 μg/l) and (C) bovine urine sample containing clenbuterol. Clenbuterol is indicated by an arrow. Cleanup and transfer conditions as in Fig. 4; M-2 = 35% methanol in buffer without formic acid (pH about 7.5). After transfer of clenbuterol from C-1 to C-2, C-1 is rinsed with 95% MeOH (solvent B) for 5 min.

sample previously analysed by the Laboratory for Residue Analysis at the RIVM by GC–MS [27] and containing 6 μg/l of clenbuterol was a nice opportunity to test the procedure. As illustrated in Fig. 5C, clenbuterol was indeed found with the direct sampling method at the expected level (7.0 μg/l, duplicate measurement).

3.3. MRM approach

In the SRM approach discussed above the use of a very small transfer volume was essential to obtain selectivity using UV detection. For the simultaneous determination of all β-agonists having a wide polarity range (see Fig. 2), a large transfer volume will be necessary. Consequently, selectivity will be significantly lower excluding, in this case, the possibility of UV detection at low levels. Therefore, this study was directed to the development of suitable separation procedure to be used in next future in combination with TSP-MS detection.

To separate adequately the analytes a gradient elution will be necessary. Usually such a separation problem is solved with the selection of an appropriate linear gradient elution. In fact, using the already obtained information of Fig. 2 one should be able to estimate a suitable gradient elution profile. Two important aspects have been considered in this case: (i) to obtain sufficient retention of the firs eluting analyte (isoprenaline) and to perform clean-up the initial mobile phase must be at a low eluotropic strength (methanol < 5%) and (ii) the application of a rather steep gradient (after elution of isoprenaline) is desired to obtain small peak volumes (sensitivity). The result of a suitable linear gradient elution obtained with trial and error optimisation and conditions given in Fig. 6, is shown in Fig. 7A.

To our experience [36,37] step-gradient elution instead of linear gradient elution is preferable especially in conjunction with column-column LC where a step gradient (nearly) always occurs on the first column. Moreover, the availability of a fully automated optimisation procedure [40] for one- and/or two-step gradient elution profiles including relevant parameters (see below) made this type of elution attractive

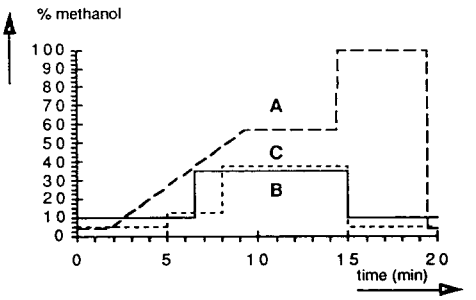
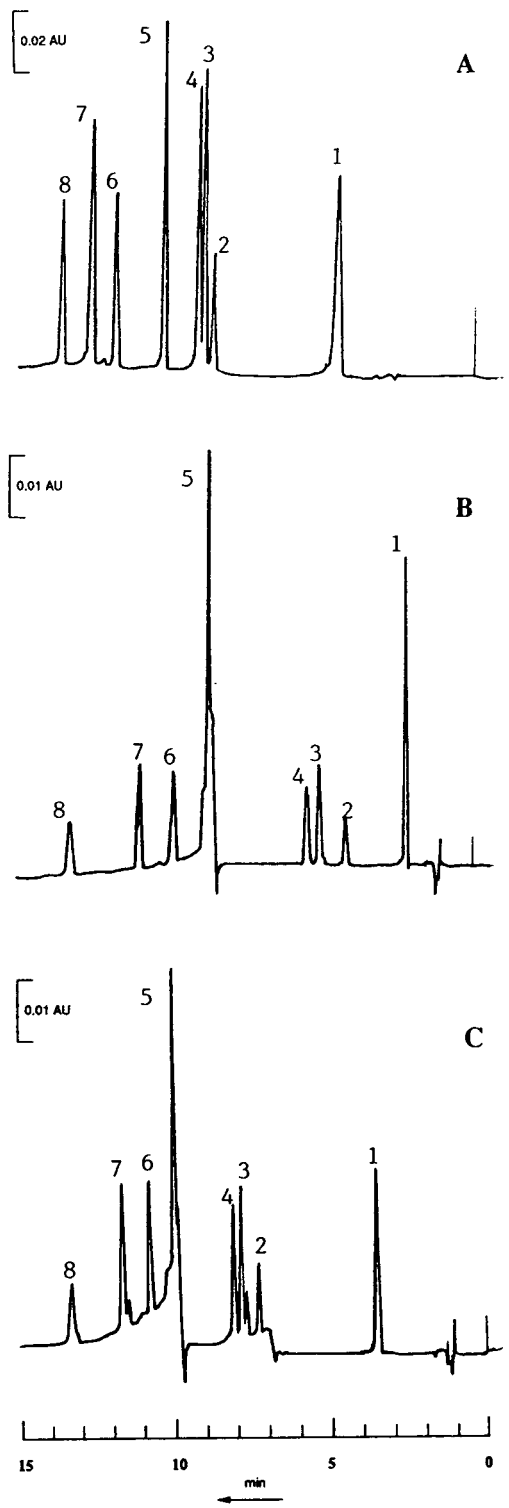


Fig. 6. Profiles of the adopted (A) linear, (B) 1-step and (C) 2-step-gradient elutions on a 3-μm Microspher C_{18} column (100 × 4.6 mm I.D.). The percentage of methanol in the mobile phase is plotted vs. time and starts with 4, 5 and 10% for A, B and C, respectively. Buffer composition as in Fig. 2.

for use. Therefore, as indicated in Fig. 2, step-gradient elution was investigated on its performance using the separation power of a 100 × 4.6 mm I.D. C_{18} column.

For the optimisation of the step-gradient elution conditions [36], the relevant parameters (i) minimal clean-up time, (ii) range eluotropic strength of mobile phases and (iii) the maximal time of analysis, were introduced into the computer program [40]. The calculated optimal conditions are presented in Fig. 6, and the corresponding chromatograms are shown in Fig. 7B en C. The comparison between calculated and experimental data is given in Table 2. The somewhat large differences for some analytes are probably caused by the use of a 100-mm column packed with material of a different batch than the 50-mm column used for the determination of the retention behaviour (Fig. 2).

As illustrated in Fig. 7, the three elution profiles provide an adequate separation of all analytes within 15 min. Concerning resolution, step gradient slightly favours linear gradient elution, while as regards the possibility to perform clean-up linear and two-step gradient elution are more suitable than one-step gradient elution. Owing to the possible negative effects of temporary very steep variations in the percentage of organic modifier in the mobile phase on the performance of the TSP interface, a linear gradient was initially selected for further experimental work.



The coupled-column RPLC system with two 50-mm columns and the selected elution conditions (see Fig. 6 and 7A) was tested for the direct injection of urine samples. Based on preliminary RPLC–TSP-MS experiments carried out at Pavia, an injection volume of 150 μl of urine sample was selected to obtain an expected sufficient sensitivity. Fig. 8 presents a chromatogram produced by the established procedure. The selected clean-up time of 1.8 min (approx. 3 times V_0) was determined by the elution time of the first analyte isoprenaline on C-1. Although UV detection reveals the presence of many co-eluting interferences, it is expected that TSP-MS will provide sufficient selectivity. Unfortunately selectivity could not be improved by using a buffer of higher pH on C-2 (see Section 3.2).

The robustness of the coupled-column procedure using acid mobile phases on both columns was tested by the injection of more than 50 urine samples, checking the performance of the columns by standard injections in between. It appeared that both retention and peak shape of the analytes were not affected, indicating that this approach is viable for the on-line determination of the β-agonists in urine samples in combination with TSP-MS.

It must be mentioned that an hydrolysis step prior to extraction is added in some published screening methods for β-agonists to include in the analysis also the fraction excreted in urine in the conjugated form (as glucuronates and/or sulphates). This fraction, though negligible for clenbuterol-like compounds, can be particularly significant for β-agonists with hydroxy groups on the aromatic ring(s) [26,29]. Although examples of notable urinary excretion of the unmodified drugs (up to 20% of total excretion) are available in the literature for terbutaline in man [18,43]

Fig. 7. RPLC–UV (285 nm) of the optimised separations obtained on a 100 × 4.6 mm, 3-μm, C_{18} column for the mixture of β-agonists with (A) linear, (B) 1-step and (C) 2-step gradient elution. Sample injection volume, 50 μl containing 1.5 μg (A) or 0.5 μg (B, C) of each analyte. Elution conditions as in Fig. 6. Peaks: 1 = isoprenaline; 2 = cimaterol; 3 = terbutaline; 4 = salbutamol; 5 = fenoterol; 6 = ractopamine; 7 = clenbuterol; 8 = mabuterol.

Table 2
Comparison of calculated (Calc.) and experimental (Exp.) chromatographic data of optimised step-gradient elution profiles given in Fig. 6 and shown in Fig. 7B and C

Compound	t_R (min)		σ (min)[a]	
	Calc.	Exp.	Calc.	Exp.
One-step gradient (Fig. 7B)				
Isoprenaline	1.54	2.18	0.020	0.025
Cimaterol	3.97	4.08	0.050	0.040
Terbutaline	5.00	4.90	0.063	0.040
Salbutamol	6.75	5.31	0.085	0.060
Fenoterol	7.97	8.41	0.014	0.020
Ractopamine	8.59	9.45	0.022	0.045
Clenbuterol	10.46	10.14	0.046	0.045
Mabuterol	16.24	12.88	0.119	0.065
Two-step gradient (Fig. 7C)				
Isoprenaline	2.78	3.45	0.035	0.040
Cimaterol	7.28	7.20	0.031	0.030
Terbutaline	7.78	7.75	0.036	0.035
Salbutamol	8.57	7.98	0.046	0.035
Fenoterol	9.46	9.82	0.014	0.025
Ractopamine	9.80	10.67	0.018	0.040
Clenbuterol	10.99	11.55	0.034	0.045
Mabuterol	14.26	13.22	0.075	0.055

[a] σ = Peak volume at 0.6 of the peak height.

and for salbutamol in calves [13], hydrolysis can be undoubtedly effective to increase sensitivity at the lower concentrations of residues in urine. Therefore, the influence of an acidic or enzymatic hydrolysis step in the presented procedure has to be evaluated. The possibility of a direct analysis of the conjugated forms was not investigated owing to the unavailability of reference standards.

4. Conclusions

The two basic approaches used in coupled-column RPLC as applied in pesticide residue analysis appeared to be effective for the analysis of β-agonists in urine samples. The usefulness of the first approach, the rapid detection of a single analyte, was clearly demonstrated for clenbuterol in urine samples. Applying large volume injection, carefully adjusted clean-up and transfer volumes, and proper mobile phase conditions, coupled-column RPLC–UV allowed the detection of clenbuterol in urine samples at the μg/l level. The short time of analysis (approximately 10 min) and the observed robustness of the LC system makes this approach highly useful for screening purposes.

The second approach, focussed at the simultaneous screening of eight β-agonists with a wide range in RPLC retention, provided easily suitable conditions for the automated processing of urine samples with coupled-column RPLC involving on-line clean-up and separation of the compounds within a time of analysis of less than 20 min. The processing of a large number of urine samples without any change in efficiency of the chromatographic system indicates that coupling of coupled-column RPLC to TSP-MS is a viable approach for the direct analysis of eight β-agonists in urine.

The applicability of the developed coupled-

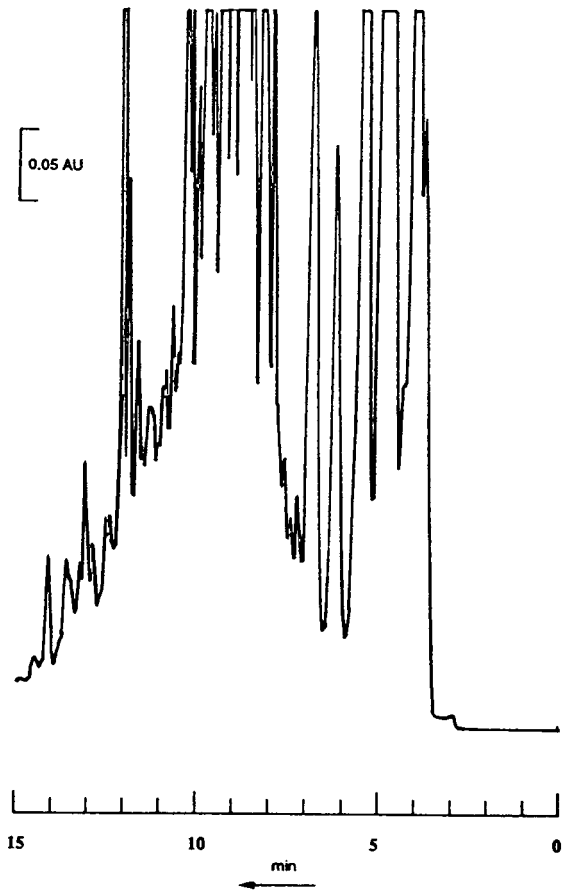


Fig. 8. Coupled-column RPLC–UV (285 nm) obtained for the injection of 150 μl of blank human urine. Clean-up time, 1.8 min; transfer time 13.2 min. For further explanation see text.

column procedures in combination with TSP-MS will be a matter of future research.

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Firefly luciferase purification using polyethylene glycol and Dyematrex Orange A

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Abstract

Efficient measurement of adenosine triphosphate by bioluminescence depends on the quality of firefly luciferase used. A rapid purification of this enzyme is reported that permits removal of enzymes interfering in the bioluminescent reaction. The enzyme was extracted from firefly tails and precipitated with PEG 20 000, and the resulting pellet was subjected to chromatography on a Dyematrex gel (Orange A), which retains the interfering enzymes but does not bind luciferase. As shown by adenylate kinase activity determination and sodium dodecyl sulfate polyacrylamide gel electrophoretic examination of the resultant preparation, partial purification of luciferase was successful in giving a preparation without interfering enzymes.

1. Introduction

The production of light catalysed by firefly luciferase depends on the presence of luciferin, adenosine triphosphate (ATP), magnesium and molecular oxygen [1]. In the crude extract of firefly tails, the presence of adenylate kinase, nucleotide diphosphate kinase and other ATP-converting enzymes [2–6] can produce light with nucleotides other than ATP. It is therefore necessary to remove these interfering enzymes during purification of firefly luciferase.

Several methods of purification have been reported [7–18]. With some of these a crystalline firefly luciferase was obtained after ammonium sulfate precipitation of the crude extract of firefly tails [7,16,17], and with others the purified luciferase was obtained after binding of the enzyme on an immobilized ligand and a long specific elution step [13–15]. The aim of all of these methods was to obtain a firefly luciferase preparation free from interfering enzymes.

Recently, Amicon (Danvers, MA, USA) developed the dye ligand Dyematrex Orange A. This matrix gel is composed of a triazinyl dye of spherical shape, of diameter 50–150 μm, immobilized on cross-linked agarose, and is remarkable for the small number of proteins that have been found to bind to it.

Firefly luciferase contains a large number of hydrophobic amino acids [19–22], making it possible to use polyethylene glycol (PEG) [23] for precipitation of the enzyme. Here we report a rapid and convenient procedure, which consists of precipitation of firefly luciferase with PEG 20 000, followed by affinity chromatography on an Orange A gel, which binds many interfering enzymes of the bioluminescent reaction but not

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luciferase, which can therefore be obtained without a specific elution step. The capacity of the method to remove interfering enzymes was demonstrated by the determination of adenylate kinase and nucleoside diphosphate kinase as references in each step of purification. The quality of the luciferase preparation was demonstrated by the very low background of luciferin–luciferase mixture. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that following this chromatography, firefly luciferase had been partially purified.

2. Experimental

2.1. Instruments and materials

An Optocomp II luminometer (Tecan, Voisins le Bretonneux, France) was used for the measurements of luciferase activity. A PhastSystem (Pharmacia–LKB Biotechnology, Uppsala, Sweden) was used for SDS-PAGE.

Desiccated firefly tails were obtained from Sigma (St. Louis, MO, USA). Dyematrex Orange A gel was purchased from Amicon (Beverly, MA, USA). All products for electrophoresis were obtained from Pharmacia–LKB Biotechnology.

2.2. Purification

Firefly luciferase extraction

A 200-mg amount of desiccated firefly tails was pounded in Fontainebleau sand (particle size 150–210 μm) (Prolabo, Paris, France) after being extracted with 1 ml of extraction buffer [10 m*M* $Mg(CH_3COO)_2$–1.5 m*M* NaN_3–5 m*M* dithiothreitol (DTT)–2 m*M* EDTA–20 m*M* Tris (pH 7.75)]. After pounding, 14 ml of same buffer were used to rinse the mortar. The mixture of sand and subcellular organelles was centrifuged at 1400 *g* for 5 min at 4°C and the supernatant solution was removed and kept on ice. The pellet obtained was washed with 5 ml of the same buffer and recentrifuged using the same conditions. The second supernatant solution was added to the first and centrifuged at 10 000 *g* for 30 min at 4°C, and the crude extract solution was recovered.

Precipitation of firefly luciferase

The clear crude extract solution obtained after the third centrifugation was mixed with 18% (w/v) of PEG 20 000 and after homogenization was kept on ice for ca. 30 min, then centrifuged at 10 000 *g* for 30 min at 4°C. The precipitate was dissolved in 5 ml of elution buffer [10 m*M* $Mg(CH_3COO)_2$–1.5 m*M* NaN_3–5 m*M* DTT–2 m*M* EDTA–20 m*M* Tris (pH 7.75) containing 20% glycerol] [16].

Dyematrex gel media preparation

A 10-ml amount of Orange A gel was mixed with 20 ml of 0.5 *M* NaCl solution and this mixture was poured into an empty PD-10 column containing a Vyon filter in the upper outlet (Pharmacia, Uppsala, Sweden; 80 × 15 mm I.D.) which had been closed. After the bed had stabilized, a second Vyon filter was settled, giving a bed height of 35 mm. The Orange A gel column was regenerated with 8 *M* urea, washed with the extraction buffer and then equilibrated with the elution buffer before use. The outlet end of the column was connected to a peristaltic pump (Pharmacia) that regulated the flow-rate at 10 ml/h.

Chromatography of firefly luciferase

Orange A gel chromatography was carried out between 4 and 10°C. After PEG precipitation, 5 ml of extract were applied to the Dyematrex Orange A gel column and eluted with the elution buffer for the first collected fractions. Foaming occurred in some fractions. In a second step, 0.5 *M* NaCl was used to strip off the bound proteins from the Dyematrex Orange A column. The volume of each fraction collected was 1 ml. After each use, the column was washed with four volumes of extraction buffer and stored capped at 4–8°C and containing a solution of 0.02% NaN_3.

2.3. Enzyme and protein assays

All assays were conducted at room temperature (22 ± 2°C).

Luciferase assay

Luciferase activity was determined by measuring light production by means of an Optocomp II luminometer. The numbers of photons were integrated in 5 s. The reaction was started by manual injection of 10 μl of sample to 100 μl of luciferase activity reagent in a polystyrene tube. The luciferase activity reagent contained 10 μl of 10^{-8} *M* ATP and 20 μl of a standard solution of luciferin in 10 ml of 5 m*M* $MgSO_4$–0.5 m*M* EDTA–0.5 m*M* DTT–25 m*M* Tris (pH 7.75) reagent buffer. The difference between the signal of each sample and the background light gave the luciferase activity expressed in units/ml, since the light intensity was expressed in relative light units (RLU). All chromatographic fractions were assayed and peak fractions containing more than 40% of the maximum luciferase activity were pooled.

Adenylate kinase assay

Adenylate kinase activity was determined according to the procedure of Bergmeyer et al. [24] at 340 nm and 25°C, and expressed in U/l (μmol/min·l), and all fractions were assayed.

Nucleoside diphosphate kinase assay

Nucleoside diphosphate kinase activity was determined according to the procedure of Bergmeyer et al., except that CDP was used instead of dTDP, at 37°C [25]. Only the crude extract, the precipitate and pooled fractions were assayed.

Protein assay

Protein concentrations were determined by the bicinchoninic acid (BCA) method [26] with bovine serum albumin (BSA) as standard, and expressed in mg/ml.

SDS-Page

SDS-PAGE was performed with PhastSystem equipment using 20% polyacrylamide homogeneous gels (PhastGel homogeneous 20; Pharmacia) [26]. Each sample (crude extract and pooled fractions from chromatography) was mixed (3:2 and 2:1, respectively) with TD 2X solution containing 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 6% (w/v) SDS and 25% (v/v) 0.5 *M* Trizma base (pH 6.8), and heated at 100°C for 2 min. A 4-μl volume of these treated samples was applied to the gel using a PhastGel applicator 6/4 μl (Pharmacia). Gels were run with SDS buffer strips (Pharmacia) at 250 V, 10 mA, 3 W, 15°C, 95 Vh. They were then fixed and stained with PhastGel Blue R (Pharmacia) according to the instructions of the manufacturer [27].

2.4. Background assay

The background was determined by measuring light production as in the luciferase activity assay. The reaction was started by injection of 10 μl of sterile injectable water instead of standard ATP in a polystyrene tube containing 100 μl of luciferin–luciferase mixture [360 μ*M* luciferin and 0.800 mg of protein (luciferase preparation) in 10 ml of 10 m*M* $Mg(CH_3COO)_2$–4 m*M* DTT–2 m*M* EDTA–0.1 g/l BSA–25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.75)]. Two luciferin–luciferase mixtures were prepared, for the preparation of luciferase precipitate and for pooled fractions. Prior to use, these mixtures were stored at room temperature and in the dark for several hours in order to reduce residual inherent light production.

After 12 h of incubation, assays were performed at intervals of 6 h for the two luciferin–luciferase mixtures.

3. Results

3.1. Purification

From 200 mg of firefly tails purified, 15 mg of total proteins were recovered in the pellet after precipitation with PEG 20 000 and 5 mg in the

Table 1
Firefly luciferase purification

Purification step	Total protein (mg)	Total activity[a] (units) · 10^5	Recovery (%)	Specific activity[a] (units/mg) · 10^4	Purification factor (-fold)	Adenylate kinase activity (U/l)	Nucleoside diphosphate kinase activity (U/l) · 10^3
Crude extract	134	1.892	100	0.140	1	1031	32.9
Precipitate after PEG	15	1.309	69	0.873	6	188	49.3
Pooled fractions from chromatography	5	1.804	95	3.608	26	3	0.39

Crude extract: 18 ml. Precipitate: 5 ml. Pooled fractions from chromatography: 3 ml.
[a] Luciferase activity is expressed in RLU/s · ml, where one unit = RLU/s.

pooled fractions after chromatography (Table 1). Fig. 1 shows that most of the proteins subjected to Orange A chromatography were washed off with the first non-specific eluent in the initial protein peak. A second protein peak corresponding to the few proteins bound to the Orange A appeared after elution with 0.5 *M* NaCl.

Firefly luciferase was purified 26-fold from the crude extract by these two purification steps.

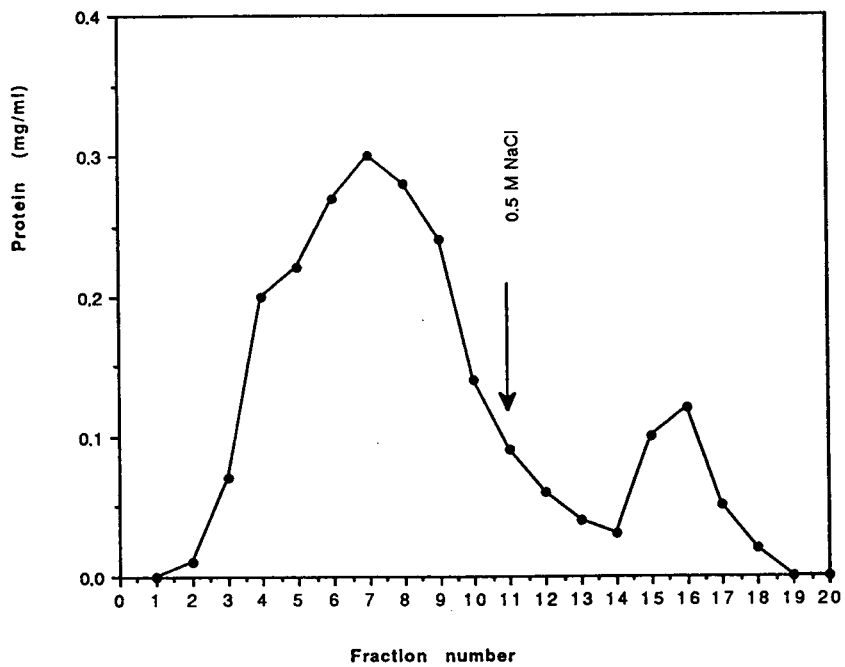

Fig. 1. Protein distribution. After the fractionation of firefly luciferase (see Fig. 2), the protein concentrations of all fractions were measured (see Experimental). Unbound and bound proteins were obtained by a non-specific eluent and 0.5 *M* NaCl, respectively (see arrow).

3.2. Enzyme activity assay

The use of 18% (w/v) of PEG 20 000 allowed the recovery of 69% of the total luciferase activity from the crude extract, with elimination of 82% of total adenylate kinase activity. The majority of the remaining adenylate kinase (18% of activity found in the crude extract) was retained by Orange A so that the final preparation had an adenylate kinase activity of only 0.3% of that of the crude extract (Table 1). Nevertheless, 18% of PEG 20 000 did not eliminate nucleoside diphosphate kinase, which was concentrated in the precipitate. As adenylate kinase, nucleoside diphosphate kinase that was present in the precipitate was retained by Orange A, and the final preparation had a nucleoside diphosphate kinase activity of 0.8% of that of the precipitate after PEG, and 1.2% of that of the crude extract (Table 1). The decrease in adenylate kinase activity, nucleoside diphosphate kinase activity and protein concentration in the pooled fractions after chromatography and the presence of glycerol [16] in the non-specific eluent buffer induced an increase in the luciferase activity, which rose to 95% of the luciferase activity of the crude extract. Fig. 2 shows one peak of luciferase activity obtained by the non-specific elution, and one peak of adenylate kinase activity obtained after the use of 0.5 *M* NaCl.

3.3. SDS-PAGE

Fig. 3 shows the results of SDS-PAGE of the final preparation in which partially purified luciferase was obtained. The molecular masses of proteins estimated from their mobility on SDS-PAGE were 66 000 for one subunit of luciferase [21,28] and 20 000 for adenylate kinase [24,29]. Most of the low-molecular-mass proteins (less than 30 000) present in the crude extract (lane 2)

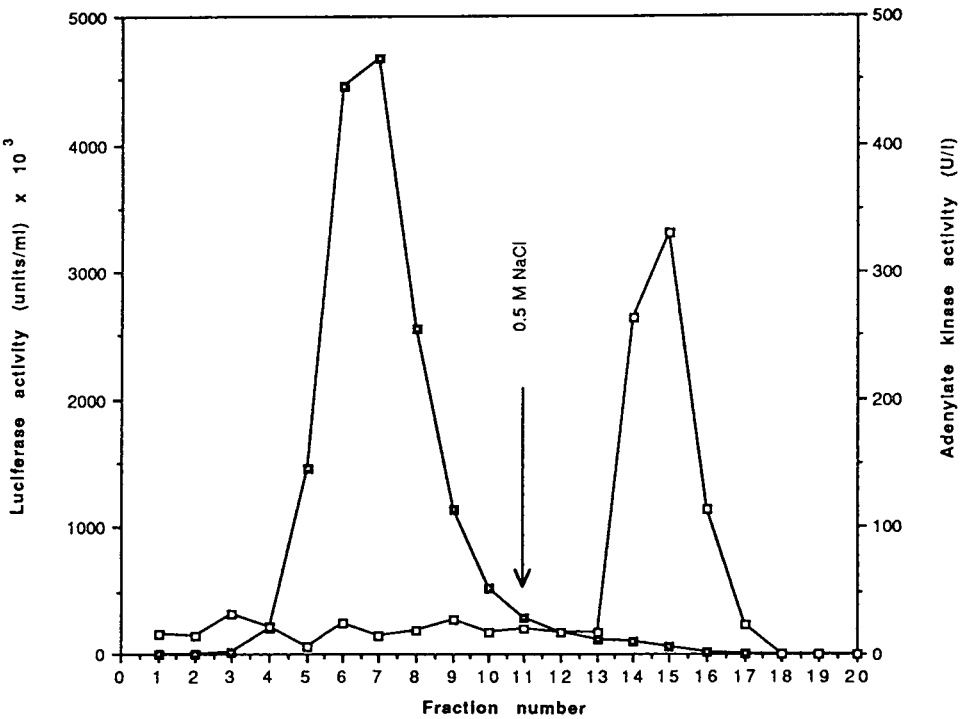

Fig. 2. Fractionation of firefly luciferase on Orange A column. The Orange A column and the conditions for the measurement of activities are described under Experimental. Fractions 6, 7, 8 were pooled (3 ml). The arrow indicates the beginning of elution with 0.5 *M* NaCl, which stripped off the bound proteins from the column, and then the appearance of the adenylate kinase activity in fractions 14, 15, 16 was observed. ■ = Luciferase activity; □ = adenylate kinase activity.

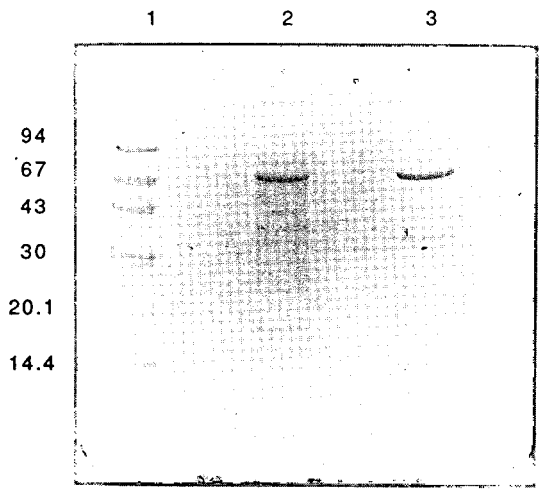

Fig. 3. SDS-PAGE of firefly luciferase in PhastGel 20% polyacrylamide homogeneous medium (Pharmacia). For the conditions of electrophoresis, see Experimental. The gels was run for 95 Vh, ca. 30 min. Staining: PhastGel Blue R (Coomassie Blue 350) (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water); destained in acetic acid–methnaol–water (1:3:6). Lanes: = 1 marker protein (M_r values $\times 10^{-3}$ are given); 2 = crude extract; 3 = pooled fractions from chromatography.

were not observed in the pooled fractions from chromatography, but another band of protein of M_r 40 000 was observed (lane 3).

3.4. Background assay

The blank value was high in the luciferase preparation before purification, and became much lower in the purified luciferase (pooled fractions). The light emitted by the purified luciferase represented 20% of the light emitted by the luciferase preparation before chromatography. Table 2 shows that the light emission and the stability of luciferase preparation depend on the presence of the contaminant enzymes.

4. Discussion

The method that we have developed, using PEG 20 000 and Orange A chromatography, permitted us to obtain a luciferase preparation with 95% of total activity, and free from interfering enzymes such as adenylate kinase and nucleoside diphosphate kinase. The activities of adenylate kinase and nucleoside diphosphate kinase after precipitation show that 18% PEG 20 000 is capable of excluding part of the adenylate kinase, but incapable of excluding nucleoside diphosphate kinase (Table 2). The presence of these interferences in the luciferase preparation was demonstrated by comparison of

Table 2
Background emission of luciferase preparations before and after chromatography on Orange A

Time (h)	Background (RLU per 5s)	
	Before chromatography[a]	After chromatography[b]
12	1676	307
	1640	287
	1616	306
18	1105	256
	1104	279
	1129	258
24	955	227
	962	215
	919	195

Each value represents the mean of three measurements, and the first measurement started 12 h after storage of luciferin–luciferase mixtures in the dark at room temperature.
[a] Luciferase preparation of precipitate after PEG.
[b] Final luciferase preparation (pooled fractions).

the backgrounds obtained with a non-purified and a purified luciferase preparation (Table 2). Moreover, it is possible to obtain a decrease in the background by dialysis before the chromatographic step, better than by chromatography without dialysis, because the presence of nucleosides is capable of interfering in the measurement of the activity of enzymes as nucleoside diphosphate kinase. The stability of the purified luciferase preparation was better than that of the non-purified luciferase preparation because of the absence of the interfering enzymes (Table 2).

In the purified luciferase preparation, another protein was observed and the nature of this contaminant protein was not established, but the nature and the role of this protein will be studied in future investigations.

Several methods have been reported for the purification of firefly luciferase. Most of these use ammonium sulfate [7,16,17] to precipitate the proteins present in the crude extract, whereas others use ammonium sulfate precipitation and classical affinity chromatography [10,12–15] with various ligands that bind luciferase. Both approaches have certain disadvantages, which are overcome by using our procedure. The first type of method requires a long dialysis and a protein concentration step. The use of PEG in our method is convenient as PEG does not need to be subsequently removed from the preparation. It is in fact possible to use PEG as an activator of luciferase activity [30], hence its presence in the precipitate is not a problem. The second type of method, classical affinity chromatography specific for luciferase, requires the use of ATP or luciferin to elute the bound enzyme [13–15], so that the fractions containing appreciable luciferase must be subjected to a long dialysis step, which is not required with our method.

Our method has the disadvantage that the final preparation obtained contains firefly luciferase and another protein (Fig. 3, lane 3). However, for the purpose of ATP measurement this luciferase preparation can be used under the same conditions as pure luciferase.

In conclusion, PEG precipitation followed by Orange A chromatography permits the removal of the enzymes, such as adenylate kinase, that interfere with the bioluminescent reaction used in the measurement of metabolites. The other contaminant enzymes, such as nucleoside diphosphate kinase, were retained by Orange A chromatography. Unlike other methods commonly used, this method is rapid and requires only a small amount of starting material (reagents and equipment), so that it could be used routinely in laboratories working with bioluminescence.

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Analytical characteristics of sample evaporation with the micro-Kuderna–Danish concentrator

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Abstract

The analytical behaviour of dichloromethane, pentane, and hexane solutions was studied through their evaporation in a micro-Kuderna–Danish concentrator. Initial sample concentration, final sample volume, matrix composition, system geometry and temperature gradients influence the process greatly. In general, solute loss increases exponentially when the final volume decreases. During the concentration process with very dilute solutions, the loss of solutes can be higher than 50% if the final volume reached is lower than 200 μl. This means a decrease in the quantification of the process, even though it is seemingly kept under control (low R.S.D.). The solute losses are not just due to the decrease in the number of plates during the reflux disappearance, but also to the probability that droplets of the evaporating sample are projected against clean and dry walls of low retention capacity. This hypothesis may explain the observed effects when concentrating more concentrated samples, or samples with a high content of some solutes. A more advantageous design for the micro-Kuderna–Danish receptor flask is also proposed. The replacement of the cylindrical flask by a flask with a conical bottom permits a decrease in the volume of losses and an improvement in the quantification of the process.

Introduction

When analysing volatile compounds at trace concentrations, it is common practice to transfer them from the starting matrix to an organic solvent, regardless of the sample characteristics and almost independently of the isolation method applied, with the exception of compounds isolated by automatic purge and trap or by automatic supercritical fluid extraction [1–4]. This organic phase must often be concentrated in order to achieve the required concentration for analysis. This is usually done by solvent evaporation within a suitable system and on very limited occasions is it possible or convenient to carry it out inside the chromatographic system [5,6]. During the development of gas chromatography, numerous concentration methods have been proposed, varying in purpose, applicability and ease of use. Classical methods when working with food matrices were proposed by Muller et al. [7], Ahrenst-Larsen and Hansen [8], Weurman [9], Blakesley and Loots [10], Guichard [11] and more recently by Boison and Tomlinson [12]. In environmental sample analysis and microanalysis, the work by Dünges [13], Grob et al. [14] and Grob and Muller [5] is particularly outstanding. Almost all the methods developed have more than satisfactory analytical characteristics. However, they must be performed in several

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steps with not readily available materials, and generally require experience and close attention to the process. In this context, the more widely used methods are concentration under a stream of nitrogen, with a Rotavapor and with a micro-Kuderna–Danish concentrator. The last approach is particularly attractive owing to its capability to concentrate relatively large volumes of sample in one step, requiring minimum attention. Among the most widely used procedures it is the only one that involves a fractional distillation. It is also the method proposed in the EPA 608 and 605 methods to determine priority pollutants, and it is used in virtually all kinds of analyses related to volatile compounds.

However, even though these methods are widely used, and are a necessary step in many analyses for trace compounds by gas chromatography, there have been hardly any studies evaluating the impact of this step of the analysis on the analytical quality of the final results. It is believed that each system must be separately studied as its behaviour cannot be extrapolated or related to any other. This situation makes it difficult to find general working rules, and to predict *a priori* the analytical behaviour of the processes. The results offered by some researchers on the recovery of solutes during the concentration steps are sometimes contradictory [5,12,15] and no conclusions except that concentration to dryness is problematic and may lead to important solute losses can be made. It is generally accepted that if the process is stopped before reaching dryness, the solute losses are reproducible and independent of the chemical nature of the solutes; therefore, they do not affect the reproducibility or accuracy of the process if the quantification is done by means of an adequate internal standard [14]. A closer look at the results reported in the literature will show that few researchers have taken precautions in checking if their sample concentration process is the most suitable or if it is dependent on the matrix composition, or to verify which percentage of the analytical variance is due to this aspect.

We are aware that each system has a particular behaviour as a consequence of its physico-chemical properties (Vapour pressure, solubility, azeotrope formation, deviation from ideal behaviour, etc.), and therefore it is difficult to develop a method that will suit all situations. Without pretending to give a global answer, this work is an attempt to understand the phenomena that take place during sample evaporation and to derive conclusions about the optimum evaporation conditions, with special emphasis on the micro-Kuderna–Danish concentrator.

2. Experimental

The standards were of analytical-reagent grade from Chemservice (West Chester, PA, USA). Standard solutions containing 200, 100, 40, 20, 10, 5, 2, 1, 0.8 and 0.08 mg l^{-1} of the following selected volatile compounds were prepared in dichloromethane or hexane: ethyl butyrate, 2-hexanone, ethyl benzene, *m*-xylene, 3-hexanol, ethyl caproate, 1-hexanol, 2-nonanone, ethyl caprylate, 1-heptanol, 1-octanol, ethyl decanoate, nerol, phenylethyl acetate and ethyl laurate.

Internal standard solutions of 2-ethylhexanol in ethanol (1 and 10 g l^{-1}) were prepared.

Dichloromethane, pentane, hexane, ethanol and 2-propanol were of analytical-reagent grade from Merck (Darmstadt, Germany). The first three were redistilled before use.

2.1. Concentration systems

A Vigreaux column (25 cm × 1.3 cm I.D.) was obtained from Afora (Barcelona, Spain). Other equipment consisted of a micro-Snyder column 158 mm long with three balls, a micro-condenser 112 mm long a 2-ml receiving vessel and a 40-ml flask from Supelco (Bellefonte, PA, USA). All joints were grease-free.

2.2. Chromatography

An HP 5890 Series II gas chromatograph (Hewlett-Packard) fitted with a Model 7673

automatic injector was used, with a split–splitless injection system and flame ionization detection. The signal was received and processed in a NEC computer equipped with the Maxima–Waters 820 program, version 3.0 (Waters Software). A Supelcowax 10 column (60 m × 0.32 mm I.D., film thickness 0.5 μm) was used.

2.3. Chromatographic conditions

With split injections, the conditions were as follows: carrier gas, hydrogen; head pressure, 50 kPa (1.5 ml min^{-1}); splitting ratio, 1 : 40; purge flow, 1.5 ml min^{-1}; injection temperature, 220°C; detector temperature, 220°C; initial column temperature, 50°C, held for 5 min and then raised at 3°C min^{-1} to 200°C. With splitless injection, the conditions were as follows: carrier gas, hydrogen; head pressure, 120 kPa (3.5 ml min^{-1}); split flow-rate 27 ml min^{-1}, splitless time, 3 min; purge flow, 1.5 ml min^{-1}; column temperature, 40°C, held for 5 min and then raised at 3°C min^{-1} to 200°C; make-up gas, nitrogen at 30 ml min^{-1}. The injection volume was 1 μl in both instances; a 5-μl Hamilton syringe was used.

Quantification with an internal standard (50 and 5 mg l^{-1} 2-ethylhexanol for split and splitless injection, respectively) was based on peak areas. The peak areas obtained from each analysis were interpolated on calibration graphs constructed with 20, 50, 100 and 200 mg l^{-1} standard solutions with split injection and 1, 2, 5 and 10 mg l^{-1} with splitless injection.

2.4. General procedure for concentration with the micro-Kuderna–Danish concentrator

The micro-Kuderna–Danish concentrator was filled, in all instances, with 30 ml of the solution to be concentrated. Two or three boiling chips no larger than 2 mm in diameter were added. The rectification column was conditioned with 2 ml of additional solvent before starting the concentration process. The concentrator was then immersed in a water-bath to the widest part of the flask body, then the evaporation was begun. When it was completed the apparatus was removed from the water-bath and immediately introduced into a water–ice bath where it was allowed to settle for 5 min. The liquid was recovered with a syringe and introduced into a 2-ml volumetric flask. The bottom of the micro-Kuderna–Danish concentrator was washed without agitation with three fractions of a volume similar to the recovered volume, and the washings were added to the volumetric flask. The internal standard was then added (10 μl) and the mixture was diluted to volume with solvent. The sample obtained was analysed by GC as indicated previously.

Study of the influence of the final volume reached and the initial sample concentration

Three sets (4, 0.8 and 0.08 mg l^{-1}) of 30 solutions in dichloromethane were prepared. Each solution was concentrated using a micro-Kuderna–Danish concentrator equipped with a Snyder column with three balls in a thermostated bath at 47°C. Six solutions from each set were concentrated to a volume of ca. 1 ml, another group of six to 0.5 ml (just before the reflux began to diminish), another group to 0.4 ml (reflux disappearance), another group to 0.2 ml (just before boiling stopped) and a last group to the point where the liquid phase disappeared at the bottom of the flask (final volume about 0.1 ml). The concentrated solutions were analysed as described previously.

Influence of the presence of third compounds

Fifteen sets of three solutions in dichloromethane containing 0.08 mg l^{-1} of the volatiles were prepared. The following compounds were added to the solutions of each set: set 1, 1 mg of ethanol; set 2, 2 mg of ethanol; set 3, 4 mg of ethanol; set 4, 6 mg of ethanol; set 5, 12 mg of ethanol; set 6, 25 mg of ethanol; set 7, 2 mg of 2-propanol; set 8, 4 mg of 2-propanol; set 9, 6 mg of 2-propanol; set 10, 12 mg of 2-propanol; set 11, 1 mg of isoamyl alcohol; set 12, 2 mg of isoamyl alcohol; set 13, 6 mg of isoamyl alcohol; set 14, 0.012 mg of heptanol and set 15, 0.12 mg of heptanol. The three solutions of each set were

concentrated to 0.1 ml and analysed as described previously.

In addition to sets 5, 10, 14 and 15, three more groups of solutions were prepared and concentrated to final volumes of 1, 0.4, and 0.2 ml.

Influence of the rectifying capacity of the column

Two other columns were used besides the Snyder three-ball column: a Vigreaux column and the standard micro-Kuderna–Danish column (microconcentrator). Sets of six concentrated and diluted solutions were prepared and concentrated in both systems as done previously, to a final volume of about 0.1 ml.

Influence of micro-Kuderna–Danish receiving flask geometry

A 2-ml flask with a conical bottom and of 1.5 cm I.D. was used to replace the cylindrical standard micro-Kuderna–Danish flask. Twelve solutions in dichloromethane containing 0.08 mg l^{-1} volatiles were concentrated in the system equipped with the new flask to final volumes of 0.2 and 0.1 ml. Also, 1-ml volume samples were directly concentrated to 0.1 ml in the new flask without fitting the rest of the microconcentrator.

Influence of the room temperature and the type of solvent containing the sample

Solutions in hexane and pentane with concentrations of volatiles of 4 and 0.08 mg l^{-1} were prepared: two sets, each of a different concentration, of six solutions in pentane were concentrated to ca. 0.1 ml in the standard system. The bath temperature was 41°C and room temperature was 24°C. Two other sets of solutions with the same characteristics were concentrated in a cold room at 15°C. In a similar manner, two sets of solutions in hexane were concentrated keeping the bath temperature at 87°C (below this temperature the reflux was not satisfactory). Two other sets of solutions in hexane were concentrated in a chamber at 45°C. On this occasion the bath temperature was kept at 76°C.

3. Results

3.1. Study of the influence of the final volume reached and the initial sample concentration (Figs. 1–4)

Losses increase exponentially with respect to the final volume, and the curve shape is a function of the starting concentration (Fig. 4). If the final volume is higher than 400 μl (concentration factor around 80), losses are kept at acceptable levels and are lower than those obtained with other systems which reach a similar concentration factor [5,13]. The results were not different on changing the initial sample concentration, as shown in Fig. 1a and b and in the first part of Fig. 4.

Below 400 μl the evaporation process depends to a great extent on the solute concentration of the media, as shown in Fig. 1a and b and 4. If the solution is somehow concentrated, solute losses are not very important until the volume approaches 100 μl (only data relating to 4.0 mg l^{-1} solutions are shown). If the solution is very dilute, the losses are substantial below 400 μl. Losses in this and for these solutions are much larger than those reported in the literature for other concentration systems. The absolute amounts of lost solute are hardly reproducible. The greater the losses, the more difficult is reproducibility. The corresponding data are shown in Fig. 2a and b. During the concentration of dilute solutions, the amount of lost matter is random when the concentration reaches final volumes lower than 0.4 ml (R.S.D. $> 10\%$ when the final volume is 0.4 ml and $>20\%$ when the final volume is 0.1 ml). On the other hand, the concentration of concentrated solutions was a much more repetitive process, the average R.S.D. being ca. 10% when the evaporation was stronger. However, the relative amounts of lost solutes are much more reproducible (see Fig. 3). It is important to point out that the low recoveries of solutes do not mean an important decrease in precision if the quantification is done with an internal standard which is also being concentrated.

The losses depend slightly on the boiling point

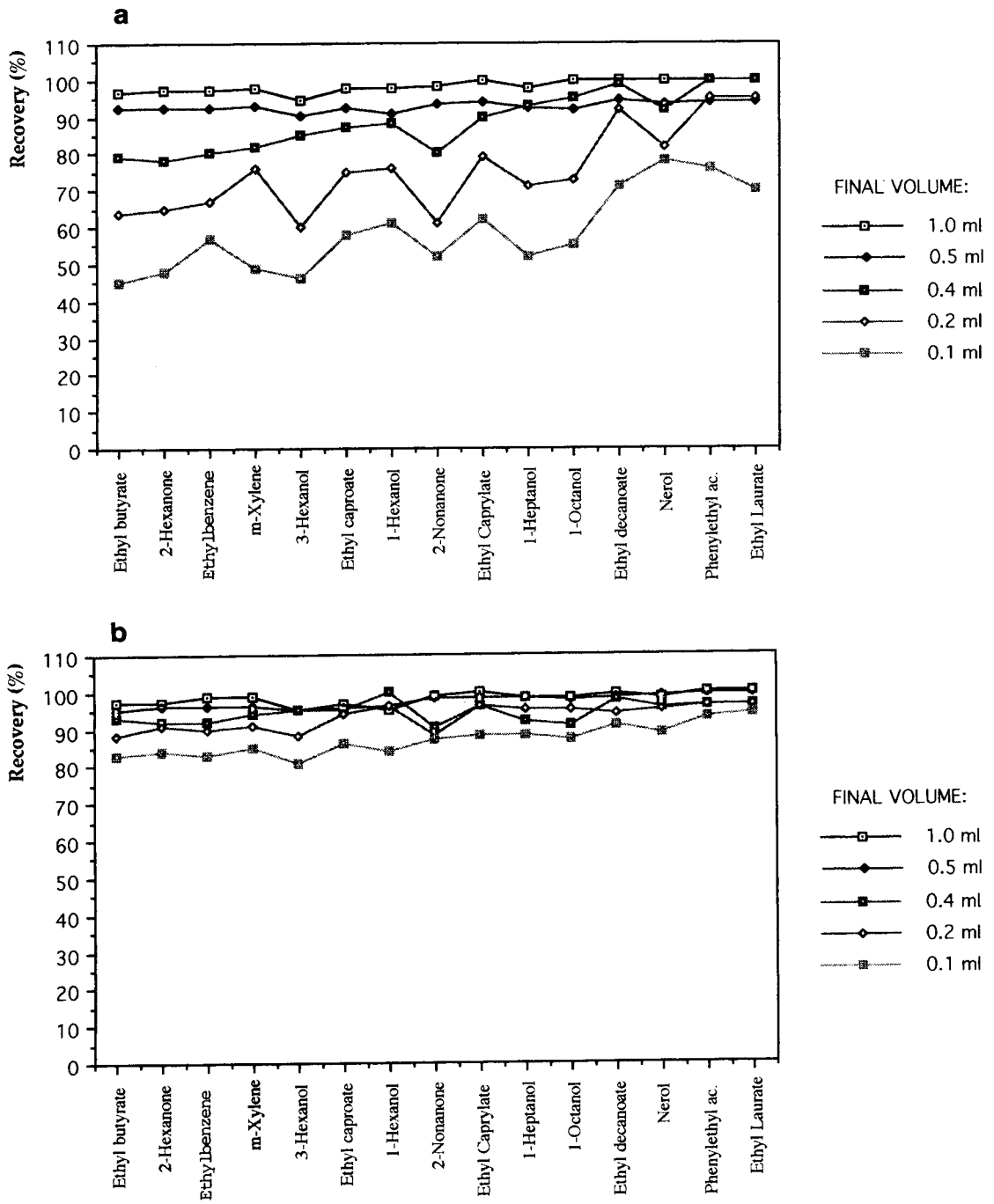

Fig. 1. Solute recovery throughout the sample concentration process: influence of final volume, (a) dilute solutions, 0.08 mg l^{-1}; (b) concentrated solutions, 4.0 mg l^{-1}.

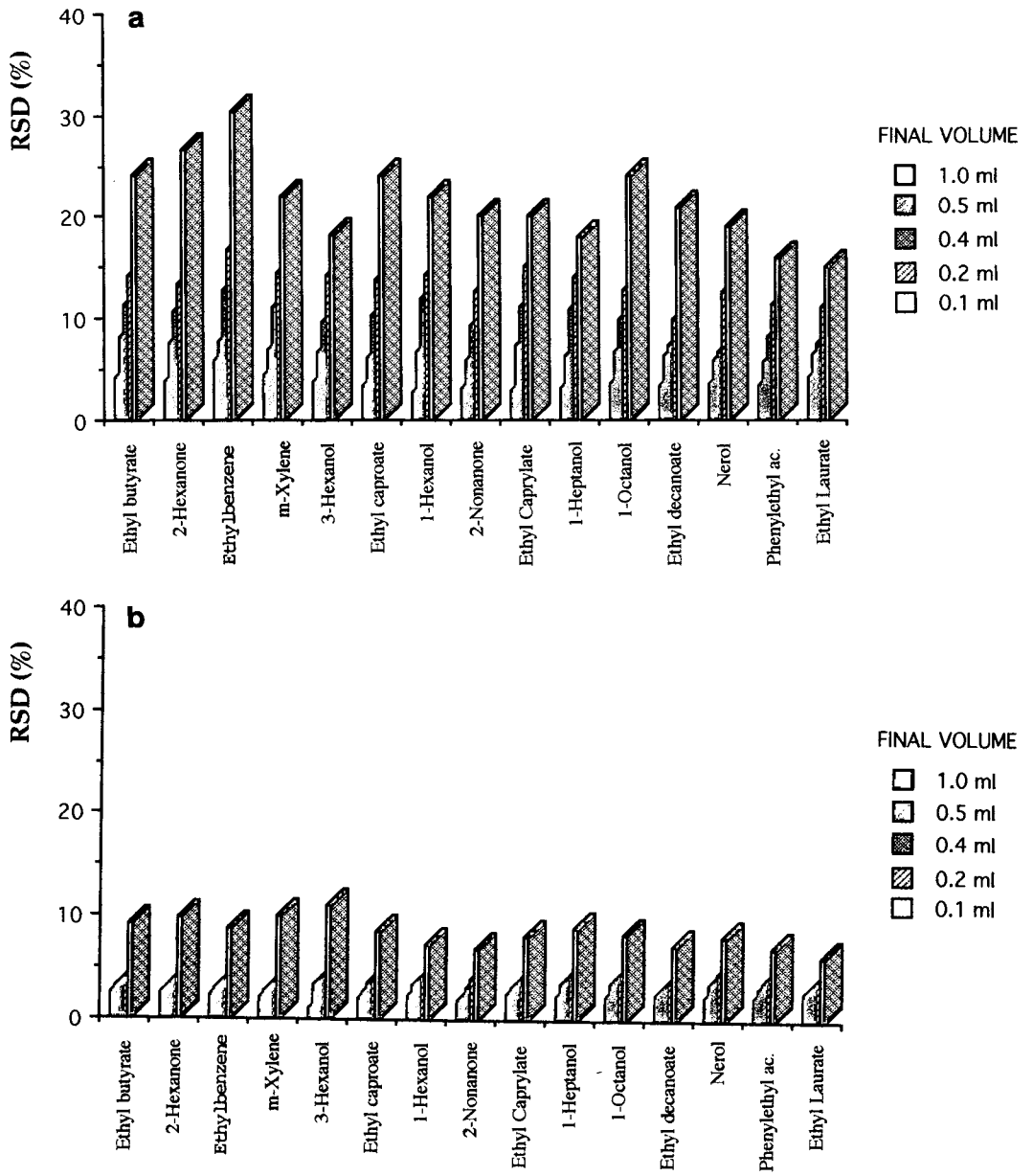

Fig. 2. Reproducibility of solute recovery throughout the sample concentration process as a function of final volume reached. (a) Concentration of 0.08 mg l^{-1}; (b) concentration 4.0 mg l^{-1}.

of the solutes: the higher the volatility, the higher the losses; however, relatively constant levels are maintained. Only when concentrating dilute solutions to 0.1 ml were the losses of the more volatile compounds almost double those observed for the less volatile compounds (see Fig. 1a). However, the differences were lower in the evaporation of the more concentrated solu-

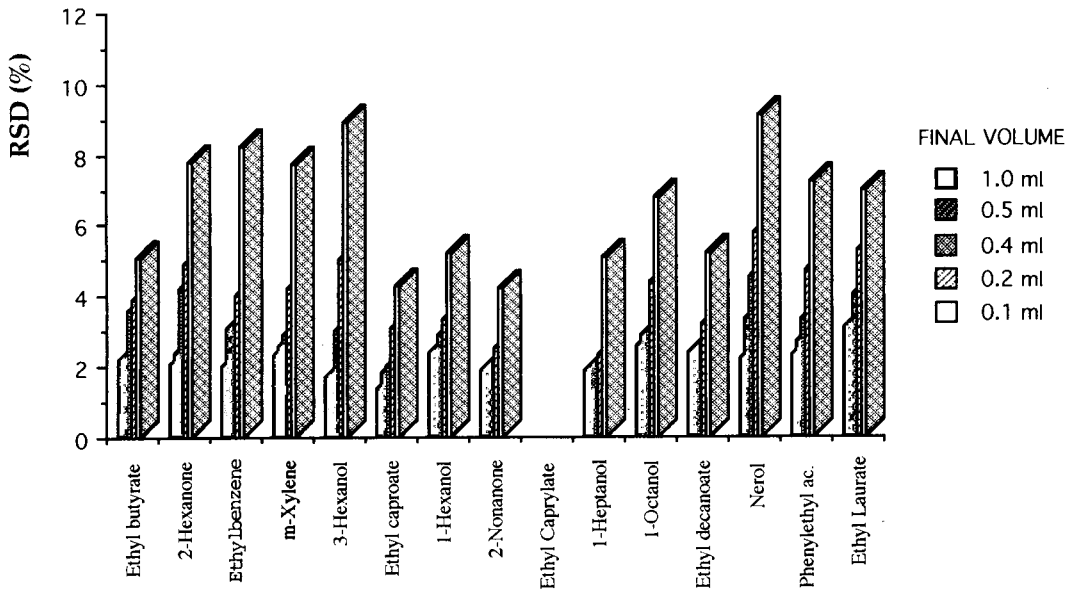

Fig. 3. Reproducibility of solute recovery relative to that of ethyl caprylate as a function of final volume reached. Concentration 0.08 mg l^{-1}.

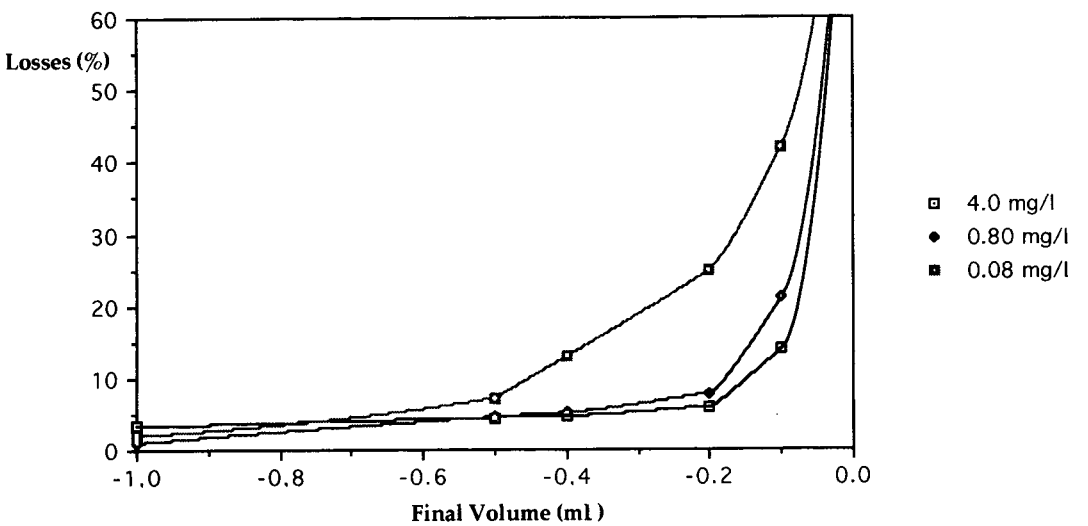

Fig. 4. Amount of ethyl caproate lost through the sample evaporation process as a function of both final volume reached and initial sample concentration.

tions (see Fig. 1b). The nature of the solute does not seem to affect the amount lost significantly.

3.2. Study of the effect of adding third compounds (Fig. 5a and b; Table 1)

After a certain critical amount, the addition of a third compound causes a severe decrease in the amount lost and a substantial improvement in the precision of the process. The critical amount of the compound to be added depends on its boiling point. The addition of four compounds was studied: ethanol, 2-propanol, isoamyl alcohol and heptanol. The minimum amounts needed to observe a significant improvement of the evaporation process were ca. 6 mg of ethanol, 4 mg of 2-propanol, 1 mg of isoamyl alcohol and 0.1 mg of heptanol. A dilute solution containing a sufficient amount of a third compound tends to behave like a concentrated solution.

3.3. Study of different rectification systems (Fig. 6)

The results obtained are diverse and are a function of the number of plates of the systems (Fig. 6). In all instances evaporation was forced almost to complete evaporation of the liquid phase, reaching a final volume of ca. 0.1 ml after its partial recondensation. In accordance with previous observations, the behaviour is strongly dependent on the initial concentration of the solution. In all instances the worst results were obtained with the microconcentrator, which is the system with the worse rectification capacity. During the concentration of more dilute solutions, some of the most volatile solutes were almost completely lost. The estimated losses of less volatile solutes were not very different from those observed with other systems. The results were slightly better for the Vigreaux column, but the losses were higher than those with the standard system.

3.4. Change in the geometry of the receptor system (Table 2)

The replacement of the cylindrical flask of the micro-Kuderna–Danish concentrator by a conical-bottomed flask lead to a great reduction in the losses during the concentration of the most diluted solutions. The results shown in Table 2 refer to strong concentration, almost to disappearance of the liquid phase. The precision of the process was improved, the average R.S.D. being ca. 7%. The behaviour of the system with the new flask is similar to that observed in the concentration of the more concentrated solutions.

3.5. Use of other solvents: pentane and hexane (Fig. 7a and b)

Fig. 7a and b show the results obtained in the concentration of solutions in pentane and hexane. The losses in both instances are higher than those when using dichloromethane as solvent. The differences found in the evaporation of the most concentrated solutions (Fig. 7a) are particularly important. The observed losses in the hexane solutions are the highest. When concentrating more dilute solutions, hexane solutions behave similarly to dichloromethane solutions, whereas the pentane solutions behave differently. The concentration of hexane solutions does not follow the same behaviour as observed so far. The losses hardly depend on the initial sample concentration under these conditions.

However, when the concentration system is kept in a chamber at 15°C for pentane or at 45°C for hexane, the results are similar to those obtained when concentrating dichloromethane solutions (data not shown). For both concentrated and dilute solutions, the best recoveries were obtained when working with pentane.

4. Discussion

Based on the results, we should consider two well differentiated steps. During the first step, an equilibrium is established between the evaporating solvent mass and the recondensing solvent mass, even though there is a net flux of solvent lost from the upper part of the column. The equipment works like a distillation system with several theoretical plates depending on the system geometry and the operating conditions. The

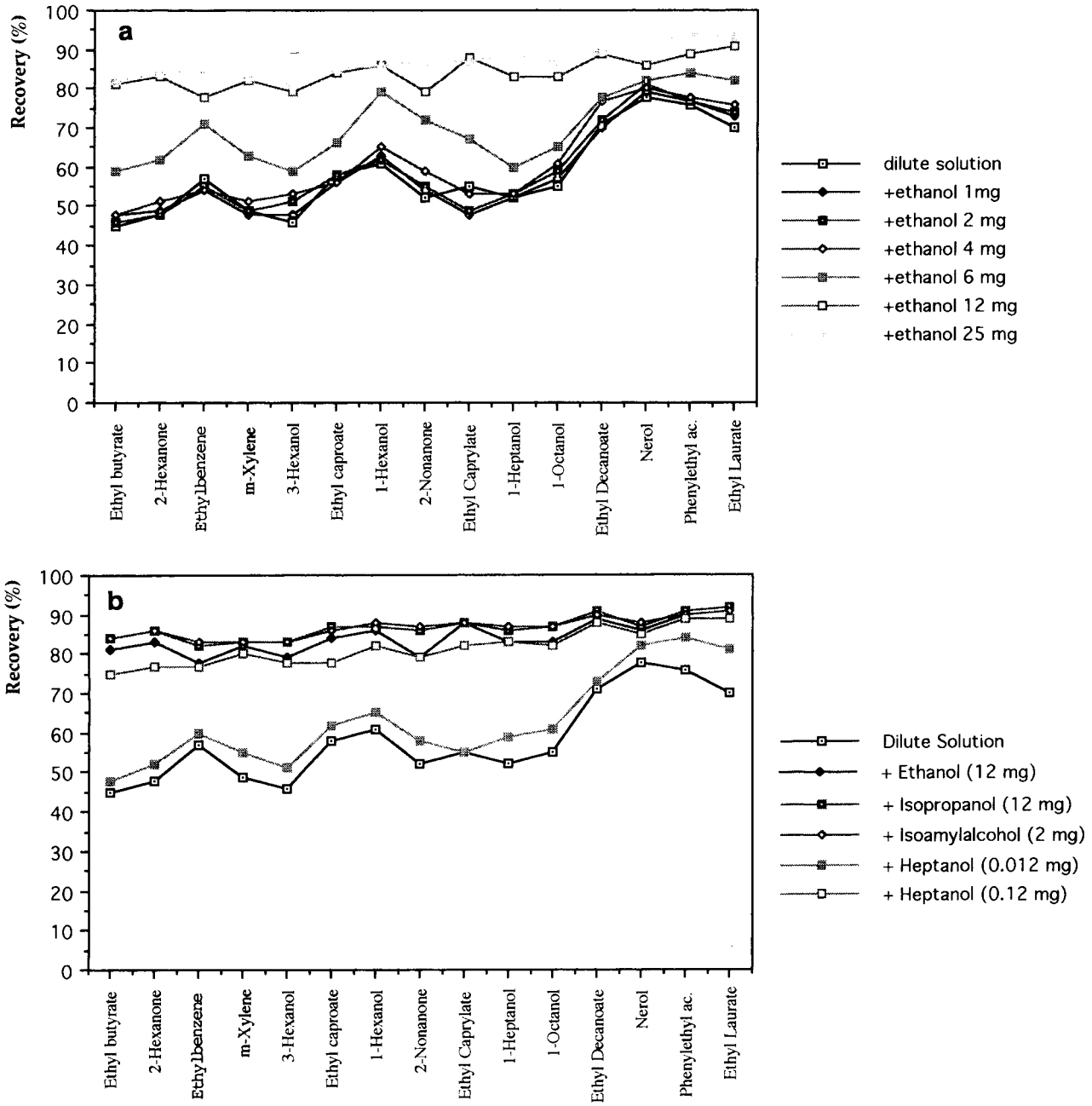

Fig. 5. Influence of the presence of a third party compound on solute recovery. (a) Study of amount of ethanol needed to achieve a significant decrease in solute losses. (b) Stabilization of matrix effect. The final point observed is independent of the added solute and tends to be similar to the evaporation process of a concentrated solution.

Table 1
Effect of addition of a third compound addition on the analytical behaviour of the evaporation process

Compound	R.S.D. (%)[a]				
	+ Ethanol (12 mg)	+ 2-Propanol (12 mg)	+ Isoamyl alcohol (6 mg)	+ Heptanol (0.012 mg)	+ Heptanol (0.12 mg)
Ethyl butyrate	14	3.1	3.4	18	11
2-Hexanone	13	3.0	3.1	14	10
Ethylbenzene	17	3.2	3.0	19	12
m-Xylene	14	2.8	3.5	21	9.1
3-Hexanol	14	3.8	3.7	18	8.5
Ethyl caproate	14	3.3	3.2	18	7.8
1-Hexanol	12	3.4	3.6	21	7.5
2-Nonanone	15	3.1	2.9	22	8.2
Ethyl caprylate	14	3.0	3.4	13	9.3
1-Heptanol	9	2.8	3.4	15	9.4
1-Octanol	11	2.9	3.0	16	8.6
Ethyl decanoate	8	4.4	4.2	11	7.2
Nerol	12	3.3	3.9	13	6.8
Phenylethyl acetate	11	3.5	3.8	14	8.0
Ethyl laurate	11	4.5	4.7	15	9.3

[a] $n = 3$.

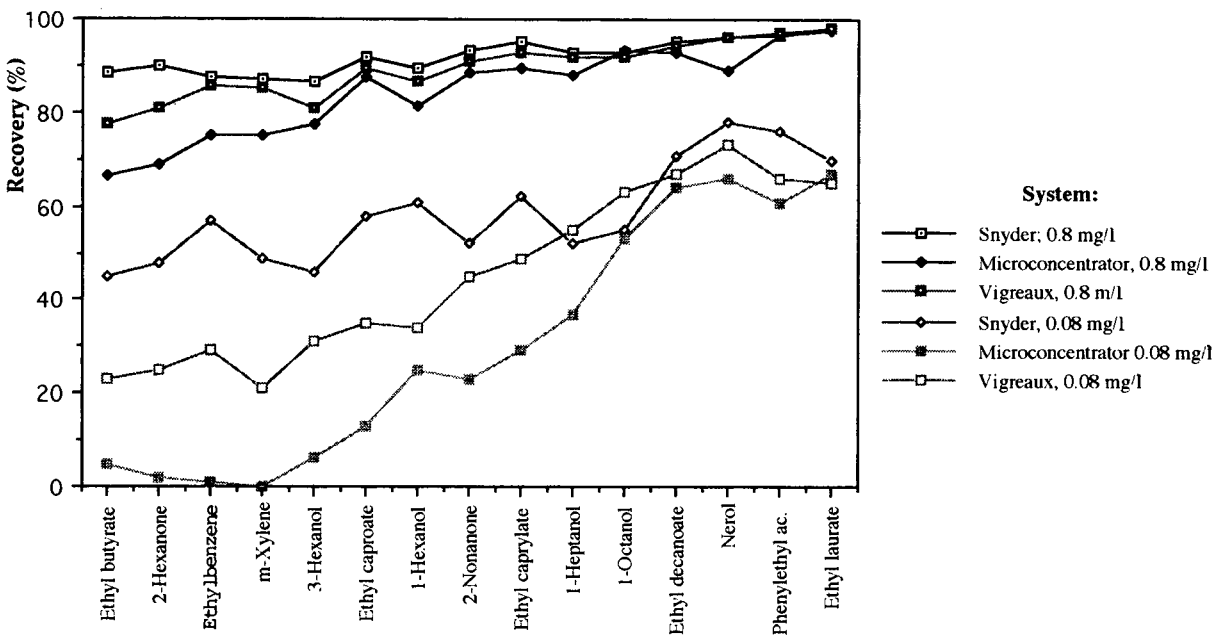

Fig. 6. Study of the influence of the rectifying system coupled to a micro-Kuderna–Danish concentrator for two different concentrations.

Table 2
Analytical results from concentration of dichloromethane solutions using a modified micro-Kuderna–Danish flask.

Compound	Recovery (%)			
	Cylindrical flask	Conical Flask	Absolute R.S.D. (%)[a]	Relative R.S.D. (%)[a]
Ethyl butyrate	45	72	9.8	5.7
2-Hexanone	48	78	8.3	7.2
Ethylbenzene	57	81	7.2	4.9
m-Xylene	49	83	8.7	5.2
3-Hexanol	46	85	7.9	5.0
Ethyl caproate	58	87	5.6	3.4
1-Hexanol	61	90	6.0	4.5
2-Nonanone	52	78	10.1	6.4
Ethyl caprylate	62	88	5.7	0.0
1-Heptanol	52	94	6.5	4.1
1-Octanol	55	91	5.6	3.9
Ethyl decanoate	71	96	4.8	2.0
Nerol	78	89	7.7	4.4
Phenylethyl acetate	76	96	5.1	3.5
Ethyl Laurate	70	97	4.3	2.7

Concentration until almost dryness. Initial concentration, 0.08 mg = l^{-1}. Final volume, 100 μl.
[a] $n = 6$.

rectification capacity of the system is sufficient and the solute losses are almost non-existent if its boiling point is above 100°C. The behaviour of the micro-Kuderna–Danish concentrator from an analytical point of view is more than satisfactory during this step. The lower limit of this step is within the range 0.4–0.5 ml, which means a concentration factor of between 60 and 80. The lack of precision introduced in the analytical result by this step is also acceptable, being in the worse scenario ca. 7% if the quantification is done without adding an internal standard before concentration. However, if an internal standard is added to the solution before the evaporation process begins, then the lack of precision introduced by the process is close to 3%, which is negligible for most trace analyses.

The second step begins when the amount of evaporated solvent is not sufficient to maintain the reflux. The differentiating characteristics of this step are the strong dependence on the sample concentration, the important solute volume losses during the concentration of diluted solutions, the way in which these losses increase when the final volume reached is decreased and the strong lack of precision associated with high losses. From an analytical point of view, the control over the process is drastically lowered and, what is worse, this fact can even go unnoticed. Examination of the data in Fig. 3 may lead one to think that the lack of precision associated with the process of evaporation is not relevant and can be accepted, given the important improvement in sensitivity. However, a closer look at Fig. 1a and 2a shows that at least three internal standards should be used to avoid these effects, and even then the process would be subject to matrix effects.

These effects are not a direct consequence of a decrease in the efficiency of the system, but to the fact that the lack of solvent makes most of the area of the glass walls dry. A drop, e.g., from a splash, that is placed on a hot and dry surface will evaporate slowly (owing to low thermal transfer from the glass walls and to the high calorific capacity of the solvent [16,17]). First the solvent will be lost, while the solutes concentrate in a progressive way and will not be

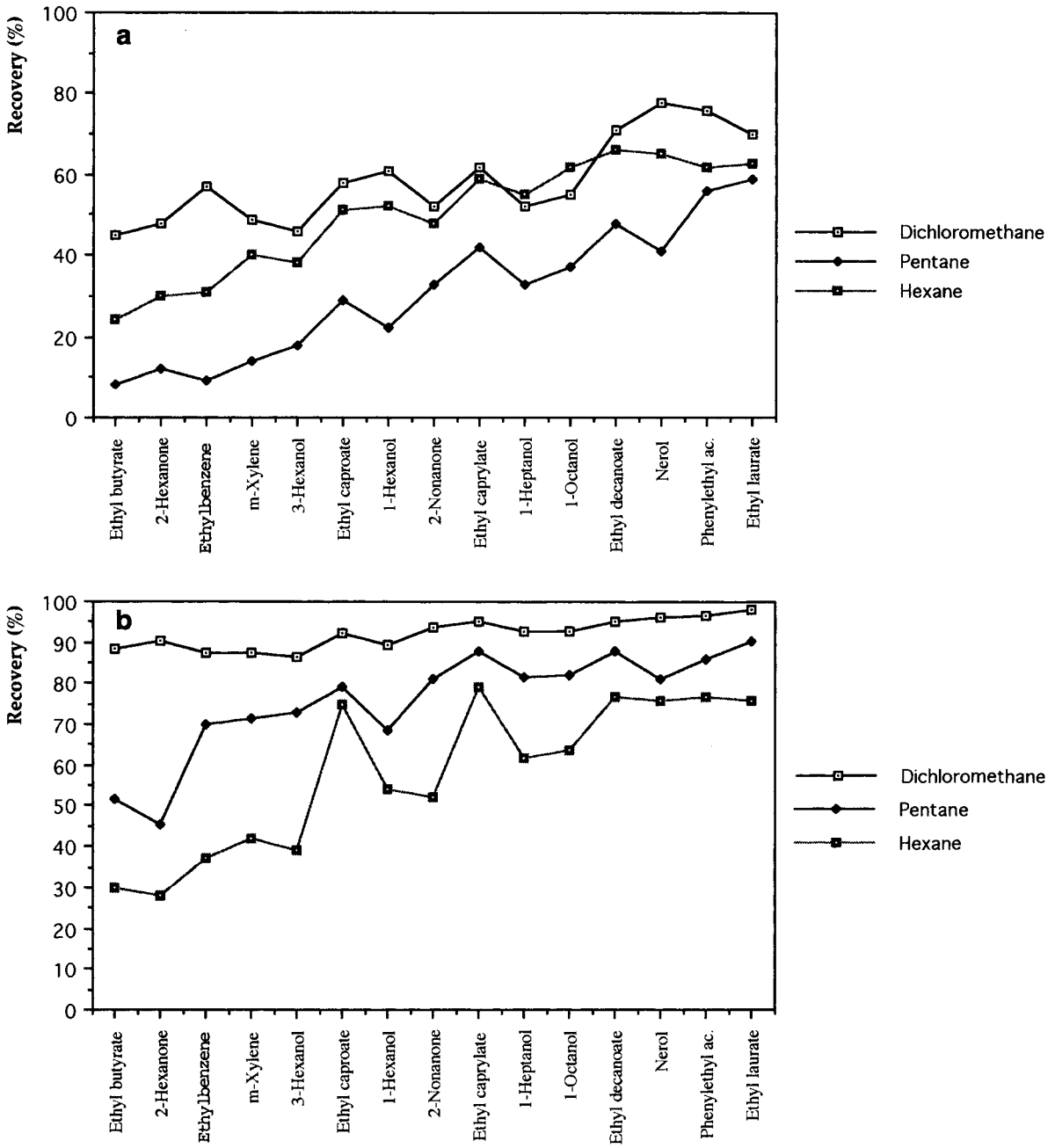

Fig. 7. Solute recovery as a function of type of solvent contained in the sample (a) Concentration 0.08 mg l^{-1}; (b) concentration 4.0 mg l^{-1}.

released until the drop of solvent that contained them has disappeared. Once it has disappeared, solutes are flushed by the solvent gas in a similar way to the evaporation of solutes in a gap of low retention surface, as has been thoroughly studied by Grob, Jr. [18, 19] and Grob [6,20].

If this previous statement is true, then it follows that the amount of lost solute depend on (1) the number of sample drop projections on dry walls and (2) the lifetime of those drops. Therefore,. all processes that contribute to increasing the number of splashes will contribute to increasing the amount of solute losses, and the shorter the life of the drop placed on the glass wall the greater is the probability of losing volatile matter. The validity of this hypothesis is discussed next based on the experimental results obtained.

4.1. Final volume dependence

Fig. 8 show two diagrams which represent the sample evaporation in the final steps of evaporation in the micro-Kuderna–Danish concentrator. They show how the violent sample boiling in the final steps of this process makes the surface constantly wet even when the volume of remaining liquid decreases drastically. According to our model, losses should be proportional to the wet surface area/remaining liquid volume ratio. In fact, this is the behaviour represented in Fig. 4. The existing parallelisms between evaporation in

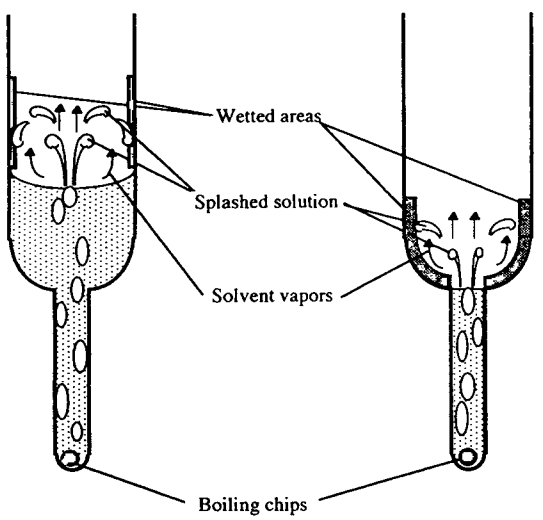

Fig. 8. Diagram of sample evaporation process in the final concentration step showing how the wet surface area/liquid volume ratio increases exponentially.

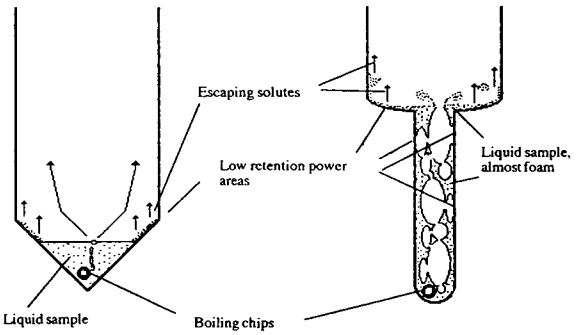

Fig. 9. Diagram of sample evaporation process in the final concentration step in two different systems. With a conical-bottomed flash the wet surface area/liquid volume ratio remains constant, whereas with a cylindrical-bottomed flash it increases exponentially. Consequently, the probability of a solute finding a low retention power area is much higher.

the cylindrical-bottomed flask and evaporation in the conical-bottomed flask are shown in Fig. 9. It can be seen that in this case the wet surface area/remaining liquid volume ratio is much lower, and this would explain the results with evaporation in this type of system which are shown in Table 2.

4.2. Effect of initial sample concentration

Studies on sample vaporization in gas chromatography with classical injectors have found that the presence of less volatile material can modulate the sample evaporation behaviour, prolonging the lifetime of solvent droplets that evaporate around a nucleus made of less volatile material [21–23]. Some of these solutes may play a similar role to non-volatile material in chromatographic injectors, adhering solvent droplets coming from splashes or recondensation. In fact, a simple observation of the behaviour of the evaporating solution shows that the size of droplets generated from solutions containing a larger amount of solutes is larger than the size of droplets generated during evaporation of dilute solutions, in spite of having a greater capacity to adhere to the glass wall and a lower mobility on it. As a result, the droplets take longer to evaporate, delaying the release of solutes contained in them, and diminishing the amount of

solute lost during this step. Once the majority of the solvent that forms the drop has evaporated, we have to consider that the thickness of the solute coating covering the glass wall makes the retention capacity of this type of stationary phase temporarily larger while avoiding solute loss, or at least delaying its release.

The addition of third components showed how important retention capacity of the pseudo-chromatographic system is. The boiling point of the compound added plays a key role in the intensity of the phenomena, as has already been mentioned. The concentration of the third component added needs to be progressively smaller as its volatility decreases in order to increase its action on the evaporation, which must be attributed to its higher capacity to form a temporary chromatographic coating. The practical importance of this observation is great, as it makes it possible to decrease the observed matrix effect and to cancel the error in quantification, and also to decrease the solute losses to acceptable levels for the concentration factors referred to here ($F_C > 400$). However, we must take into consideration that these "boiling regulator" compounds could form azeotropes with the solvents, which could lead to different results from those presented here, and should be considered with caution in each instance.

4.3. *Irreproducibility of the process*

This observation fits the proposed model. In fact, the number of projections is highly unpredictable and imprecise because it depends on uncontrolled factors such as the glass surface behaviour or the boiling of chips. Since solute losses are a direct consequence of these projections, the absolute solute losses are highly irreproducible. On the other hand, and according to the simplified model, the solutes contained in a droplet from a projection will be completely evaporated almost independently of their boiling point. This implies that losses will be constant from a relative point of view, and therefore reproducible.

4.4. *Concentration of solutions contained in other solvents*

The results with other solvents (see Fig. 7) are worse than those obtained with dichloromethane. With pentane under conditions used, almost no recondensation of pentane took place in the rectifying column, and evaporation occurred at high speed. In contrast, with hexane, solvent rectification at room temperature was so intense that an increase in the temperature of the thermostated bath to well above the solvent boiling point was needed. The higher losses in the case of poor refluxing are easily explained and must be attributed to a low process efficiency. Losses when the reflux is excessive are due to the large amount of energy needed to achieve a constant release of solvent at a reasonable speed from the rectifying system. This aggravates the coevaporation problems, as droplet lifetime decreases while their number increases as boiling becomes more violent. Experiments with both solvents were repeated at different external temperatures (15°C with pentane and 45°C with hexane). The behaviour of these systems was then very similar to that observed previously, supporting the hypothesis and demonstrating that there is an optimum concentration point for each rectification system depending on the boiling point of the solvent and its calorific capacity.

5. Conclusions

Micro-Kuderna–Danish concentration is a very suitable approach for the concentration of average sample volumes (10–40 ml) by solvent evaporation, providing the process with very good analytical characteristics of precision and accuracy.

Solute losses in systems that do not form azeotropes are mainly due to flushing of solute layers from dry surfaces (coevaporation) during the final sample evaporation step (below 0.5 ml). Therefore, special precautions are needed at this stage. Very dilute solutions should not be concen-

trated below that volume, but concentrated solutions or solutions containing a large amount of a third, miscible component can be concentrated below that limit. If the cyclindrical collector flask is replaced by a flask with a conical bottom, even dilute solutions can be concentrated below 100 μl without losing the analytical characteristics. The use of rectifying systems less efficient than the Snyder column leads to higher solute losses.

There are optimum operating conditions for each system. The temperature gradient between the reflux system and the ambient air around it should not be below 20°C (no reflux) or above 40°C (excessive risk of coevaporation).

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JOURNAL OF CHROMATOGRAPHY A

Identification of the isomers from mono- and dinitration of α-hydroxydiphenylacetic acid by capillary gas chromatography with Fourier transform infrared and mass spectrometric detection

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Abstract

All three mononitro and all six heteronuclear dinitro isomers from nitration of α-hydroxydiphenylacetic (benzilic) acid were separated as methyl esters by capillary gas chromatography and their retention indices were measured. Heteronuclear dinitro substitution of α-hydroxydiphenylacetic acid was confirmed by inspection of the MS fragmentation patterns of the eluted compounds. The mononitro and dinitro derivatives were identified on the basis of their Fourier transform IR spectra and, in part, of the MS spectra recorded in the positive-ion mode.

1. Introduction

In previous work [1] we solved the problem of the separation and identification of all the isomers from the mono- and dinitration of phenyl- and diphenylacetic acids, as their corresponding methyl esters, by capillary gas chromatography (GC) with Fourier transform infrared (FT-IR) and mass spectrometric (MS) detection.

The use of an OV-1 coated capillary column working between 150 and 300°C with a gradient 10°C min^{-1} allowed the separation of all the isomers actually present in the available mixtures. GC–MS established that all the dinitro derivatives had both rings bearing a nitro group. The positional assignments were essentially established by extrapolation of the observations of the IR stretching vibrations of both the nitro group and the benzene ring in the methyl esters of mononitrophenylacetic acid isomers.

In continuation of synthetic work on the mono- and dinitration of α- and α,α-substituted toluenes and diphenylmethanes, we encountered the problem of the prompt identification of the isomers produced in the nitration of α-hydroxydiphenylacetic (benzilic) acid and its methyl ester under a variety of experimental conditions.

This paper describes the investigation of this problem by the procedure successfully used previously [1], i.e., the separation of the nitration products of α-hydroxydiphenylacetic acid (after methylation with diazomethane) by capil-

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lary GC and identification of the separated peaks by FT-IR spectrometry, in part supported by MS. However, the introduction of the hydroxyl group into the structure of phenylacetic acid derivatives causes a significant change in both the symmetry and polarity of the molecule, creating a new identification problem in respect of the nitro derivatives of diphenylacetic acid. This problem has not been reported previously; moreover, all these nitro derivatives are unknown or poorly characterized compounds.

2. Experimental

2.1. Materials

α-Hydroxydiphenylacetic acid (benzilic acid) was provided by Chimica del Friuli (Torviscosa, Udine, Italy). Nitration experiments on α-hydroxydiphenylacetic acid and its methyl ester, obtained by Fisher methylation [2], yielding the corresponding mono and dinitro derivatives, were carried out in a standard fashion. Mixtures of nitrated acids were methylated by the action of diazomethane in diethyl ether. Pure *o*-nitrobenzilic acid was obtained by crystallization from a mononitration reaction mixture; *p,p'*-dinitrobenzilic acid was analogously obtained from a dinitration mixture.

2.2. GC–FT-IR and GC–MS measurements

GC separations were carried out on a capillary column (8 m × 320 μm I.D.) coated with HP-5 as the stationary phase with a 0.17-μm film thickness (Hewlett-Packard, Avondale, PA, USA). The carrier gas was helium at an inlet pressure of 50 kPa and a linear velocity of 44 cm s^{-1}. Samples were injected on to the column with a 1-μl syringe with the aid of a splitter operating at a splitting ratio of 1:50. The column efficiency was 20 000 effective plates for *m,m'*-dinitrobenzilic acid methyl ester with $k' = 30$ at 200°C. *n*-Alkanes were added to the mixtures of nitration products in order to evaluate retention indices in isothermal and temperature-programmed experiments. Retention indices were measured with an average repeatability of ±1 index unit (i.u.). The column temperature was programmed from 110 to 300°C at 10°C min^{-1}.

A Hewlett-Packard (Palo Alto, CA, USA) Model 5890 Series II gas chromatograph equipped with an HP Model 5965 A IR detector was used to record FT-IR spectra.

Mass spectra were recorded using a Fisons VG TRIO 2000 gas chromatograph–mass spectrometer (VG Biotech, Cheshire, UK), equipped with automated data retrieval, under electron impact (EI) (70 eV) conditions working in the positive-ion mode. Each individual GC peak was carefully inspected for constancy of the MS pattern in order to detect possibly overlapping components and to ensure homogeneity of the eluates.

3. Results and discussion

3.1. Gas chromatography

The chromatogram of the methyl esters of the nitration products of α-hydroxydiphenylacetic acid obtained with temperature programming on a capillary column with HP-5 as the stationary phase is shown in Fig. 1. In a short capillary column with an efficiency of 20 000 plates, complete separation of the isomeric mononitro and dinitro derivatives was obtained. The measured values of their retention indices with temperature programming [3] and isothermally at 200°C, and their temperature coefficients, are given in Table 1.

The retention orders of the isomeric mononitro (*ortho* < *meta* < *para*) and dinitro (*ortho*, *ortho'* < *ortho,meta'* < *ortho,para'* < *meta*, *meta'* < *meta,para'* < *para,para'*) methyl esters of α-hydroxydiphenylacetic acid were identical with those of the mono- and dinitromethyl esters of diphenylacetic acid [1], and also with those of methyl- and dimethylbiphenyls [4]. When a second methyl group is introduced in the second ring of a methylbiphenyl, additivity of the retention index contribution acts in a range of a few units. Similarly, with a change in substitution from mono- to dinitromethyl esters of α-hydroxydiphenylacetic acid the measured values

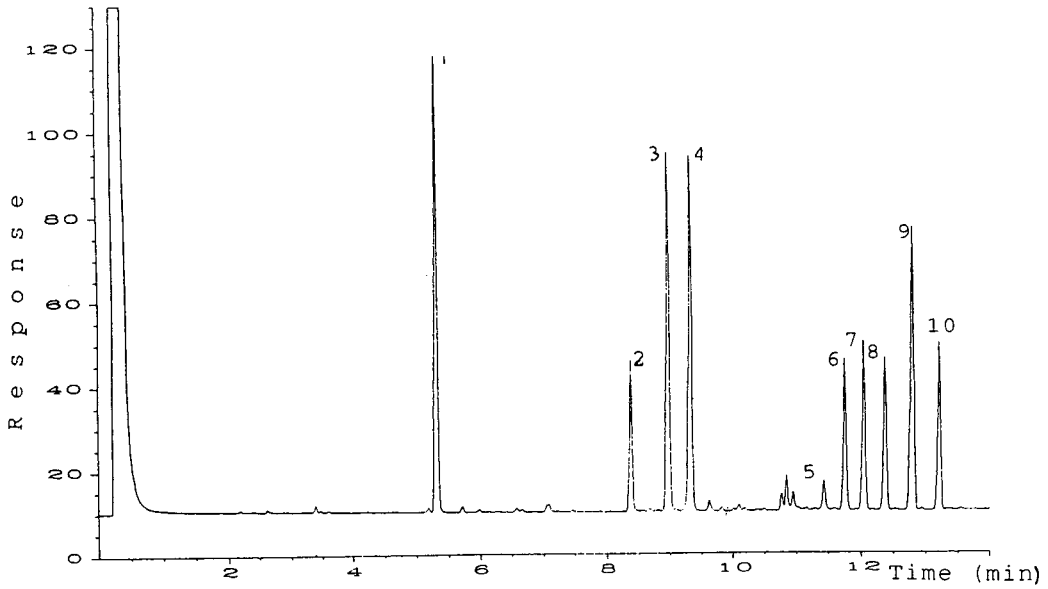


Fig. 1. Chromatogram of the methyl esters of the products from the nitration of α-hydroxydiphenylacetic acid. Peak identification as in Table 1.

are on average only 11 i.u. higher than those calculated from the retention index contribution of mononitro derivatives. This value is about five times smaller than the value of the retention index difference of neighbouring eluted isomers, which for mononitro isomers is 41–68 i.u. and for dinitro isomers 38–57 i.u. It follows from the retention index data in Table 1 that substitution

Table 1
Temperature-programmed retention indices (TP-*I*), isothermal (200°C) retention indices (*I*) and temperature coefficients (d*I*/d*T*) of methyl esters of products from the nitration of α-hydroxydiphenylacetic acid on HP-5

GC peak No.[a] (Fig. 1)	Identification	TP-*I*	*I*	d*I*/d*T*
1		1825	1868	0.8
2	*ortho*	2152	2183	1.0
3	*meta*	2220	2245	1.0
4	*para*	2261	2285	1.0
5	*ortho,ortho'*	2516	2519	1.1
6	*ortho,meta'*	2557	2558	1.2
7	*ortho,para'*	2595	2592	1.2
8	*meta,meta'*	2638	2632	1.1
9	*meta,para'*	2693	2677	1.4
10	*para,para'*	2750	2732	1.3

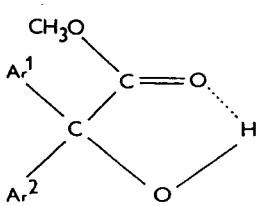
[a] 1 = Methyl ester of α-hydroxydiphenylacetic acid; 2–4 = methyl esters of α-hydroxymononitrodiphenylacetic acid; 5–10 = methyl esters of α-hydroxydinitrodiphenylacetic acid with one nitro group in each phenyl ring.

of one NO_2 group in the parent compound increased the retention index of the *ortho* isomer by 315 i.u., of the *meta* isomer by 377 i.u. and of the *para* isomer by 417 i.u. Heteronuclear substitution of two NO_2 groups in the parent compound increased the retention indices by 651–864 i.u. and substitution of NO_2 in mononitro derivatives by 336–447 i.u.

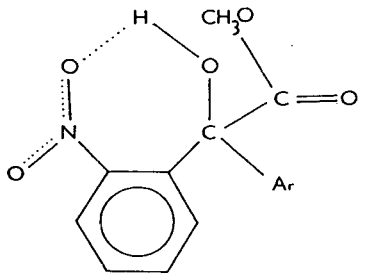
None of the products is commercially available for comparison purposes and they do not appear to be easily produced by any reported method. Positive identification of isomeric nitration products was achieved with the highly informative detection of capillary column eluates both by FT-IR spectrometry and EI-MS.

3.2. GC–FT-IR

The GC peak identification and the FT-IR data for the methyl esters of α-hydroxydiphenylacetic acid and the products of its mononitration are given in Table 2. The carbonyl stretching frequencies of *m*- and *p*-nitro derivatives of the methyl esters of α-hydroxydiphenylacetic acid are observed in a region close to the ν(C=O) value of the parent methyl ester and can be assigned to the molecules stabilized by an intramolecular hydrogen bond between the C=O and OH groups [5], as illustrated.



The structure shown is also reflected by absorption in the O–H stretching frequency region, the Δν(OH) value being 75–80 cm^{-1}, which corresponds to a hydrogen bond energy of 7.6–8.0 kJ mol^{-1}. On the other hand, the carbonyl stretching frequency of the *o*-nitro derivative of the methyl ester of α-hydroxydiphenylacetic acid is 11–14 cm^{-1} higher than those of α-hydroxydiphenylacetic methyl ester derivatives lacking any NO_2 group in the *ortho* position of the benzene ring. It follows from the above data that with an *o*-nitro derivative of the methyl ester of α-hydroxydiphenylacetic acid the C=O group becomes free of intramolecular hydrogen bonding as the equilibrium is shifted towards the formation of a new hydrogen bond between the OH and the NO_2 groups [6], as shown.



The above results show that the IR frequency of the carbonyl stretching vibration in α-hydroxydiphenylacetic acid esters can be advantageously used as a reliable criterion for identification and distinction of the *o*-nitro derivatives. The second characteristic feature of an *o*-nitro derivative in comparison with *para*- and *meta*-substituted nitro compounds is the increase in $\nu_s(NO_2)$ frequency by 7 cm^{-1}, as a consequence of a decrease in coplanarity, i.e., deconjugation between the NO_2 and OH groups. It must be pointed out that the FT-IR spectrum of the *o*-nitro derivative of the methyl ester of α-hydroxydiphenylacetic acid obtained from the mixture by GC separation was identical with that measured for the isolated pure compound. Analogously to the nitration products of phenyl- and diphenylacetic acids [1], the *p*- and *m*-nitro derivatives of the methyl ester of α-hydroxydiphenylacetic acid can be clearly distinguished comparing the values of $\nu_{as}(NO_2)$ frequencies, and in the case of *para* substitution, by the occurrence of the characteristic aromatic absorption ν(arom.) at ca. 1600 cm^{-1}. Finally, the *para* position of the nitro group on the benzene ring of the methyl ester of α-hydroxydiphenylacetic acid was also assigned using the characteristic position of the absorption band belonging to the out-of-plane wagging deformation of the benzene ring ω(CH).

Table 2
FT-IR data for the methyl esters of α-hydroxydiphenylacetic acid and its products of mononitration

GC peak No. (Fig. 1)	Position of NO_2 group on benzene ring	ν (cm^{-1})							ω(CH)
		$\nu(OH)_{free}$	$\nu(OH)_{bond}$	$\Delta\nu(OH)$[a]	$\nu(C{=}O)$	$\nu_{as}(NO_2)$	$\nu_s(NO_2)$	$\nu(C{-}O)$	
1	–[b]	3623	3548	75	1744	–	–	1243	–[c]
2	*ortho*	3622	3536	86	1758	1547	1359	1241	763
3	*meta*	3622	3543	79	1747	1546	1352	1245	–[c]
4	*para*[d]	3622	3542	80	1746	1538	1352	1245	851, 754

[a] $\Delta\nu(OH) = \nu(OH)_{free} - \nu(OH)_{bond}$.
[b] Methyl ester of α-hydroxydiphenylacetic acid.
[c] Unresolved.
[d] Additional absorption band of ν(arom.) = 1599 cm^{-1}.

The identification rules discussed above for simple mononitration products of α-hydroxydiphenylacetic acid enabled a strategy for the identification of structurally more complex dinitro derivatives to be elaborated. The GC peak assignments and the FT-IR features of the dinitration products of methyl esters of α-hydroxydiphenylacetic acid are listed in Table 3. The knowledge of the position of the stretching vibration of free and hydrogen-bonded C=O groups was implemental for identification and distinction of the *ortho* substitution. Further, the $\nu_{as}(NO_2)$, ν(arom.) and, in some instances, also the ω(CH) bands were successfully employed to identify *meta* and *para* substitution.

In addition, it has been observed that for all the dinitro derivatives the intensity ratio $\nu_{as}(NO_2):\nu(C{=}O)$ is always >1, whereas the same ratio for mononitro compounds is ≤1. This general rule allows one to distinguish reliably all the products of dinitration from those of mononitration. Finally the identity of the *p,p'*-dinitro derivative was confirmed by recording the FT-IR spectrum of the isolated substance. It must also be mentioned that these results are in good agreement with those obtained previously [1] for the corresponding nitration products of phenyl- and diphenylacetic acids.

3.3. GC–MS

The mass spectral patterns of the three methyl mononitrobenzilates **1**, **2** and **3** (Fig. 2) showed essentially the same main features, i.e., a base peak for the loss of the methyl carboxylate function from the parent ion [*m/z* 228 (**4**)] and an intense ion (**5**) at *m/z* 150, corresponding to the composition $O_2NC_6H_4CO$, believed to originate from the former by elimination of a benzene molecule. This could be formed by the acidic proton on the oxygen in **4** being transferred to the more electron-rich phenyl group rather than to the less electron-rich nitrophenyl group; eventually, both **5** and **6** should lose carbon monoxide, but only the latter did. This was completely unexpected behaviour, and we therefore have to assume that the true nature of the fragment had by this time changed to that of a mesomerically favourable nitrite derivative **7** from that of a nitro derivative **5**. We also noticed the presence of an ion at *m/z* 104 (**8**) for the *meta* and *para* isomers (**2** and **3**); the ion at *m/z* 77 is always present and larger than any at *m/z* 76. In the spectrum of the *para* isomer **3**, the ion at *m/z* 150 (**7**) underwent loss of a nitrogen monoxide molecule (*m/z* 120, **9**) to some extent, which eventually collapsed to **10** (*m/z* 92). The direct inlet mass spectrum of the isolated *ortho* isomer **1** was identical with that obtained for the first GC peak of the mononitro isomers.

The six dinitro derivatives of the methyl ester of α-hydroxydiphenylacetic acid (**12–17**) present in the nitration mixture also exhibited mass spectral patterns (Fig. 3) that were so similar that they could not be used even as fingerprints to distinguish them one from another. Their parent ions were absent or barely observable from a regular spectrum recorded at 70 eV; the

Table 3
FT-IR data for the methyl esters of the products of the heteronuclear dinitration of α-hydroxydiphenylacetic acid

GC peak No. (Fig. 1)	Position of NO_2 group on the benzene rings	ν (cm^{-1})					ω(CH)
		ν(C=O)	ν(arom.)	$\nu_{as}(NO_2)$	$\nu_{s}(NO_2)$	ν(C–O)	
5	*ortho,ortho'*	1760	1603[a]	1548	1359	1243	–[b]
6	*ortho,meta'*	1760	–	1547	1353	1244	–[b]
7	*ortho,para'*	1759	1602	1546	1352	1248	851
8	*meta,meta'*	1749	–	1547	1351	1247	–[b]
9	*meta,para'*	1749	1600	1546	1351	1248	851
10	*para,para'*	1749	1600	1543	1351	1250	848

[a] Exception: see also the methyl ester of 2-nitrobenzeneacetic acid [1].
[b] Unresolved.

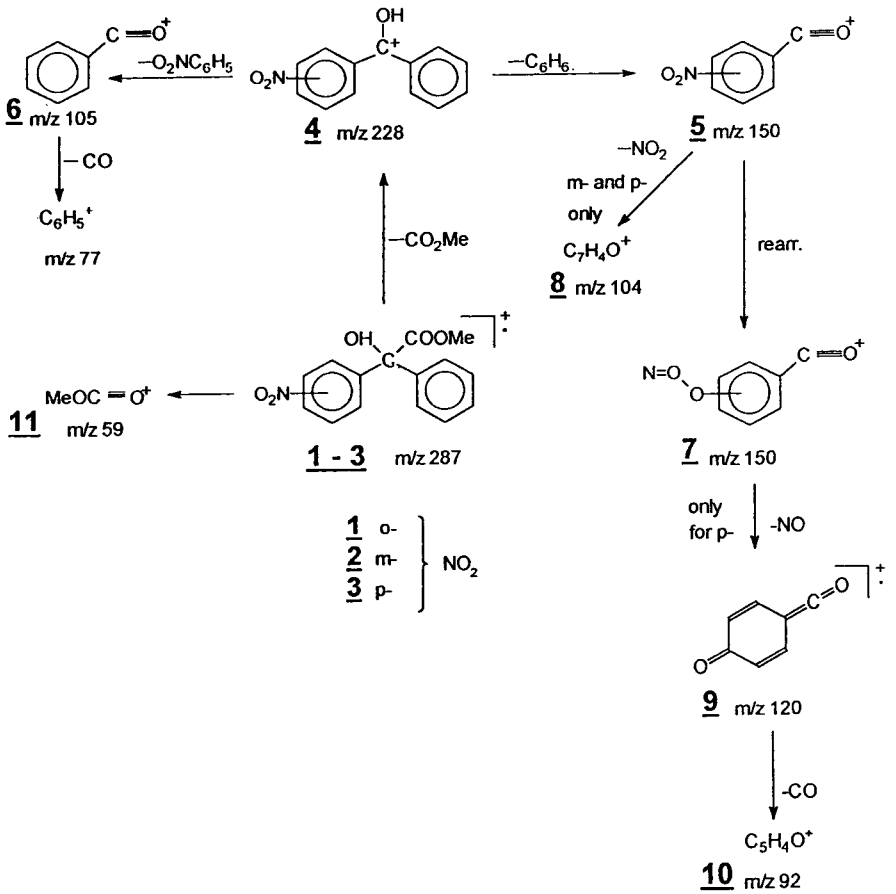


Fig. 2. Mass spectral patterns of the mononitro derivatives of the methyl ester of α-hydroxydiphenylacetic acid.

ion at *m/z* 59 for the $MeCO_2$ ion (**11**) was constantly present, but of definitively small intensity. The common feature was a fragment ion at *m/z* 273 (loss of $MeCO_2$ from the parent ion, **18**), which corresponded in all instances to the base peak. A peak at *m/z* 150 (**5** and/or **7**) was the second highest (65–80%), accompanied by weaker ions at *m/z* 151 and 152 (**19** and **20**). The weaker peak at *m/z* 105 could be ascribed to a different route, namely loss of NO_2 from **19**. A weak ion at *m/z* 180 (**21**) was equally ubiquitous and is believed to yield the ion at *m/z* 152 (**20**) by loss of carbon monoxide. Loss of nitrogen dioxide from the ion at *m/z* 150 (**5** and/or **7**) yielded the ubiquitous ion of medium intensity at *m/z* 104 (**8**), as we saw before. The *para* derivatives **14**, **16** and **17** all showed a small but distinct ion at *m/z* 120 (**9**), a feature observed in the *p*-mononitro isomer **3**.

The above observations, coupled with the peak homogeneity for all the dinitro isomers **12**–**17**, allowed us to discard the possibility of the presence of any significant concentrations of isomers with both the nitro groups in a single phenyl ring in the analysed mixture. The direct inlet mass spectrum obtained for isolated pure methyl *p,p'*-dinitrobenzilate (**17**), independently identified by ^1H NMR and IR spectrometry, was identical in all details with the mass spectrum of the sixth GC peak of the dinitro isomers GC sequence.

Nitration of methyl *o*-nitrobenzilate (**1**) with

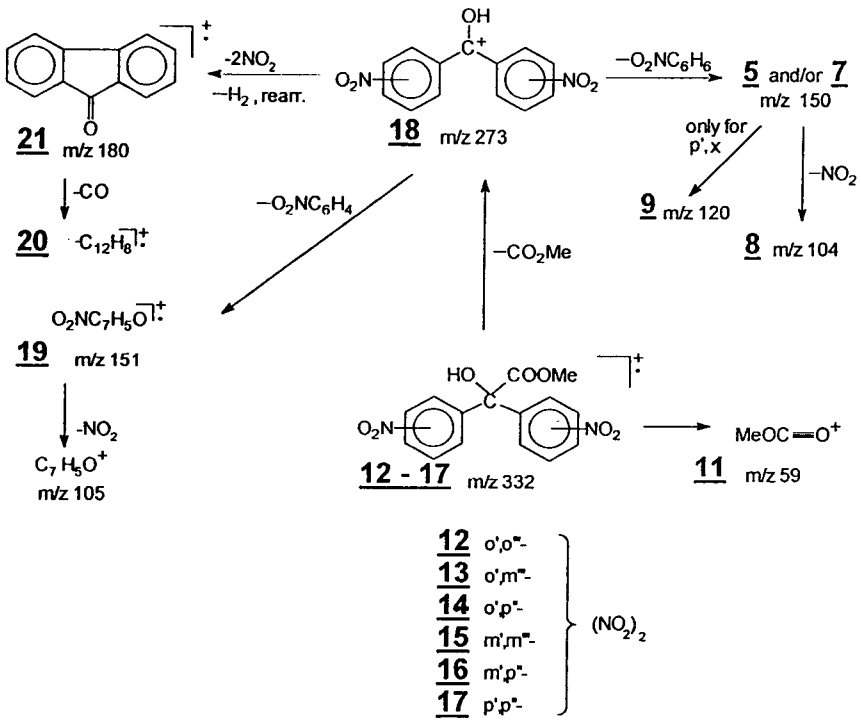


Fig. 3. Mass spectral patterns of the dinitro derivatives of the methyl ester of α-hydroxydiphenylacetic acid.

100% nitric acid yielded three *ortho,x*-isomers, which overlapped the first three GC peaks of the whole nitration mixture and exhibited identical mass spectra.

The minor peaks detected in the chromatogram (Fig. 1) were found to be side-products of a different nature.

4. Conclusions

The coupling of capillary GC with FT-IR spectrometry and EI-MS allowed the identification of all the isomers arising from the mononitration and dinitration of α-hydroxydiphenylacetic acid.

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Analysis of irritants by capillary column gas chromatography–tandem mass spectrometry

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Abstract

Daughter spectra were obtained for the molecular and principal electron impact fragmentation ions of four irritants, 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin, during capillary column GC–MS/MS analysis. The use of standardized collisional activated dissociation cell conditions resulted in the acquisition of daughter spectra suitable for identification and data-base generation purposes. Daughter operation detection limits of 100 pg ($S/N > 10:1$), for the highest molecular mass irritant, dibenz[*b*,*f*]-1,4-oxazepin, were obtained. This level of sensitivity was approximately the same as that routinely obtained for chemical warfare agents during capillary column GC–MS analysis under electron impact ionization conditions. The specificity of GC–MS/MS was demonstrated by spiking a complex diesel exhaust extract, with 1-methoxycycloheptatriene.

1. Introduction

Chemical warfare agents can be classified into two general categories, those that exert a lethal effect and those that act in an incapacitating manner. Lethal chemical warfare agents include nerve agents such as sarin, soman and tabun, while incapacitating agents include irritants (tear gases or riot control agents). Acute exposure to irritants causes a number of incapacitating effects including burning or irritation of the skin and eyes, coughing, nausea and vomiting. The incapacitating nature of these chemicals has led to the development of dispersal devices for their use in riot control situations, during military training exercises and to a lesser extent as chemical weapons on the battlefield [1]. The most commonly employed irritants are *o*-chlorobenzylidenemalononitrile, often referred to as tear gas, and 2-chloroacetophenone. Dibenz[*b*,*f*]-1,4-oxazepin has been used less frequently and 1-methoxycycloheptatriene was evaluated as a possible military training agent.

The text of the 1994 "Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and their Destruction" states in Article 1 that: "Each state party undertakes not to use riot control agents as a method of warfare". United Nations peacekeeping forces could encounter use of irritants during active duty in regions of the world where there is a threat of chemical warfare agent use. Intelligence gathering, through the collection of contaminated samples, and subsequent analysis of the samples would enable identification of the suspect chemical and con-

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firm use of a controlled chemical for warfare purposes. The results of such analyses would likely contribute to the development of appropriate strategic and political positions.

Gas chromatographic (GC) methods, including methods based on GC retention indices [2–7], have been used for the detection of irritants in suspect samples. These methods and others involving the use of chromatographic techniques for the detection of irritants and other chemical warfare agents have recently been reviewed [8]. In cases where positive identification is required, most laboratories analyse suspect extracts by GC–MS, as this technique offers the greatest sensitivity and certainty for chemical warfare agent identification. Electron impact (EI) ionization has been used frequently for irritant identification and the EI data for the common irritants, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin [9–14], and related benzylidenemalononitriles [12,15] have been reported.

However under an allegations of use scenario, collected samples would typically be environmental samples such as soil, water and air, or man-made materials such as paint, concrete, and munitions or munition fragments from the scene of an attack. In many cases the samples taken may have been exposed to rain, heat, sunlight or wind, for days or weeks and much of the original chemical warfare agent may have evaporated or undergone degradation (e.g., hydrolysis of *o*-chlorobenzylidenemalononitrile). This "weathering" process makes identification of the chemical warfare agent that much more difficult. The levels of contamination could be extremely low in these cases and require sophisticated methods for the detection and identification of these compounds in the presence of naturally occurring chemical interferences. Similar difficult analytical situations could also be envisioned during the analysis of forensic samples for irritants.

GC–MS, while generally accepted as the technique of choice for the confirmation of irritants, has limitations in the presence of high levels of chemical interferences. Tandem mass spectrometry offers considerable advantages over traditional mass spectrometry under this scenario, as it is a more specific and sensitive technique for the identification and confirmation of chemical warfare agents and related compounds [16–21] in complex environmental samples [17–19]. Daughter spectra of lethal chemical warfare agents have been published in these papers, but they do not include tandem mass spectrometric data for common irritants.

GC–MS/MS characterization of irritants was investigated, since this technique may enable selective identification of these compounds in complex samples. This report summarizes the daughter spectra obtained for the irritants, 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin, during GC–MS/MS analysis of these compounds and illustrates the utility of GC–MS/MS for the specific identification of 1-methoxycycloheptatriene in the presence of numerous interfering hydrocarbons collected in a diesel exhaust environment.

2. Experimental

2.1. Samples

Four irritants, 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin were provided by the DRES Organic Chemistry Laboratory. Standard solutions containing the irritants were prepared at the 10 and 0.2 ng/μl level in dichloromethane for use during capillary column GC–MS/MS analyses.

Air from a diesel exhaust environment was sampled through a R51A charcoal chemical cartridge (American Optical Corp., Southbridge, MA, USA) for 2 h at a rate of 20 l/min. The charcoal (50 g) was Soxhlet extracted overnight with dichloromethane (250 ml) and concentrated by nitrogen blowdown to 10 ml. 1-Methoxycycloheptatriene was spiked into a portion of the extract at the 0.5 ng/μl level to evaluate the detection of an irritant in a complex environmental matrix, similar to what would be expected during battlefield sampling in the presence of diesel powered vehicles.

2.2. Instrumental

Capillary column GC–MS/MS analyses were performed with either a VG AUTOSPEC-Q or VG-70SQ hybrid tandem mass spectrometer, each of which was equipped with a Hewlett Packard Model 5890 gas chromatograph. Either a 15 m × 0.32 mm ID DB-1701 or DB-5 J&W capillary column (0.25 μm film thickness) was used for GC–MS/MS analyses with the following temperature program: 40°C (2 min hold), 10°C/min to 280°C (5 min hold). GC injections were cool on-column using an injector of our own design [2] with the AUTOSPEC-Q instrument and were splitless with the VG-70SQ instrument.

EI-MS operating conditions were similar for both mass spectrometers: source pressure, $3 \cdot 10^{-6}$ Torr (1 Torr = $1.3 \cdot 10^{2}$ Pa); source temperature, 200°C; electron energy, 70 eV; electron emission, 100 or 200 μA and accelerating voltage, 8 kV. CAD (collisional activated dissociation) cell conditions [CAD cell argon pressure of $8–9 \cdot 10^{-7}$ Torr and an energy of 25 eV (laboratory scale)], based on the best compromise between sensitivity and spectral content were used for all daughter analyses [20,21]. This argon pressure reduced the intensity of the perfluorokerosene ion at *m/z* 219 to 50% of its intensity with residual air in the CAD cell. A typical daughter spectra for *m/z* 219 at 30 eV (with no detectable signal below 12 eV) under this CAD cell condition follows:

Argon (50% reduction): *m/z* 219:*m/z* 131:*m/z* 69 = 1:0.25:0.15

Daughter spectra were obtained under these CAD cell conditions for the molecular and principal EI fragmentation ions for each of the four irritants during capillary column GC–MS/MS analysis with the AUTOSPEC-Q instrument. The quadrupole was operated at unit resolution and scanned from 250 to 50 u at 0.7 s/scan and, the sector resolution was set at 1000 (10% valley definition). Daughter spectra for all four irritants were obtained during each chromatographic analysis (DB-1701 column) by monitoring for 1-methoxycycloheptatriene from 1:00 to 6:00 min, 2-chloroacetophenone from 6:00 to 12:00 min, *o*-chlorobenzylidenemalononitrile from 12:00 to 14:00 min and dibenz[*b*,*f*]-1,4-oxazepin from 14:00 and 17:00 min.

Similar GC–MS/MS conditions were employed with the VG-70SQ during multiple reaction ion monitoring analyses of the diesel exhaust extract and 1-methoxycycloheptatriene spiked diesel exhaust extract. Both the *m/z* 122 to 107 and *m/z* 122 to 92 processes were alternately monitored for 80 ms (20 ms delay) to provide data from three characteristic 1-methoxycycloheptatriene ions, during chromatographic analyses.

A sector resolution of 10 000 (10% valley definition) was employed during high resolution GC–MS analyses with the AUTOSPEC-Q instrument.

3. Results and discussion

Fig. 1 illustrates typical capillary column GC–MS/MS chromatograms obtained during the

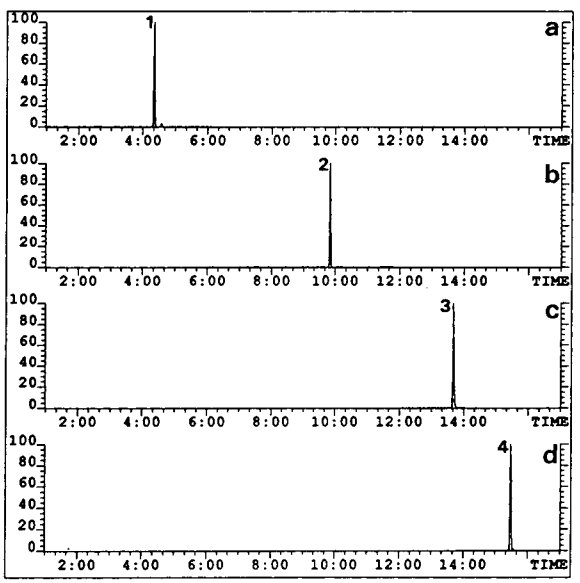

Fig. 1. Capillary column GC–MS/MS chromatograms for daughters of (a) *m/z* 122 [$M^{+\cdot}$ for 1-methoxycycloheptatriene, (1)], (b) *m/z* 154 [$M^{+\cdot}$ for 2-chloroacetophenone, (2)], (c) *m/z* 188 [$M^{+\cdot}$ for *o*-chlorobenzylidenemalononitrile, (3)] and (d) *m/z* 195 [$M^{+\cdot}$ for dibenz[*b*,*f*]-1,4-oxazepin, (4)]. [Time scale in minutes, DB-1701 column]

analysis of a 10-ng standard containing the four irritants, 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin with the DB-1701 column. Daughter spectra for the molecular ions obtained during this analysis are illustrated in Figs. 2a, 3a, 4a and 5a. The reproducibility of the daughter spectra obtained were found to be similar to that obtained for phosphate esters, with the standard deviation of the daughter ion relative intensities being approximately 30% [21].

Typical sensitivity of daughter detection was evaluated by monitoring the molecular ion (*m*/*z* 195, EI relative intensity: 100%) of the highest molecular mass irritant, dibenz[*b*,*f*]-1,4-oxazepin (Fig. 6). An interpretable daughter spectrum (inset in Fig. 6) was obtained for 100 pg of dibenz[*b*,*f*]-1,4-oxazepin ($S/N > 10{:}1$). This spectrum contains two characteristic ions, and while this may be satisfactory for the detection of this compound for some applications, it should be noted that confirmation under MS/MS conditions requires the acquisition of a minimum of three characteristic ions. To achieve this level of certainty, acquisition of an additional daughter

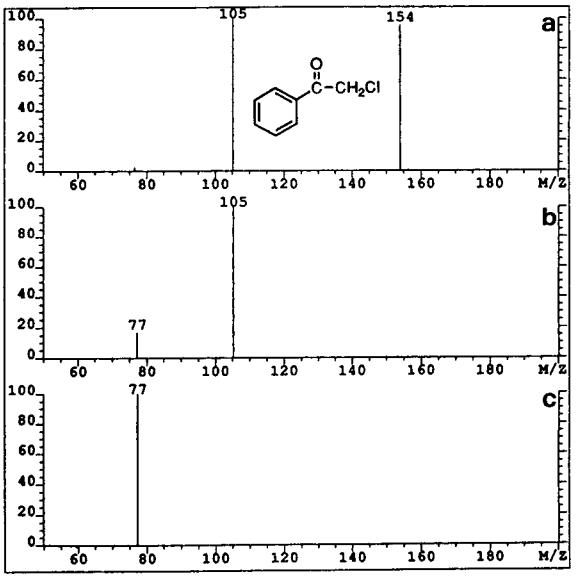

Fig. 3. Daughter spectra of (a) *m*/*z* 154, (b) *m*/*z* 105 and (c) *m*/*z* 77 for 2-chloroacetophenone.

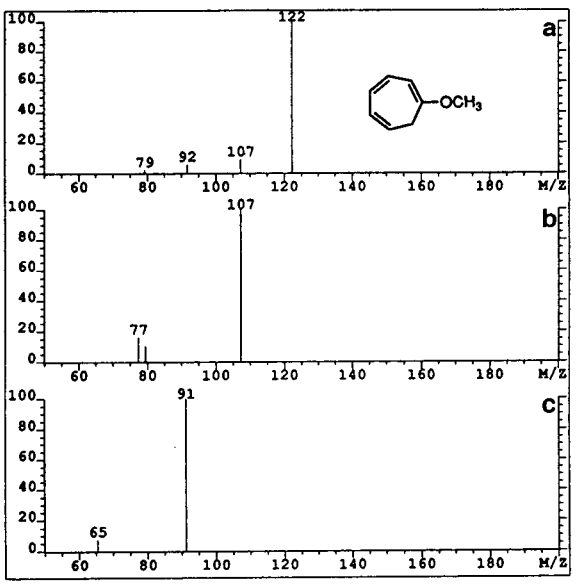

Fig. 2. Daughter spectra of (a) *m*/*z* 122, (b) *m*/*z* 107 and (c) *m*/*z* 91 for 1-methoxycycloheptatriene.

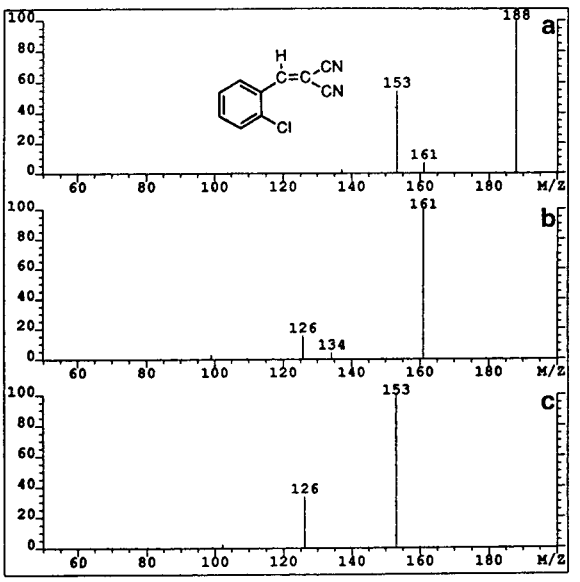

Fig. 4. Daughter spectra of (a) *m*/*z* 188, (b) *m*/*z* 161 and (c) *m*/*z* 153 for o-chlorobenzylidenemalononitrile.

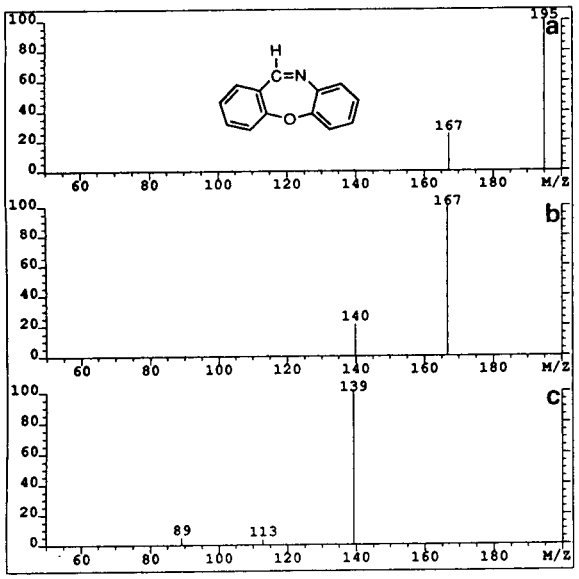

Fig. 5. Daughter spectra of (a) m/z 195, (b) m/z 167 and (c) m/z 139 for dibenz[b,f]-1,4-oxazepin.

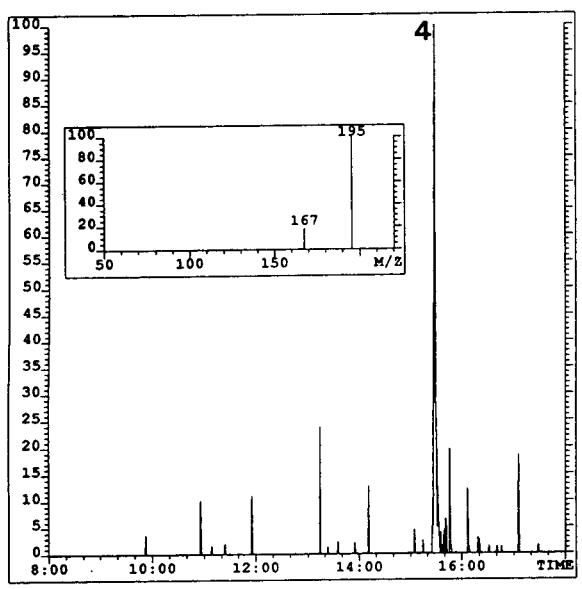

Fig. 6. Capillary column GC–MS/MS chromatogram for daughters of (a) m/z 195 ($M^{+\cdot}$ for 100 pg of dibenz[b,f]-1,4-oxazepin, [4]). Daughter spectrum obtained for 100 pg of dibenz[b,f]-1,4-oxazepin inset on Figure. [Time scale in minutes, DB-1701 column]

spectrum for another parent ion (e.g., m/z 167 or m/z 139) would be required.

Daughter sensitivity depends directly on the relative abundance of the parent ion, as these compounds have similar EI detection limits. As a result, sensitivities would be similar for most of the irritant ions monitored during daughter study, with the exception of the molecular ion of 2-chloroacetophenone due to its very low EI relative intensity (1.8%).

In general, an interpretable full scanning EI mass spectrum may be acquired for 100 pg to 500 pg of chemical warfare agent, depending on the compound, during capillary column GC–MS analysis. Similar sensitivity has been observed during daughter study and the following has been noted. If an interpretable full scanning EI mass spectrum can be acquired, then the analyst can expect to acquire an interpretable daughter spectra for the compound at the same level, provided the parent ion has an EI relative abundance above 25%.

Table 1 lists the EI ions monitored during the acquisition of daughter spectra, their relative intensity and the elemental composition of each ion, as confirmed during high-resolution GC–MS analysis. Figs. 2 to 5 illustrate typical daughter spectra for the molecular and principal EI fragmentation ions of 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[b,f]-1,4-oxazepin, respectively.

1-Methoxycycloheptatriene exhibits a molecular ion at m/z 122 and two higher mass fragmentation ions at m/z 107 and m/z 91 during EI-MS operation. The molecular ion at m/z 122 fragments in the CAD cell to form daughter ions at m/z 107, m/z 92, m/z 79 and m/z 77, due to $[M-CH_3]^+$ and $[M-CH_2O]^+$, $[M-CH_3-CO]^+$ and $[M-CH_3-CH_2O]^+$, respectively (Fig. 2a). The EI fragmentation ion at m/z 107, $[M-CH_3]^+$, produced daughters at m/z 79 and m/z 77 due to neutral loss CO and CH_2O, respectively (Fig. 2b). Only one daughter at m/z 65, due to neutral loss of C_2H_2 was observed in the daughter spectrum of m/z 91 (Fig. 2c).

The EI mass spectrum of 2-chloroacetophenone contains a weak molecular ion (refer to

Table 1
Ions monitored during GC–MS/MS study

Irritant[a]	EI mass measured[b]	EI mass calculated	Error (mmu)	% R.I.[c]	Composition
a) CH	122.0722	122.0732	−1.0	100	$C_8H_{10}O$
	107.0501	107.0497	+0.4	55	C_7H_7O
	91.0536	91.0548	−1.2	70	C_7H_7
b) CN	154.0198	154.0185	+1.3	1.8	C_8H_7OCl
	105.0324	105.0340	−1.6	100	C_7H_5O
	77.0343	77.0391	−4.8	54	C_6H_5
c) CS	188.0132	188.0141	−0.9	54	$C_{10}H_5N_2Cl$
	161.0033	161.0032	+0.1	17	C_9H_4NCl
	153.0457	153.0453	+0.4	100	$C_{10}H_5N_2$
d) CR	195.0709	195.0684	+2.5	100	$C_{13}H_9NO$
	167.0730	167.0735	−0.5	52	$C_{12}H_9N$
	139.0540	139.0548	−0.8	25	$C_{11}H_7$

[a] CH = 1-methoxycycloheptatriene; CN = 2-chloroacetophenone; CS = *o*-chlorobenzylidenemalononitrile; CR = dibenz[*b*,*f*]-1,4-oxazepin
[b] Obtained during GC–MS analysis at 10 000 resolution (10% valley definition).
[c] %Relative intensity.

Table 1) and two higher mass EI fragmentation ions at *m/z* 105 and *m/z* 77. Daughters at *m/z* 105 and *m/z* 77, due to $[M - CH_2Cl]^+$ and $[M - CH_2Cl - CO]^+$, respectively, were observed during GC–MS/MS analysis of the molecular ion at *m/z* 154 (Fig. 3a). The EI fragmentation ion at *m/z* 105 produced a single daughter ion at *m/z* 77 due to neutral loss of CO (Fig. 3b) and this ion did not produce any significant daughter ions (Fig. 3c).

o-Chlorobenzylidenemalononitrile, the most commonly used irritant, contains a molecular ion and two higher mass EI fragmentation ions at *m/z* 161 and *m/z* 153. Significant daughter ions due to loss of HCN and Cl, at *m/z* 161 and *m/z* 153, respectively, were observed in the daughter spectrum of the *o*-chlorobenzylidenemalononitrile molecular ion at *m/z* 188 (Fig. 4a). Daughter ions at *m/z* 134 and *m/z* 126, due to loss of HCN and Cl, respectively, were detected in the daughter spectrum of *m/z* 161, $[M - HCN]^{+\cdot}$ (Fig. 4b). An ion at *m/z* 126, due to loss of HCN, was the principal daughter ion in the spectrum acquired for $[M - Cl]^+$ at *m/z* 153 (Fig. 4c). Several minor daughter ions at *m/z* 137, *m/z* 126 and *m/z* 102, detected in the acquired daughter data for *o*-chlorobenzylidenemalononitrile, were likely due to C_7H_4NCl, C_9H_4N and C_4H_5NCl, respectively.

The molecular ion of dibenz[*b*,*f*]-1,4-oxazepin, at *m/z* 195, and two higher mass EI fragmentation ions (*m/z* 167 and *m/z* 139) were investigated during GC–MS/MS operation. An ion at *m/z* 167, due to loss of CO, was the principal daughter in the spectrum acquired for *m/z* 195 (Fig. 5a). The daughter spectrum of *m/z* 167, due to $[M - CO]^{+\cdot}$, exhibited a significant daughter ion at *m/z* 140 due to neutral loss of HCN (Fig. 5b). Two daughters at *m/z* 113 and *m/z* 89, likely due $[C_9H_5]^+$ and $[C_7H_5]^+$ were observed in the daughter spectrum of $[C_{11}H_7]^+$ at *m/z* 139 (Fig. 5c).

Fig. 7 illustrates the capillary column GC–MS chromatogram of the dichloromethane extract of the collected diesel exhaust sample. This complex extract, containing numerous hydrocarbons (refer to Table 2), was selected to evaluate the selectivity of GC–MS/MS, as it is typical of what one might collect during battlefield sampling in the presence diesel powered vehicles. 1-Methox-

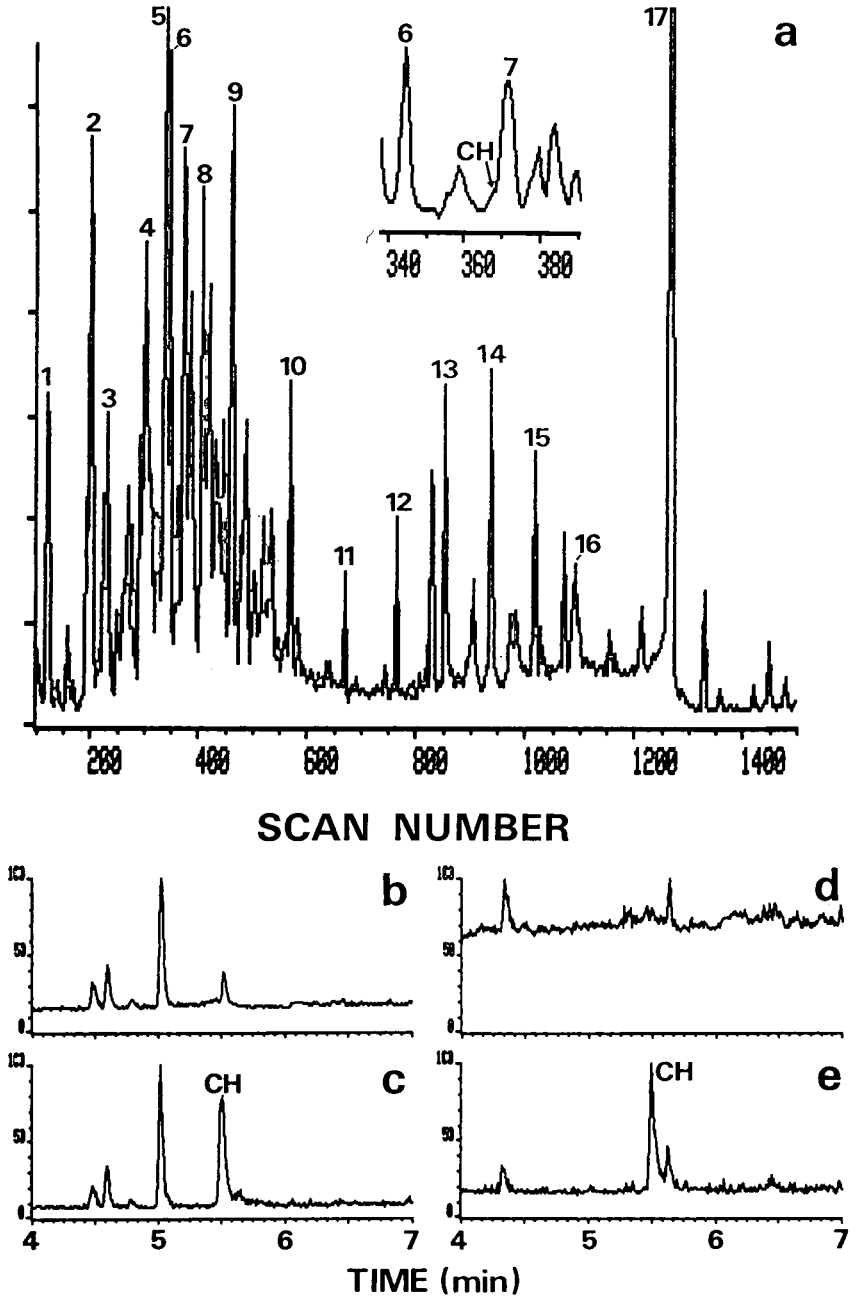

Fig. 7. (a) Capillary column GC–MS total ion-current chromatogram (300 to 40 u) of the diesel exhaust extract (Numbers of sample components are listed in Table 2). The expanded insert illustrates the expected retention time for 1-methoxycycloheptatriene (Each scan number represents about 0.6 s, DB-5 column). Reaction ion monitoring chromatograms for m/z 122 to 107 obtained during analysis of the (b) diesel exhaust extract and (c) the same diesel exhaust extract spiked at the 500 pg level with 1-methoxycycloheptatriene. Reaction ion monitoring chromatograms for m/z 122 to 92 obtained during analysis of the (d) diesel exhaust extract and (e) the same diesel exhaust extract spiked at the 500 pg level with 1-methoxycycloheptatriene.

Table 2
Major diesel exhaust extract components

Peak No.[a]	Compound
1	Methyl benzene
2	C_2-Substituted benzene
3	nC_9-Alkane
4,5,7	C_3-Substituted benzene
6	nC_{10}-Alkane
8	C_4-Substituted benzene
9	nC_{11}-Alkane
10	nC_{12}-Alkane
11	nC_{13}-Alkane
12	nC_{14}-Alkane
13	nC_{15}-Alkane
14	nC_{16}-Alkane
15	nC_{17}-Alkane
16	nC_{18}-Alkane
17	Sulfur (S_8)

[a] Refer to Fig. 7.

ycycloheptatriene, at the apex of the hydrocarbon envelope, was selected to illustrate the specificity of GC–MS/MS as it was completely masked by chemical interferences and could not be detected at the 5 ng spiked level during GC–MS analysis under EI conditions.

Multiple reaction ion monitoring of m/z 122 to 107 and m/z 122 to 92 processes resulted in the detection of 500 pg of 1-methoxycycloheptatriene with a $S/N > 10{:}1$ in the presence of numerous interfering hydrocarbons in the diesel exhaust extract (Fig. 7). A minor interference was observed for the m/z 122 to 107 process. This contribution was likely due to loss of CH_3 from a C_9H_{14} ion in the diesel exhaust extract. No interferences were detected for the m/z 122 to 92 process.

4. Conclusions

Daughter spectra were obtained for the molecular and principal electron impact fragmentation ions of four irritants, 1-methoxycycloheptatriene, 2-chloroacetophenone, *o*-chlorobenzylidenemalononitrile and dibenz[*b*,*f*]-1,4-oxazepin, during capillary column GC–MS/MS analysis. The use of standardized collisional activated dissociation cell conditions resulted in the acquisition of daughter spectra suitable for identification or data-base generation purposes. Daughter operation detection limits of 100 pg ($S/N > 10{:}1$), for the highest molecular mass irritant, dibenz[*b*,*f*]-1,4-oxazepin, were obtained. This level of sensitivity was approximately the same as that routinely obtained for other chemical warfare agents during capillary column GC–MS analysis under electron impact ionization conditions. The specificity of GC–MS/MS was demonstrated by detection of 1-methoxycycloheptatriene in the presence of numerous chemical interferences in the diesel exhaust extract.

Tandem mass spectrometry may be used for the acquisition of complementary daughter data for the identification of irritants and other chemical warfare agents. Use of MS/MS data as well as traditional MS data would increase the level of confidence in the identification of irritants in samples suspected to contain these compounds.

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Multi-residue matrix solid-phase dispersion method for the determination of six synthetic pyrethroids in vegetables followed by gas chromatography with electron capture detection

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Abstract

An effective multi-residue matrix solid-phase dispersion (MSPD) extraction and gas chromatographic–electron-capture detection method for the determination of six synthetic pyrethroids (fenpropathrin, cyhalothrin, permethrin, cypermethrin, fenvalerate and deltamethrin) in 5 g of vegetables (West Indian gherkin, eggplant, pak-choi, cabbage and garden peas) is described. The method uses a Florisil-based MPSD column for direct in-line clean-up with *n*-hexane–acetone (9:1). Recoveries calculated at 0.1 and 0.5 μg/g fortification levels were between 92 and 113%. The method detection limits were between 5.1 and 91.5 ng/g. The method compared favourably with the traditional method in terms of the sample size, analysis time and overall cost. The method may serve as a screening protocol for the determination of pyrethroids in vegetables.

1. Introduction

Synthetic pyrethroids are increasingly being used for insect control on field crops because of their advantageous environmental properties such as short field life and relatively low mammalian toxicity [1,2]. Nevertheless, pyrethroid residues in vegetables after application to the crops still pose risks to human health and other species. Therefore, monitoring of pyrethroid residue levels in vegetables is of particular concern for human health.

Current pyrethroid residue screening methods usually require extraction with a polar solvent, followed by liquid–liquid partitioning. The sample is concentrated by evaporation, often with a solvent exchange, and subjected to clean-up before the final determination. Extraction solvents such as acetonitrile [3,4], acetone [5,6], *n*-hexane–acetone (9:1) [7,8], acetone–methanol [9], cyclohexane–chloroform (4:1) [10] and ethanol [11] have been used for pyrethroid analysis. The clean-up is generally based on column chromatography using sorbents such as Florisil [3,6,11], a mixture of active carbon, magnesia and diatomaceous earth [5], silica gel [8] and

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active acidic alumina [7,9]. Final determinations are carried out using gas chromatography with electron-capture detection (GC–ECD) [3,5, 9,11,12], liquid chromatography [8,12], thin-layer chromatography [7], differential-pulse polarography [10] and gas chromatography–mass spectrometry (GC–MS) [4,6].

The general drawbacks, such as the use of large amounts of solvent, the occurrence of troublesome emulsions with certain vegetables and slowness associated with liquid–liquid extraction [3,5–9] are avoided by using solid-phase extraction (SPE) cartridges [4,10]. SPE is a simple and rapid technique in comparison with liquid–liquid extraction and offers the advantages of a shorter analysis time, the consumption of smaller volumes of organic solvents and lower cost [13–15]. However, large amounts of solvent are still consumed during the extraction step. Appropriate treatment and activation of the sorbents are also mandatory.

The aim of this work was to develop an SPE-like method to overcome the above shortcomings. The method described in this paper is based on a recently developed multi-residue extraction technique called matrix solid-phase dispersion (MSPD) [16–19] to separate simultaneously six synthetic pyrethroids (fenpropathrin, cyhalothrin, permethrin, cypermethrin, fenvalerate and deltamethrin) in the vegetables West Indian gherkin, eggplant, pak-choi, cabbage and garden peas. These pyrethroids are among the most commonly used pyrethroids for insect control on vegetable fields in Taiwan. MSPD extracts trace organic compounds from homogeneously dispersed solid matrices by adsorbing them on suitable solid adsorbents, followed by desorption with a small amount of organic solvent. In-line clean-up or further purification is feasible by the simultaneous use of a co-column packed with a clean-up sorbent. Existing MSPD methods were mostly developed for biological matrices of tissues [17,18]. To our knowledge, this work represents the first attempt to extend the application fields of MSPD to vegetable samples. The method was applied to some vegetable samples obtained from a local market.

2. Experimental

2.1. Materials

All solvents used were of HPLC grade from Tedia (Fairfield, OH, USA). Standard pyrethroids with purity >99% were obtained from Dr. S. Ehrenstorfer (Augsburg, Germany). Standard calibration mixtures containing 100 μl of 3.9 ppm 2,4,6-tribromobiphenyl (from UltraScientific, North Kingstown, RI, USA) as an internal standard were prepared by diluting a stock standard solution in acetone. C_{18} (40 μm, Superclean LC-18) and Florisil (60–100 mesh) were obtained from Supelco (Bellefonte, PA, USA). Neutral alumina (70–230 mesh, activity I) was obtained from Merck (Dermstadt, Germany). Vegetable samples (West Indian gherkin, eggplant, pak-choi, cabbage and garden peas) used for blank, fortified and real studies were obtained from a local market. The C_{18} material was prewashed by continuously refluxing it in *n*-hexane–acetone (1:1) for 24 h in a Soxhlet apparatus followed by oven drying at 150°C for 1 h. Florisil and neutral alumina were activated by oven drying at 150°C for 12 h before use.

2.2. Preparation of sample extracts

The percentage of moisture in the vegetables was determined by placing 50 g of homogeneously mixed vegetable sample in an ignited and weighed beaker, drying at 110°C for 24 h and weighing the cooled beaker. The moisture content was calculated using moisture (%) = (g of as-received sample − g of oven-dried sample) /(g of as-received sample) · 100.

A 100-g amount of vegetable sample was chopped in a food chopper and 5 g of chopped and homogeneously mixed sample were weighed into a glass mortar. For the preparation of fortified samples, 50 μl of 50 (or 100) ppm stock standard solution were added to different portions of the sample to produce sample equivalents of 0.1 (or 0.5) μg/g, respectively. An 8-g portion of Florisil was then added. The Florisil–vegetable mixture was gently blended in the

mortar for ca. 2 min using a glass pestle with a clockwise circular motion to yield a semi-dry, homogeneous-appearing material. The homogeneous mixture was placed in a glass column (30 cm × 15 mm I.D.) containing a Pyrex frit, 1 cm of anhydrous sodium sulfate and 0.5 g of Florisil packed at the bottom. The additional 0.5 g of Florisil was used to trap possible co-eluting interfering compounds. The column was lightly tapped to remove air pockets, then tightly compressed to ca. 13 cm using a syringe plunger. Finally, a 0.1–0.2-cm layer of anhydrous sodium sulfate was placed at the top of the column.

A 500-ml round-bottomed flask was positioned below each column to collect the eluate. The column was eluted with 100 ml of eluent by gravitational flow. Eluting efficiencies were studied using ethyl acetate, *n*-hexane–acetone (4:1) and *n*-hexane–acetone (9:1) as eluents. The flow usually ceased after 30 min. The eluate was then transferred into a concentration tube, concentrated using a rotary vacuum evaporator and finally purged with nitrogen to a volume of less than 1.0 ml. A 100-μl volume of 3.9 ppm tribromobiphenyl was added as an internal standard. For the analysis of real samples, the optimum combination of a Florisil co-column and 60 ml of *n*-hexane–acetone (9:1) eluent was used. The final volume of the eluate was adjusted to 1.0 ml and subjected to GC–ECD analysis. Pyrethroid residues detected in real samples were confirmed by GC–MS with selected ion monitoring (SIM).

2.3. Apparatus

GC–ECD analyses were carried out using an HP-5890 Series II gas chromatograph equipped with a J & W DB-5MS capillary column (30 m × 0.25 mm I.D., 0.25-μm film thickness) and a ^{63}Ni electron-capture detector. Samples were introduced into the GC column via an on-column injector system. The injector temperature was programmed similarly to the column, except that it was maintained 3°C higher. The detector was operated at 320°C. Helium and nitrogen were used as the carrier and make-up gas at flow-rates of 1 and 70 ml/min, respectively. The column temperature was initially held at 60°C, then programmed at 30°C/min to 220°C and at 3°C/min to 300°C.

GC–MS analyses were carried out using a Shimadzu QP-1000 EX mass spectrometer equipped with a Shimadzu GC-14A gas chromatograph. The injector was operated in the splitless mode at 250°C. The effluent from the GC column was transferred via a transfer line held at 250°C and fed into a 70 eV electron impact ionization source held at 250°C. Helium was used as the carrier gas at a flow-rate of 1 ml/min. The column type and column temperature used were the same as those used in GC–ECD.

3. Results and discussion

3.1. Sample pretreatment and clean-up

The MSPD method used in this study differs from traditional methods in that the sample is dispersed over a large surface area on Florisil by the mechanical shearing force of blending. Polar materials such as chlorophylls, triglycerides and phytosterols, which are the common components in vegetables [20], are associated with the surface of the Florisil. This is ascribed to the lipid-absorbing capacity of the activated magnesium silicate solid support that might mimic the lipid-solubilizing character of the octadecylsilane liquid phase used in MSPD. The hydrophobic pyrethroids remain weakly bound on the adsorbent surface. These combined effects assist the unfolding and dispersion of the sample matrix. Subsequent elution using a solvent of high pyrethroid solubility could effectively extract the weakly bound pyrethroids from the adsorbent. However, certain co-extracted compounds might interfere. These interferences were minimized in this study via the use of an appropriate solvent system and clean-up adsorbent. Because the analytes and the matrices involved in this study are different from those in the previous studies [16–19], the need for a new

dispersed adsorbent, eluting solvent and clean-up adsorbent was expected and found to be necessary for the best recoveries of the determined pyrethroids.

The moisture content in vegetables is usually higher than that in biological tissues. To facilitate the preparation of a semi-dry and homogeneously appearing sample, the approximate moisture content in the vegetable is preferably known in advance. The moisture contents were found to be 96% in West Indian gherkin, 94% in eggplant, pak-choi and cabbage and 91% in

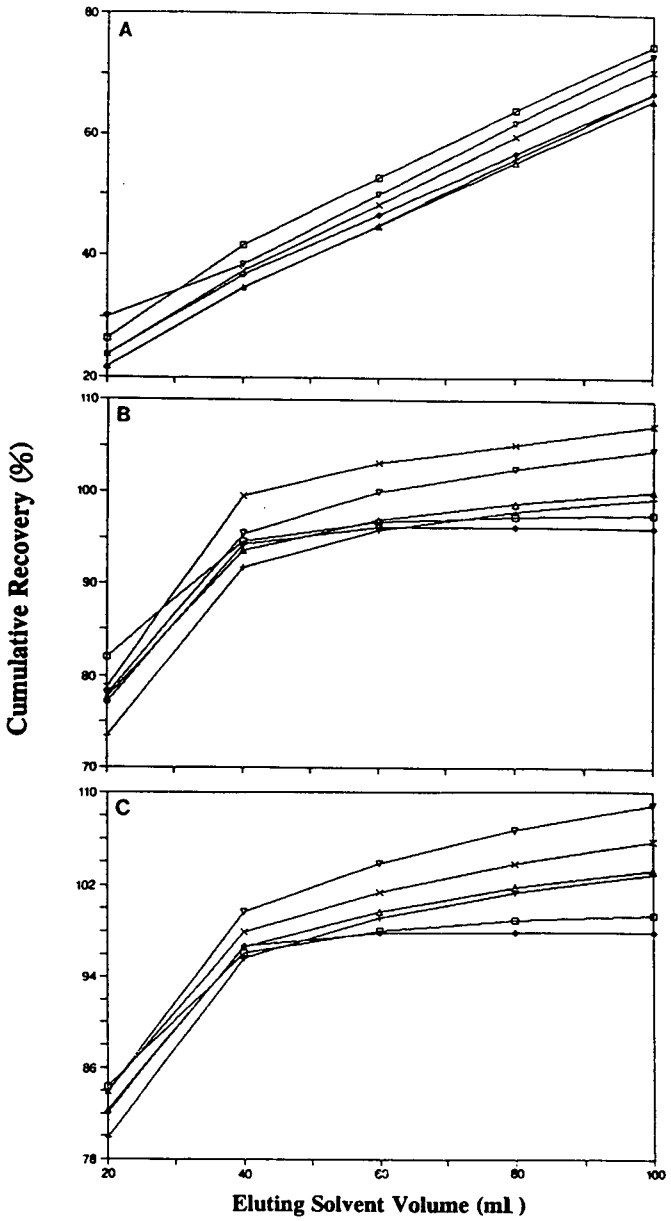

Fig. 1. Cumulative recoveries of the six pyrethroids in West Indian gherkin fortified at 2 μg/g as a function of eluent volume. Eluents: (A) ethyl acetate, (B) *n*-hexane–acetone (4:1) and (C) *n*-hexane–acetone (9:1). ◇ = Permethrin; △ = cypermethrin; × = fenvalerate; □ = fenpropathrin; + = cyhalothrin; ▽ = deltamethrin.

Table 1
Percentage recoveries and method detection limits for the six pyrethroids in fortified West Indian gherkin, eggplant, pak-choi, cabbage and garden peas

Sample	Concentration (μg/g)	Fenpropathrin	Cyhalothrin	Permethrin	Cypermethrin	Fenvalerate	Deltamethrin
West Indian gherkin	0.1[a]	106 ± 4	102 ± 5	95 ± 5	113 ± 5	101 ± 4	104 ± 5
	0.5[b]	102 ± 2	96 ± 3	99 ± 2	104 ± 2	98 ± 2	99 ± 2
	MDL[c](ng/g)	11.3	14.3	14.8	14.2	12.1	15.2
Eggplant	0.1[a]	104 ± 2	100 ± 2	96 ± 4	100 ± 2	98 ± 2	102 ± 6
	0.5[b]	102 ± 4	97 ± 3	100 ± 4	100 ± 3	96 ± 3	102 ± 3
	MDL[c](ng/g)	5.1	6.5	12.1	7.6	6.0	18.3
Pak-choi	0.1[a]	101 ± 3	102 ± 3	101 ± 6	96 ± 2	97 ± 2	100 ± 3
	0.5[b]	96 ± 2	98 ± 2	101 ± 1	94 ± 2	96 ± 2	94 ± 2
	MDL[c](ng/g)	10.2	8.4	17.4	6.6	7.3	9.2
Cabbage	0.1[a]	104 ± 5	101 ± 2	95 ± 6	94 ± 3	97 ± 2	137 ± 6
	0.5[b]	97 ± 2	97 ± 2	100 ± 2	93 ± 2	95 ± 2	99 ± 4
	MDL[c](ng/g)	16.3	7.6	17.7	9.9	7.0	89.2*
Garden peas	0.1[a]	100 ± 2	98 ± 3	96 ± 3	213 ± 16	97 ± 3	102 ± 3
	0.5[b]	95 ± 3	92 ± 4	95 ± 2	118 ± 4	95 ± 3	98 ± 2
	MDL[c](ng/g)	6.1	8.8	9.9	91.5*	9.3	10.9

[a] $n = 7$.
[b] $n = 4$.
[c] Values without asterisks, 3.14 times the standard deviation of seven replicate analyses of vegetable fortified at the 0.1 μg/g level. Values with asterisks, 4.52 times the standard deviation of four replicate analyses of vegetable fortified at the 0.5 μg/g level.

garden peas. Using 5 g of West Indian gherkin, with the highest moisture content, the amount of dispersed adsorbent needed to prepare a semi-dry sample was 30 g of anhydrous sodium sulfate, 20 g of C_{18}, 8 g of Florisil or 12 g of neutral alumina. In the initial study, it was observed that most of the chlorophylls could be retained on Florisil or neutral alumina, but not on anhydrous sodium sulfate or C_{18}. Because the cost of the Florisil used per run was less than that of neutral alumina, we selected Florisil as the dispersed adsorbent. To ensure the complete removal of the chlorophyll, an additional 0.5 g of Florisil was packed below the Florisil-blended sample in the MSPD column. No visible colour was observed in the eluate. The interfering compounds appeared to be trapped by the Florisil as a green colour developed in the underlying Florisil during elution.

The effect of eluting solvent and its volume was studied by collecting each pyrethroid in every 20-ml fraction during elution using a sample fortified at 2 μg/g. The cumulative recoveries on eluting with 100 ml of ethyl acetate, *n*-hexane–acetone (4:1) and *n*-hexane–acetone (9:1) are shown in Fig. 1. The best recoveries were obtained with *n*-hexane–acetone (4:1) (Fig. 1B) and *n*-hexane–acetone (9:1) (Fig. 1C). The recovery for each pyrethroid increased rapidly to 92% after elution with 40 ml of eluent. The recovery then increased slowly with increasing eluent volume and reached an equilibrium value with 60 ml of eluent. The eluent volume used for subsequent studies was therefore set at 60 ml. Considering the potential of eluting the retained polar chlorophyll and other polar interfering compounds with the more polar *n*-hexane–acetone (4:1), we selected *n*-hexane–acetone (9:1) as the eluent for subsequent studies.

3.2. Quantification

The fortification levels used in this study were selected because they covered the ranges of tolerance levels (0.1–0.5 μg/g) set by the ROC Department of Health [21]. The recoveries and detection limits using pyrethroid-fortified vegetables at levels of 0.1 and 0.5 μg/g are summa-

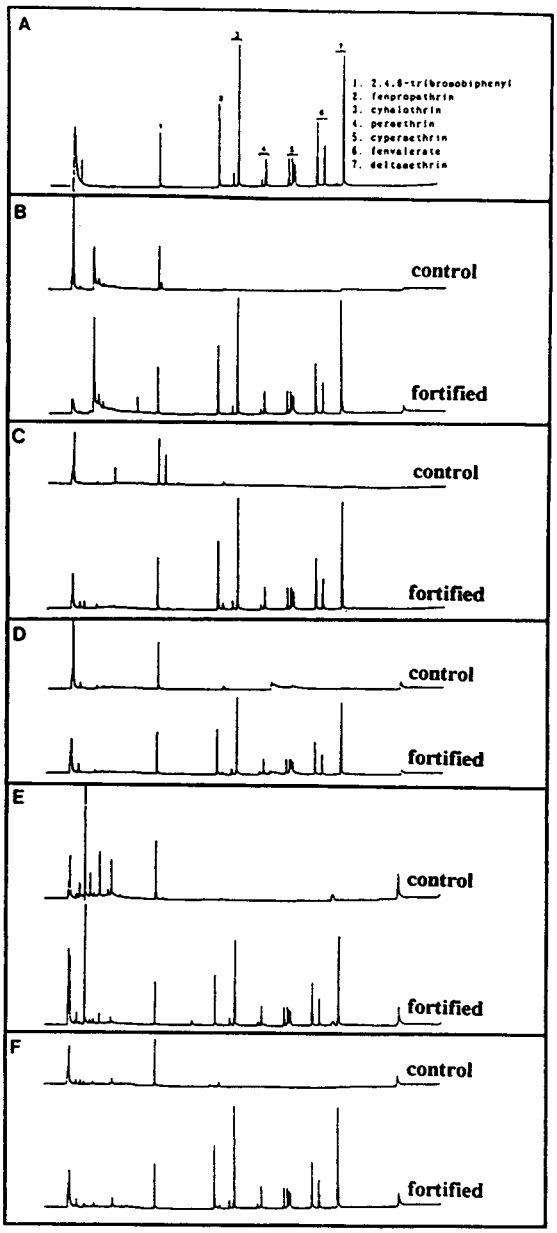

Fig. 2. Representative gas chromatograms from (A) the standard and (B–F) control and fortified vegetable samples of (B) West Indian gherkin, (C) eggplant, (D) garden peas, (E) cabbage and (F) pak-choi.

rized in Table 1. The recoveries were between 92 and 113%. The reproducibility expressed as standard deviation was between 1 and 6%. Representative GC chromatograms from the standard, control and fortified samples of each vegetable are shown in Fig. 2. No attempt has been made to assign isomer identities to the peaks. Several small interfering peaks appeared in the chromatogram of West Indian gherkin, eggplant and pak-choi (Fig. 2B, C and F); these peaks were located outside the retention time window of the analytes and did not jeopardize subsequent quantification work. In the chromatogram of cabbage (Fig. 2E), small interfering peaks appeared in the retention time window of deltamethrin. This might explain the unusually high recovery (137%) of deltamethrin in cabbage sample fortified at the 0.1 μg/g level. This interference became insignificant in samples fortified at the 0.5 μg/g level as the recovery decreased to 99%. The interfering peaks located in the retention time window of cypermethrin in the chromatogram of garden peas (Fig. 2D) were more visible. This might explain the unusually high recoveries (213 and 118%) of cypermethrin sample fortified at both the 0.1 and 0.5 μg/g level. Therefore, the method detection limits (MDLs) for deltamethrin in cabbage and cypermethrin in garden peas were calculated at the 0.5 μg/g level. Other MDLs shown in Table 1 were calculated at the 0.1 μg/g level. The MDLs thus obtained were between 5.1 and 91.5 ng/g and were below the tolerance levels (0.1–0.5 μg/g) set by the ROC Department of Health [21]. The

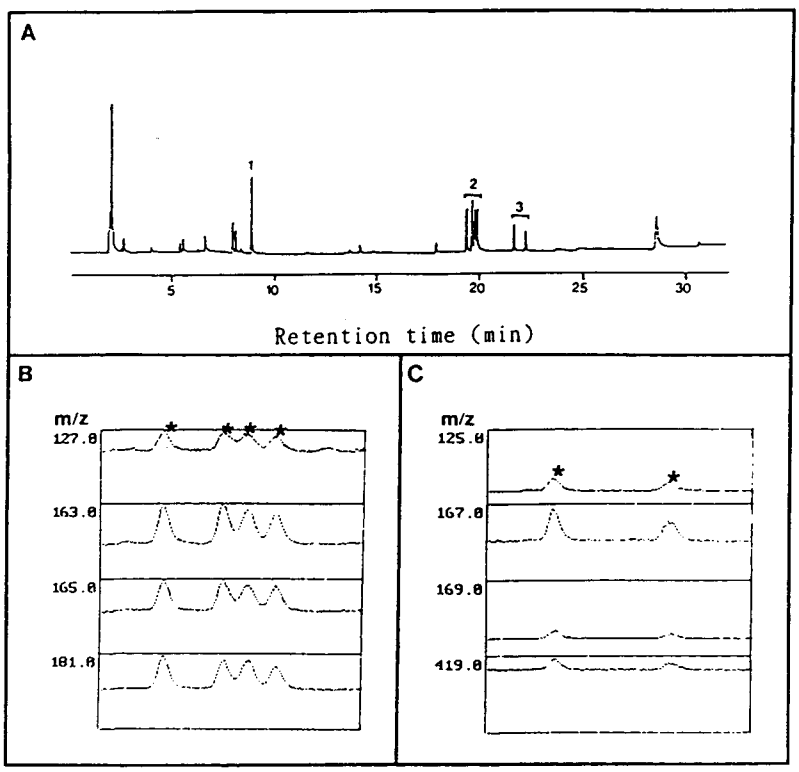

Fig. 3. (A) Gas chromatogram (peaks: 1 = 2,4,6-tribromobiphenyl; 2 = cypermethrin; 3 = fenvalerate) of a cabbage sample with an identifiable level of cypermethrin and fenvalerate, and corresponding mass chromatograms obtained from GC–MS-SIM analysis for (B) cypermethrin and (C) fenvalerate.

results indicate that the proposed MSPD method yields satisfactory extraction and determination of pyrethroids in vegetable samples.

The proposed MSPD method was applied to the determination of pyrethroids in vegetables obtained from a local market. Among the ten vegetable samples, pyrethroid residues were detected in one cabbage sample and confirmed by GC–MS-SIM (Fig. 3). They were 0.58 μg/g of cypermethrin and 0.18 μg/g of fenvalerate, respectively. The characteristic masses [m/z with relative abundance (%) in parentheses] used were 163 (100), 181 (88), 165 (67), and 127 (32) for cypermethrin and 125 (100), 167 (86), 169 (45), and 419 (28) for fenvalerate.

4. Conclusions

The proposed MSPD method can be readily applied to the extraction of six synthetic pyrethroids (fenpropathrin, cyhalothrin, permethrin, cypermethrin, fenvalerate and deltamethrin) in 5 g of vegetables (West Indian gherkin, egg plant, pak-choi, cabbage, and garden peas). The method uses a Florisil-based MSPD column for direct in-line clean up with *n*-hexane–acetone (9:1). The procedures are simple and rapid and require only small amount of samples and solvents. The method may serve as a screening protocol for the determination of pyrethroids in vegetables.

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Capillary electrophoresis of carboxylated carbohydrates I. Selective precolumn derivatization of gangliosides with UV absorbing and fluorescent tags☆

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Abstract

We demonstrate that the precolumn derivatization reaction, recently introduced by our laboratory for the selective labeling of carboxylated monosaccharides, can be readily transposed to other glycoconjugates containing carboxylated sugar residues, namely sialogangliosides. The selective derivatization reaction described here involved the attachment of sulfanilic acid (a UV-absorbing tag) or 7-aminonaphthalene-1,3-disulfonic acid (a UV-absorbing and also fluorescing tag) to the sialic acid moiety of the gangliosides via the carboxylic group in the presence of water-soluble carbodiimide. This labeling of the sialic acid moiety of the gangliosides with a chromophore and/or fluorophore leads to the formation of an amide bond between the carboxylic group of the sugar residue and the amino group of the derivatizing agent, thus replacing the weak carboxylic acid group of the carbohydrate species by the stronger sulfonic acid group which is ionized over the entire pH range. Furthermore, novel electrolyte systems were introduced and evaluated for the separation of the derivatized and underivatized gangliosides. The addition of acetonitrile or α-cyclodextrin (α-CD) to the running electrolyte was necessary to break-up the aggregation of amphiphilic gangliosides and allowed for their efficient separation as monomers in aqueous media using capillary electrophoresis. Several operating parameters were investigated with these electrolyte systems including the additive concentration as well as the ionic strength, pH and nature of the running electrolyte. Acetonitrile at 50% (v/v) in 5 m*M* sodium phosphate at high and low pH or 15 m*M* α-CD in 100 m*M* sodium borate, pH 10.0, proved ideal, in terms of resolution and separation efficiency, for the group separation of mono-, di- and trisialogangliosides. On the other hand, the complete resolution of disialoganglioside isomers (e.g., G_{D1a} and G_{D1b}) necessitated the superimposition of a chromatographic component on the electrophoretic process. This was achieved by adding either a hydrophobic (e.g., decanoyl-N-methylglucamide–borate surfactant complex) or hydrophilic [e.g., poly(vinyl alcohol) or hydroxypropyl cellulose] selectors to the running electrolyte.

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1. Introduction

Gangliosides are sialic acid-containing glycosphingolipids found in nearly all vertebrate tissues [1,2]. They reside in the plasma membrane

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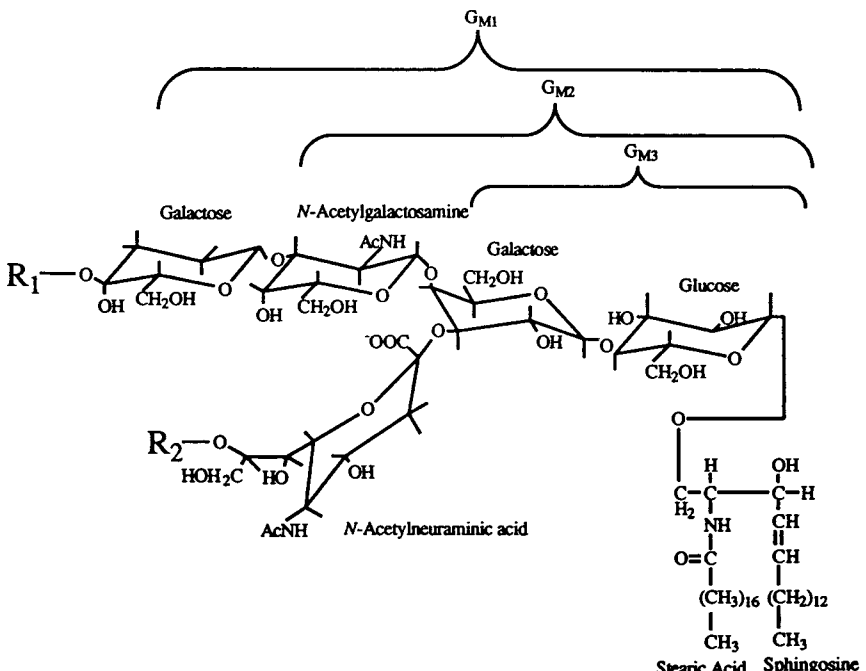
and can act as receptor binding sites on the cell surface. A ganglioside molecule has a hydrophilic sialooligosaccharide chain and a hydrophobic moiety, i.e. ceramide, that consists of a sphingosine and fatty acid [3] (see Fig. 1). The sialic acid residues, which determines the negative charge of the ganglioside molecule, seems to be involved in regulating the physiological capabilities of gangliosides such as reactivity with toxins, viruses, antibodies and growth factors [4–6].

Growing interest in the numerous and important biological functions of gangliosides has engendered the need for capable separation techniques for their isolation and determination. Chromatography in its various forms has been a widely used technique in the isolation of gangliosides [3,7–10]. As with most of other carbohydrate species, one of the major difficulties encountered in the analysis of gangliosides is the lack of chromophores in their molecules. This inherent property hinders their determination at low levels since their UV detection at wavelengths lower than 200 nm is met with relatively low sensitivity. The low detectability of underivatized gangliosides is also problematic because most gangliosides are often only available in minute amounts. Thus, besides the need for sensitive detection methodologies there is also a strong demand for microcolumn separation methods of high resolving power and small sample requirements. In this regard, high-performance capillary electrophoresis (HPCE) is the alternative technique for the analytical separation and determination of gangliosides. In fact, HPCE has already been briefly explored in the separation of underivatized gangliosides using low-wavelength UV detection [11,12].

To overcome the detectability problem, several precolumn derivatization reactions have been



G_{M1}, $R_1 = R_2 = H$
G_{M2}, $R_2 = H$
G_{M3}, $R_2 = H$
G_{D1a}, R_1 = N-Acetylneuraminic acid (NeuNAc), $R_2 = H$
G_{D1b}, $R_1 = H$, R_2 = NeuNAc
$G_{D3} = G_{M3}$, R_2 = NeuNAc
G_{T1b}, $R_1 = R_2$ = NeuNAc

Fig. 1. Structures of the gangliosides.

already reported for the tagging of gangliosides, and in particular prior to separation by high-performance liquid chromatography (HPLC). UV-absorbing perbenzoyl [13,14] and *p*-nitrobenzyloxyamine derivatives [15] are typical examples. Although the sensitivity of these procedures was sufficiently high, the reactions were not specific for gangliosides and moreover, prewashing to remove reagents and by-products was necessary prior to separation. Nakalayashi et al. [10] introduced a specific precolumn derivatization of gangliosides based on the formation of an ester bond between *p*-bromophenancyl bromide reagent and the carboxylic group of the sialic acid moieties, and demonstrated its suitability for the analysis of the derivatives by HPLC. Although convenient to regenerate the original molecule, the susceptibility of the ester bond to hydrolysis makes this specific derivatization [10] rather unsuitable for the production of stable derivatives.

Similar to HPLC, with HPCE it is preferable that precolumn derivatization be selective. In addition, the tag should be charged or if it is neutral, its attachment to the carbohydrate molecule should not eliminate the already existing charge of the solute molecule so that separation in an electric field would be possible. Very recently, we have shown that these two requirements are readily met by exploiting the reactivity of the carboxylic group of acidic monosaccharides [16]. Since gangliosides are sialic acid-containing glycolipids, the carboxylic groups of the sialic acid residues can also be readily tagged as in the case of carboxylated monosaccharides. The tagging procedure [16] involves the formation of a stable amide bond between the carboxylic group of the sugar analyte and the amino group of the derivatizing agent, e.g. 7-aminonaphthalene-1,3-disulfonic acid or sulfanilic acid, in the presence of water-soluble carbodiimide. Besides providing the chromophore or fluorophore, the derivatization reaction utilized here replaces the weak carboxylic acid group of the gangliosides by the strong sulfonic acid group, thus ensuring permanent negative charges on the derivatives over the entire pH range.

This study is a logical continuation to our recent contribution to the area of separation of minute amounts of acidic carbohydrates by HPCE. Herein we report (i) the evaluation of the new derivatization procedure in the selective tagging of carboxylated glycoconjugates, e.g. gangliosides, and (ii) the introduction of novel electrolyte systems for the separation of gangliosides in capillary electrophoresis.

2. Experimental

2.1. Instruments and capillaries

The instrument for capillary electrophoresis was assembled in the laboratory from commercially available components [16–18]. It comprised two high-voltage power supplies of positive and negative polarity, Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a UV–Vis variable-wavelength detector Model 200, equipped with a cell for on-column capillary detection from Linear Instruments (Reno, NV, USA). The detection wavelength was set at 247 nm for UV detection. Fluorescence detection of the derivatized gangliosides utilized a spectrofluorescence detector Model FL-750 BX from McPherson (Acton, MA, USA) with variable excitation wavelength. The excitation wavelength was set at 315 nm for fluorescence detection and 400 nm sharp cut-off long-wavelength absorption filter was used for collecting the emission wavelength. Electropherograms were recorded with a Shimadzu computing integrator Model CR5A (Columbia, MD, USA).

The absorption spectra of the derivatizing agents were performed on a UV–Vis spectrophotometer Model Lambda Array 3840 from Perkin-Elmer (Norwalk, CT, USA) by scanning from 190 to 350 nm.

Fused-silica capillary columns of 50 μm I.D. × 365 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). All capillaries used in this study were uncoated since the analytes are negatively charged and would repulse from the like-charged capillary surface. The derivatized gangliosides were introduced

into the capillary as thin plugs by means of hydrodynamic injection for approximately 5 s at 15 cm differential height between inlet and outlet ends of the capillary.

2.2. Reagents and materials

Gangliosides G_{M1}, G_{D1a}, and G_{D3} were extracted from bovine brain as described below and elsewhere [19]. Ganglioside type III mixture (containing approx. 20% N-acetylneuraminic acid) and standard monosialoganglioside (G_{M1}) from bovine brain, as well as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma (St. Louis, MO, USA). Standard disialogangliosides (G_{D1a}, G_{D1b}, and G_{D3}) and trisialoganglioside (G_{T1b}) were obtained from Sigma and from Matreya (Pleasant Gap, PA, USA). Neuraminidase from *Clostridium perfringens* and MEGA 10 (decanoyl-N-methylglucamide) surfactant were purchased from Calbiochem (LaJolla, CA, USA). The derivatizing agents 7-aminonaphthalene-1,3-disulfonic acid (ANDSA) and sulfanilic acid (SA) were purchased from TCI America (Portland, OR, USA). α-Cyclodextrin (α-CD) was donated by the American Maize-Products Co. (Hammond, IN, USA). Reagent-grade sodium phosphate monobasic, hydrochloric acid, sodium hydroxide, boric acid granular, and HPLC-grade acetonitrile (ACN) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Poly(vinyl alcohol) (PVA), 87–89% hydrolyzed (M_r 124 000–186 000) and hydroxypropyl cellulose (HPC) of average M_r 100 000 were purchased from Aldrich (Milwaukee, WI, USA). A reversed-phase HPLC column, C_{18}-silica Microsorb-MW, with 5 μm mean particle diameter and 100 Å mean pore diameter was purchased from Rainin (Woburn, MA, USA). This column was used in purifying the side products of the derivatization reactions. Deionized water was used to prepare the running electrolytes, buffers and the sample solutions. The running electrolytes were filtered with 0.2-μm Whatman syringeless filters obtained from Baxter Diagnostics (McGaw Park, IL, USA) to avoid column plugging and minimize baseline noise.

2.3. Extraction of gangliosides

Approximately 500 g of bovine brain tissues were extracted. Tissues were homogenized and extracted twice with 1500 ml of chloroform–methanol (2:1, v/v) and once with isopropanol–hexanes–water (55:25:20, v/v/v). Following Folch's partition [20] the upper layer glycolipids were subjected to DEAE-Sephadex column chromatography to separate neutral and acidic glycolipids (sulfatide and gangliosides) [21]. The acidic glycolipids were eluted in three fractions with increasing salt concentration. The monosialogangliosides were eluted with 0.05 *M* NH_4OAc in MeOH, sulfatide and the disialogangliosides with 0.15 *M* NH_4OAc in MeOH and polysialogangliosides with 0.45 *M* NH_4OAc in MeOH. The lower-phase glycolipids were acetylated and subjected to column chromatography with Florisil [22]. Further purification of the gangliosides was performed by HPLC with a Varian Model 9012 HPLC system using a column (100 × 1 cm) of Iatrobeads (6RS-8010, Iatron, Tokyo, Japan) and eluted at 2.0 ml/min with a 200-min gradient of isopropanol–hexanes–water [19,23] from 55:44:1 (v/v/v) to 55:20:25 (v/v/v). Further purification of individual glycolipids was accomplished by preparative high-performance thin-layer chromatography (HPTLC) with the use of chloroform–methanol–water (55:40:10, v/v/v containing 0.02% $CaCl_2$). Separated glycolipids were visualized with UV light after being sprayed with 0.1% primuline in 80% aqueous acetone. Once visualized, individual glycolipids were recovered from HPTLC plates by scraping followed by sonication in isopropanol–hexanes–water (55:25:20, v/v/v).

2.4. Derivatization of gangliosides

Gangliosides were tagged with ANDSA or SA as previously described [16]. Briefly, an aliquot of 50 μl of 100 m*M* aqueous solution of EDAC, pH 5.0, was initially added to sub-microgram amounts of solid gangliosides and the mixture was stirred for 1 h. Then, 50.0 μl of 100 m*M* aqueous solution of the derivatizing agent were added and the mixture was stirred for an addi-

tional 1.5 h. Subsequently, the entire reaction mixture containing the derivatized gangliosides, excess derivatizing agent and other components of the reaction mixture was analyzed by capillary zone electrophoresis (CZE).

2.5. Neuraminidase treatment

An aliquot of 20.0 μl of the SA derivative of G_{T1b} aqueous solution was added to 7.0 μl of neuraminidase (i.e., 0.028 units), dissolved in 50.0 μM sodium acetate buffer, pH 5.0, and the mixture incubated at 37°C for 72 h [24–26]. Aliquots of the reaction mixture were taken at various time intervals and analyzed by CZE.

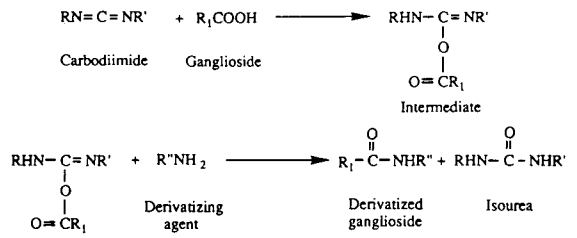
3. Results and discussion

Throughout the various studies concerning the electrophoretic behavior of derivatized gangliosides, the results were compared to those obtained with their underivatized counterparts in order to assess the various changes that might have been caused by the precolumn labeling of the different gangliosides.

In evaluating the suitability of the various electrophoretic systems under consideration, gangliosides type III mixture, which was purchased from Sigma, as well as individual gangliosides were utilized. The type III mixture may contain essentially G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} since it is known that mammalian brains generally contain these four major gangliosides [3,27] in the concentration ratios of 2:3.5:1:1.5, respectively [27].

3.1. Precolumn derivatization of gangliosides

The use of low wavelengths (e.g., 195 or 185 nm) for the detection of underivatized gangliosides impairs the utility of many buffer systems because at these wavelengths the components of the electrolyte systems starts to absorb substantially in the UV, thus leading to high background signals. The lack of suitable chromophores in ganglioside molecules makes their detection impossible at high wavelengths where many useful running electrolytes are UV transparent. To overcome this difficulty, and permit the sensitive detection at higher wavelengths, the gangliosides were derivatized with SA and ANDSA according to established procedures for carboxylated carbohydrates which were recently introduced by our laboratory [16]. The precolumn derivatization reaction involves a condensation reaction between the free amino group of the derivatizing agent and the free carboxyl group of the ganglioside to form a peptide link by acid-catalyzed removal of water in the presence of EDAC. The carbodiimide EDAC, which is required to promote the formation of the peptide bond [28,29], was selected because of its high solubility in aqueous solutions.The derivatization proceeds according to the following scheme:



where $R''NH_2$ is either SA or ANDSA, and R_1COOH can be any ganglioside (e.g., Fig. 1).

Individual gangliosides as well as Sigma type III ganglioside mixture were derivatized according to the above scheme. Typical electropherograms of both SA and ANDSA derivatives of type III mixture are displayed in Fig. 2. The excess of derivatizing agent eluted at 23 min for SA (peak not shown) while it did not elute in reasonable time in the case of ANDSA (peak not shown) when 15 kV were used as the running voltage. The detection wavelength was set at 247 nm since the UV spectra of both derivatizing agents, i.e., SA and ANDSA, as well as the gangliosides derivatives showed maximum absorbance at 247 nm. Returning to Fig. 2, the signal of disialogangliosides (G_Ds) is much greater than that of G_{M1} and G_{T1b}. As stated above, there are more G_Ds in the mixture than mono- and trisialogangliosides. Since G_{T1b} and G_Ds have three and two derivatization sites, respectively, under exhaustive precolumn derivatiza-

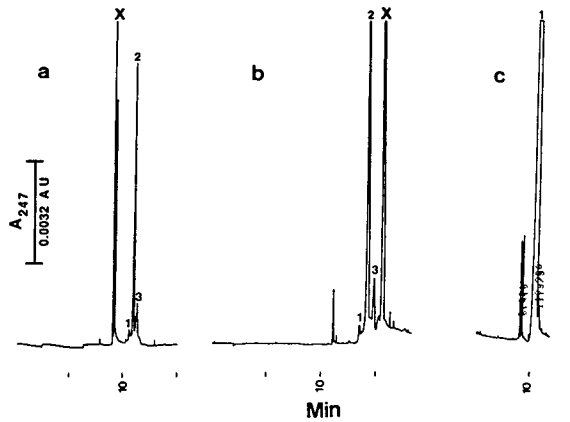


Fig. 2. Typical electropherograms of SA (a) and ANDSA (b) derivatives of the ganglioside mixture type III from Sigma and ANDSA derivative of standard G_{D1a} (c). Capillary, 80.0 cm (50.0 cm to detection) × 50 μm I.D.; running voltage, 15.0 kV in (a) and (b) and 20.0 kV in (c). Running electrolytes: (a) and (b): 150 m*M* borate containing 15.0 m*M* α-CD, pH 10.0; (c) 10 m*M* sodium phosphate, pH 7.0, containing 15.0 m*M* α-CD. Peaks in (a) and (b): 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}, × = by-product; peak in (c): 1 = G_{D1a}.

tion conditions two and three chromophores could be attached to G_{T1b} and G_Ds, respectively. This would explain the difference between peak height ratios of derivatized and underivatized gangliosides (compare Figs. 2 and 6c).

As can be seen in Fig. 2a and b, in addition to the derivatized gangliosides, by-products are also produced in the precolumn derivatization reaction. The by-products were detected by UV at 247 nm (in the case of SA and ANDSA) and by fluorescence in the case of ANDSA. This suggests that both by-products possess the derivatizing agents as part of the molecule. This was further confirmed by the electrophoretic behaviors of the by-products when varying the pH of the running electrolyte. The SA by-product seems to be neutral at pH ≥ 5 and positively charged at pH < 5 as inferred from its migration with or ahead of the marker of the electroosmotic flow (EOF), respectively. The by-product of ANDSA has a net negative charge at pH ≥ 5 and neutral at pH < 5 as indicated by the migration time of its peak with respect to that of the EOF marker. The pK_a values of the amino groups of SA and ANDSA are in the range of 3–4 [30]. Together, these observations imply that the by-product of SA has involved reaction with the sulfonic acid group of the SA molecule while leaving its amino group unsubstituted. The same conclusion could be drawn regarding the ANDSA by-product whereby one sulfonic acid group of the molecule has been involved in the reaction while the other sulfonic acid group and the amino group were left unsubstituted. In fact, studies made by ^{1}H NMR and ^{13}C NMR on an HPLC purified fraction of the by-product of ANDSA agreed with these observations. Both modes of NMR suggested the presence of alkylated moieties attached to the derivatizing agent which may indicate that the by-product is the result of the reaction between the derivatizing agent and the carbodiimide.

The by-products were seen when the amount of ganglioside added to the reaction mixture was not well controlled. With almost all ganglioside samples we did not have sufficient quantities that would allow the accurate weighing of a known and desirable amount. In other words, the side product was observed only when the amount of carbodiimide far exceeded the amount of analyte in the reaction mixture. In fact, when the reaction was made such that for each mole of carboxylic group, one mole of EDAC and two moles of SA or ANDSA were added, the derivatization reaction did not lead to the formation of detectable amount of side product as was the case of G_{D1a} (Fig. 2c). The absence of by-products was also observed with acidic monosaccharides reported earlier [16] whenever the ratio of 1:1:2 of sugar:carbodiimide:derivatizing agent was utilized.

To confirm the occurrence of the derivatization at the sialic acid site, the derivatized trisialoganglioside SA-G_{T1b} was treated with neuraminidase (for structure, see Fig. 1). Neuraminidase is an exoglycosidase which catalyzes the hydrolysis of the linkage joining a terminal sialic acid residue to a D-galactose or a D-galactosamine residue [31]. Since G_{M1} is resistant to neuraminidase, and G_{T1b} is hydrolyzed more rapidly than G_Ds [32], the cleavage of G_{T1b} with neuraminidase should result in the production of G_{M1} and G_{D1b} [25,26]. This is shown in Fig. 3

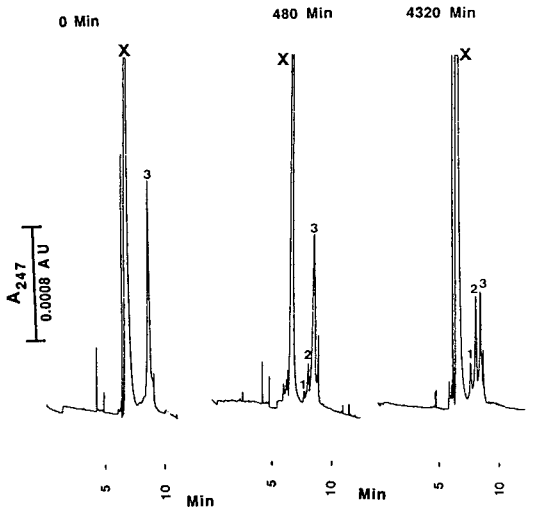


Fig. 3. Monitoring of the neuraminidase action on the SA derivative of GT1b by capillary electrophoresis. Conditions as in Fig. 2a and b. Peak: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}; × = by-product.

where SA-derivatized G_{T1b} gave one peak before treatment with neuraminidase and three peaks after exhaustive treatment with neuraminidase. Two additional peaks (not shown) have also appeared which were assumed to correspond to the cleaved mono- and disialic acid residues. The relatively intense peak that appeared at 19.2 min may be the SA derivative of sialic acid cleaved from G_{T1b} to yield G_{D1b} (i.e., the R_1 residue, see Fig. 1) while the less intense peak that eluted at 13.1 min may correspond to the SA derivative of disialic acid cleaved from G_{T1b} to yield G_{M1} (i.e., the R_2-sialic acid residues, see Fig. 1). The assumption that the less intense peak is that of the disialic acid and the more intense peak is that of the sialic acid monomer, corroborates well with the fact that the amount of G_{M1} produced is much less than that of the G_{D1b}. The excess SA was observed at 23.1 min.

The progress of the derivatization reaction with SA and ANDSA was monitored by CZE using the type III ganglioside mixture. Typical time course plots for SA-G_{T1b} and ANDSA-G_{T1b} are shown in Fig. 4 in terms of integrated peak heights versus time. In all cases, the precolumn derivatization reaction proceeded at a faster rate with SA than with ANDSA. The derivatization

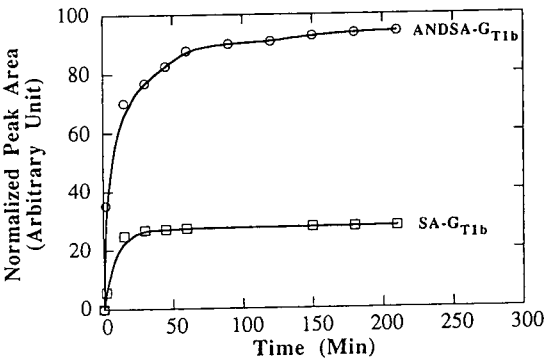


Fig. 4. Time course for the derivatization reactions of G_{T1b} with ANDSA and SA. The reaction mixture was subjected to CZE at different time intervals using conditions of Fig. 2a and b.

with SA reached a steady state in almost 1 h, while that with ANDSA required almost 2.5 h to reach the steady state. These results corroborate earlier findings with carboxylic monosaccharides [16], and may be attributed to differences in collision rates arising from differences in the molecular masses of the two derivatizing agents.

3.2. Capillary electrophoresis of gangliosides

It is well established that gangliosides exist as stable micelles in aqueous solutions with critical micellar concentration (CMC) in the range of 10^{-10}–10^{-8} *M* [10,33]. This phenomenon hinders the efficient separation of gangliosides as monomeric species in neat aqueous media. Consequently, an organic solvent or an additive capable of breaking the micelles is needed. In this study, acetonitrile and other additives were added individually to the running electrolytes used in separating the gangliosides. One virtue of the precolumn derivatization is that SA and ANDSA derivatives have maximum absorptivity at 247 nm far removed from low UV wavelengths (e.g., 195 nm) where only few solvents and light buffers can be used. In addition, ANDSA derivatives are readily detected by fluorescence.

HPLC-grade ACN was used as the organic modifier for the separation of gangliosides because its UV cut-off is 185 nm. Fig. 5 shows the separation of standard derivatized gan-

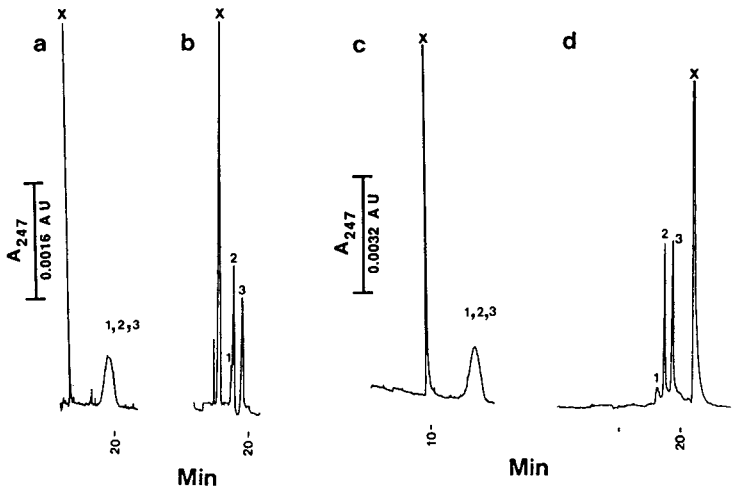


Fig. 5. Electropherograms of standard gangliosides derivatized with ANDSA at neutral (a and b) and high pH (c and d) in the presence (b and d) and absence (a and c) of acetonitrile in the running electrolyte. In (a) and (b): running electrolyte, 25 m*M* sodium phosphate, pH 7.0, at 0% (a) and 50% (v/v) (b) acetonitrile; running voltage, 25.0 kV. In (c) and (d): running electrolyte, 10 m*M* sodium phosphate, pH 10.0 at 0% (c) and 50% (v/v) (d) acetonitrile; running voltage, 20 kV. Samples: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}. Other conditions as in Fig. 2

gliosides at basic and neutral pH. At both pH values, and in the absence of ACN in the running electrolyte the ANDSA-gangliosides eluted as a single broad peak with no separation (see Fig. 5a and c). The inclusion of 50% ACN in the running electrolyte has apparently brought about the break up of aggregation and in turn the separation of the gangliosides. The same trend was also observed with the underivatized gangliosides (type III mixture) shown in Fig. 6, where 50% (v/v) ACN concentration seems to be the optimum amount which yielded the best separation in terms of resolution and sharpness of the migrating zones. The derivatization of the carbohydrate moiety of the gangliosides seems not to introduce undesirable effects as far as the aggregation of these species is concerned. In fact, both derivatized and underivatized gangliosides behaved similarly when electrophoresed with acetonitrile-rich electrolytes. In all cases, at ACN concentration below 30% (v/v), the gangliosides co-migrated indicating that the different analytes traveled through the capillary as a mixed micelle. As the ACN concentration increased above 30%, the different gangliosides migrated as monomers or oligomers and their migration mobility was greatly affected by their charge-to-mass ratio (Fig. 6). As can be seen in Fig. 6, only three different peaks were detected, which were identified by spiking the mixture with standard samples of the different gangliosides. The disialoganglioside isomers, G_{D1a} and G_{D1b},

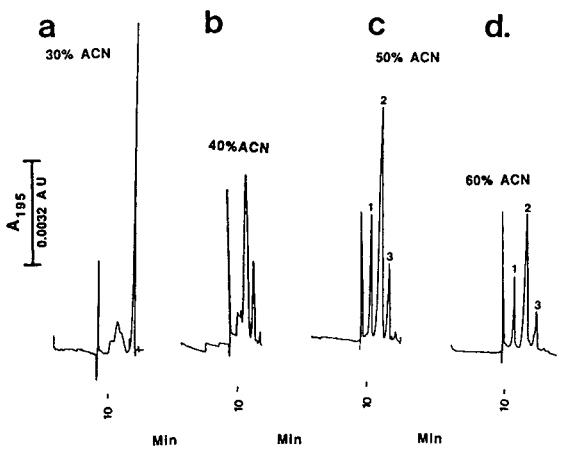


Fig. 6. Electropherograms of underivatized gangliosides (mixture type III). Running electrolytes: 5.0 m*M* sodium phosphate, pH 7.50, at various % (v/v) acetonitrile; running voltage, 15.0 kV. Peaks: 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}. Other conditions as in Fig. 2.

co-migrated since both lecules have the same charge-to-mass ratio. (mum separation conditions were achieved a( )% (v/v) ACN since at 60% ACN the gain in resolution was on the expense of broader peaks and at 70% ACN the efficiency dropped by a factor of almost 2.

To further optimize the hydro–organic electrolyte system just described, the effects of phosphate concentration and pH of the running electrolyte were examined. As expected, increasing the ionic strength of the running electrolyte decreased the electroosmotic flow which resulted in increasing the migration time of the gangliosides through the capillary (Fig. 7). More importantly, for amphiphilic gangliosides increasing the ionic strength of the running electrolyte reduces the CMC value. In fact, it has been shown that the CMC of G_{M1} decreased by a factor of three when going from pure water to 50 m*M* sodium acetate buffer [34]. As can be seen in Fig. 7, the effect of increasing buffer ionic strength seems to be more pronounced for the monosialoganglioside G_{M1} since electrostatic repulsion between the polar head groups of singly charged amphiphilic species can be shielded at much lower salt concentration, a phenomenon that favors aggregation and reduction of CMC in aqueous solution. This may explain the decrease in the sharpness of the G_{M1} peak as the concentration of phosphate buffer increased. Optimum separation efficiencies in terms of number of theoretical plates were achieved at 5.0 m*M* phosphate buffer concentration.

Increasing the pH of the running electrolyte from 5.0 to 8.0 did not greatly affect the electroosmotic flow (fluctuated between 8.5 and $9.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}), nor the electrophoretic mobility of the different gangliosides (results not shown). For instance, the electrophoretic mobility of G_{M1} only increased from $2.0 \cdot 10^{-4}$ to $2.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. This might be due to the presence of the acetonitrile in the running electrolyte.

One of the shortcomings of the hydro–organic buffer systems evaluated above is that G_{D1a} and G_{D1b} were not separated. To provide other buffer systems that are useful for the separation of gangliosides in their monomeric forms, α-CD was used as a buffer additive. It has been shown that α-CD has the ability to break up the micellar form of the different gangliosides mixtures in a borate electrolyte [12]. In fact, and as can be seen in Fig. 8a, the ganglioside mixture type III could be separated into its mono-, di- and trisialogangliosides in the presence of α-CD. Although 7 m*M* α-CD was sufficient for keeping the different gangliosides as monomers, a 15 m*M* α-CD concentration was used because this

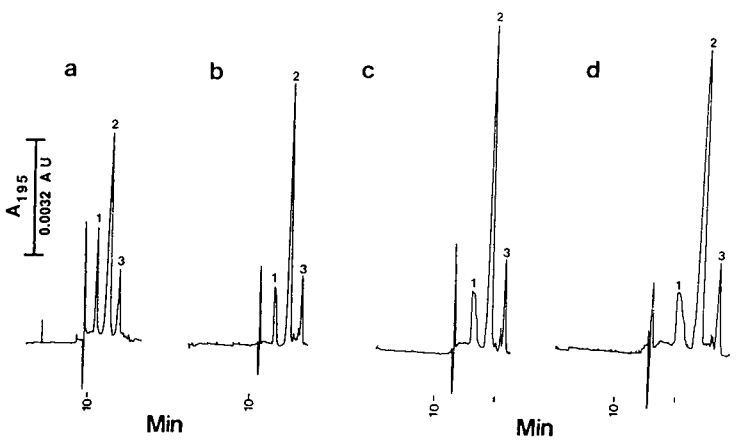


Fig. 7. Electropherograms of standard underivatized gangliosides. Running electrolytes: sodium phosphate buffers, pH 7.50, containing 50% (v/v) acetonitrile at 5.0, 15.0, 25.0 and 35.0 m*M* sodium phosphate in (a), (b), (c) and (d), respectively. Average number of theoretical plates per meter: 25 200, 14 400, 13 200 and 16 700 in (a), (b), (c) and (d), respectively. Peaks: 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}. Other conditions as in Fig. 2.

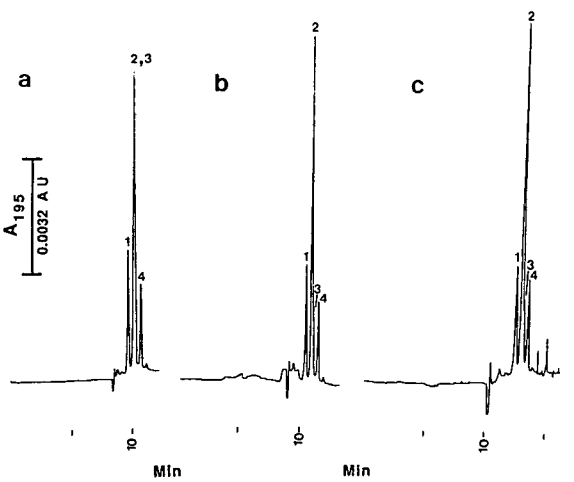


Fig. 8. Electropherograms of underivatized gangliosides (mixture type III). Running electrolytes, borate buffers containing 15.0 m*M* α-CD, pH 10.0, at 50, 100 and 150 m*M* borate in (a), (b) and (c), respectively. Peaks: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{D1b}; 4 = G_{T1b}. Other conditions as in Fig. 2.

amount yielded the highest plate count as shown in Fig. 9.

The effect of borate complexation with the oligosaccharide moiety of gangliosides was studied at three different borate concentrations to determine the best conditions for the separation of the two disialoganglioside isomers, G_{D1a} and G_{D1b}. As can be seen in Fig. 8, 100 m*M* borate provided partial separation between the two isomers and a further increase in borate concentration did not improve the resolution (Fig. 8c). This may indicate that the changes in the charge density between the isomers due to borate complexation may not be sufficiently high to allow their complete separation. As expected, increasing the borate concentration increased the migration time due to increasing the extent of complexation of the carbohydrate moieties of the gangliosides with borate and to decreasing the EOF. It should be noted that the electrophoretic velocity of the solutes is in the opposite direction to the EOF. The same trend was also observed with ANDSA-gangliosides when the borate concentration was varied in the presence of α-CD (Fig. 10). As with the underivatized gangliosides, increasing the borate concentration resulted in a partial separation between the isomers but at the expense of longer analysis time. As can be seen in Fig. 10, the complexation between borate and the individual gangliosides occurs to the same extent as indicated by the parallel increase of the migration time with the increase in borate concentration. This may indicate that the sialic acid does not contribute significantly to borate complexation since the only difference in the carbohydrate moieties of the various gangliosides is the number of sialic acids.

ANDSA is a fluorescent tagging agent. It has a maximum excitation signal at 315 nm and a maximum emission signal at 420 nm [35]. This

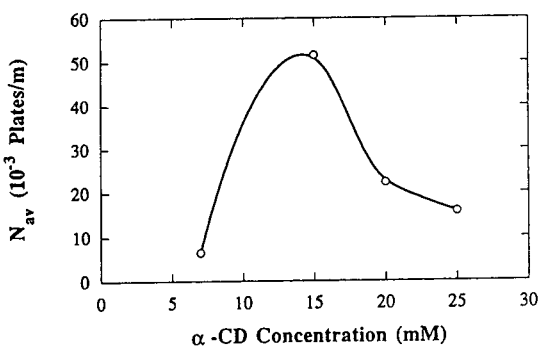


Fig. 9. Plot of average plate counts per meter at various α-CD concentrations in the running electrolyte. Running electrolytes, 150 m*M* borate, pH 10.0, at various concentration of α-CD. Other conditions as in Fig. 2.

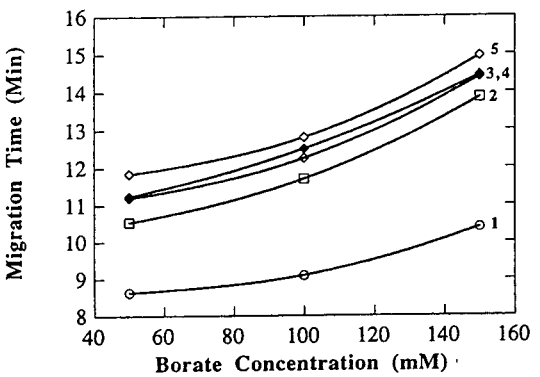


Fig. 10. Typical plots of migration time versus borate concentration in the running electrolyte obtained with ANDSA derivatives of standard gangliosides. 1 = Dimethyl sulfoxide; 2 = G_{M1}; 3 = G_{D1a}; 4 = G_{D1b}; 5 = G_{T1b}. Other conditions as in Fig. 8.

allowed the fluorescence detection of the ANDSA derivatives of gangliosides as shown in Fig. 11.

As shown above, the hydro–organic electrolyte system containing ACN and the α-CD buffer system did not provide adequate resolution for the disialoganglioside isomers, i.e., G_{D1a} and G_{D1b}, despite the fact that they were very efficient in breaking up micelle formation. Therefore, there is a need to add hydrophobic or hydrophilic selectors to the electrolyte to provide sufficient selectivity. To evaluate some potential additives, three model disialogangliosides were used including G_{D1a}, G_{D1b} and G_{D3}. G_{D3} has a smaller molecular mass than the two isomers G_{D1a} and G_{D1b} since its carbohydrate moiety is a tetrasaccharide with two sialic acid residues (Fig. 1). The charge-to-mass ratios of G_{D1a}, G_{D1b} and G_{D3} are $1.09 \cdot 10^{-3}$, $1.09 \cdot 10^{-3}$ and $1.35 \cdot 10^{-3}$, respectively.

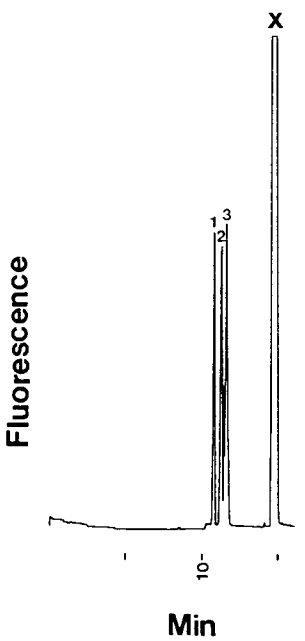


Fig. 11. Typical fluorescence electropherogram of mono-, di- and trisialogangliosides derivatized with ANDSA. Running electrolyte, 10 m*M* sodium phosphate, pH 12.0, containing 15.0 m*M* α-CD; running voltage, 18.0 kV. Peaks: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}; × = by-product. Other conditions as in Fig. 2.

In addition to the fact that the α-CD and ACN electrolyte systems provided marginal or no resolution between G_{D1a} and G_{D1b}, they did not allow the complete resolution of G_{D1a} and G_{D1b} from G_{D3} as shown in Fig. 12a and b. As can be seen in this figure, phosphate electrolyte containing 50% ACN yielded a better separation between G_{D3} and the other two disialoganglioside isomers. Similar results were obtained with the derivatized G_{D1a}, G_{D1b} and G_{D3}.

To enhance the selectivity of the electrophoretic system and to achieve separation of the G_Ds, hydrophilic interaction was superimposed on the electrophoretic process by including HPC or PVA in the running electrolytes in addition to α-CD. The amount of these additives were varied, and best results were attained at 0.5% (v/v) (see Fig. 13).

In another set of experiments, hydrophobic interaction was exploited to achieve the separation of the G_Ds. In this regard, the possibility of using micellar electrokinetic capillary chromatography was considered by investigating the usefulness of in situ charged micelles recently introduced by our laboratory [36,37]. In situ

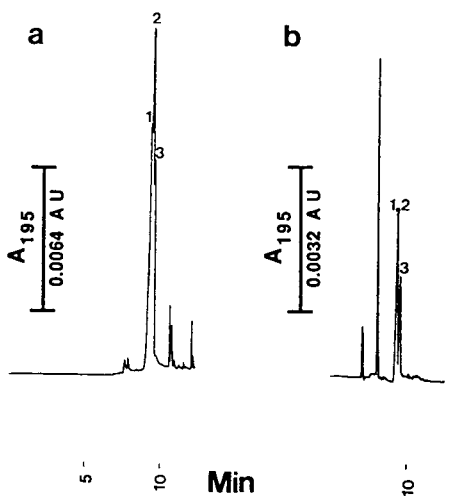


Fig. 12. Electropherograms of standard underivatized gangliosides. (a) Running electrolyte, 50 m*M* borate containing 15 m*M* α-CD, pH 10.0; running voltage 20.0 kV. (b) Running electrolyte, 10 m*M* sodium phosphate, pH 7.0 containing 50% (v/v) acetonitrile; running voltage, 25.0 kV. Samples: 1 = G_{D1a}; 2 = G_{D1b}; 3 = G_{D3}. Other conditions as in Fig. 2.

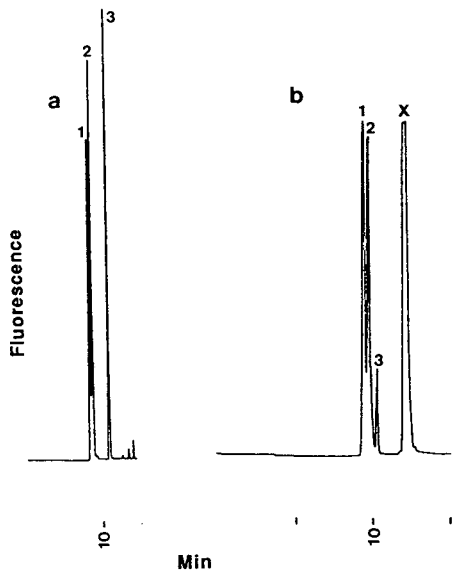


Fig. 13. Electropherograms of standard ANDSA derivatives of disialogangliosides. Running electrolytes, 10 m*M* sodium phosphate, pH 12.0, containing 15 m*M* α-CD and 0.5% HPC in (a) or 0.5% PVA in (b); running voltage, 18.0 kV. Peaks: 1 = G_{D1a}; 2 = G_{D1b}; 3 = G_{D3}; × = by-product. Other conditions as in Fig. 2.

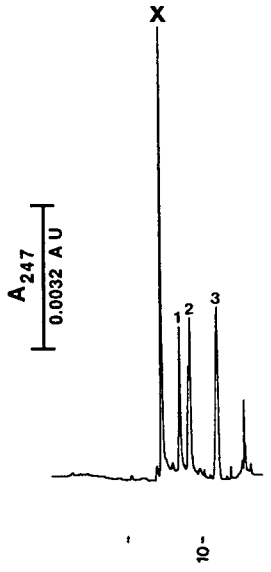


Fig. 14. Electropherogram of standard ANDSA-disialogangliosides. Running electrolyte, 50 m*M* borate, pH 6.0 containing 5.0 m*M* MEGA surfactant and 15.0 m*M* α-CD. Peaks and other conditions as in Fig. 13.

micellar systems are based on the complexation of alkylglycoside surfactants and borate, a phenomenon that provides micelles with adjustable surface charge density. As seen in Fig. 14, a micellar electrolyte system consisting of 50 m*M* borate, 5 m*M* MEGA 10 and 15 m*M* α-CD gave a baseline separation and a high plate count for the G_Ds. The pH of the electrolyte was adjusted to 6 to ensure a different migration time between the analytes and the by-product.

In conclusion, the selective precolumn derivatization of gangliosides involving the carboxylic acid groups of the sialic acid residues has proved suitable for the analysis of small amounts of these sialoglycolipids by HPCE. This attractive feature in conjunction with the unique selectivities offered by the various electrolyte systems developed in the present studies are expected to facilitate the determination of gangliosides in small tumors.

Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Separation and simultaneous determination of the components in an analgesic tablet formulation by micellar electrokinetic chromatography

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Abstract

The separation and simultaneous determination of the active ingredients of an analgesic tablet formulation composed of caffeine, paracetamol, dextropropoxyphene, acetylsalicylic acid and chlorpheniramine was investigated by micellar electrokinetic chromatography (MEKC). As the use of sodium dodecyl sulphate could not resolve the two basic compounds, the separation was completed by using a mixture of the bile salts sodium cholate and sodium deoxycholate. The determination of the five ingredients together with salicyclic acid could be performed with acceptable accuracy and precision.

1. Introduction

Micellar electrokinetic chromatography (MEKC), one of the most important modifications of capillary electrophoresis (CE), has been proved to be an excellent alternative to LC in drug analysis. In some respects there are advantages of MEKC over LC including the ability to perform simultaneous analysis of drug combinations with different polarity and hydrophobicity [1–5], although CE is often considered less sensitive than LC owing to the minute amount of sample introduced into the capillary and the small volume of the detector cell [6]. There are many possibilities for manipulating the CE operating parameters in order to enhance the sensitivity, as suggested by Altria [7]. In this paper, the optimization of the separation of a selected analgesic tablet formulation, consisting of caffeine 36 mg, paracetamol 120 mg, acetylsalicylic acid 250 mg, dextropropoxyphene hydrochloride 30 mg and chlorpheniramine maleate 1.6 mg, is described. The conventional simultaneous determination of the active ingredients together with salicylic acid, a degradation product of acetylsalicylic acid, is then evaluated from an analytical point of view.

2. Experimental

2.1. Equipment

Electrophoresis was carried out on a P/ACE System 2100 fitted with a UV detector (Beck-

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man, Palo Alto, CA, USA). Separations were performed in a 570 mm × 0.075 mm I.D. fused-silica capillary tube (Beckman). The integration of the electropherograms was achieved by the Chromatography Software System Gold V.711 (Beckman).

2.2. Drugs, chemicals and reagents

The drug substances used in the work were of European Pharmacopoeia quality. Acetylsalicylic acid (ASA), caffeine monohydrate (C) and propyl hydroxybenzoate were purchased from Merck (Darmstadt, Germany), paracetamol (P) from Bios (Leuven, Belgium), salicylic acid (SA) from Aldrich (Bornem, Belgium) and dextropropoxyphene hydrochloride (D) and chlorpheniramine maleate (Ch) from Thissen (Br-l'Alleud, Belgium). The surfactants sodium dodecyl sulphate (SDS), sodium deoxycholate (SDC) and sodium cholate (SC) were purchased from Sigma (St. Louis, MO, USA) and used as received. All the other reagents were of analytical-reagent grade. The solvent used for preparing standard and sample solutions was 10 m*M* hydrochloric acid containing 20% (v/v) acetonitrile. The composition of the solvent mixture was intended to achieve complete dissolution and stability of the solutes. The separating buffer solutions were prepared by mixing an appropriate volume of 20 m*M* sodium tetraborate solution with 20 m*M* sodium dihydrogenphosphate solution. The water used for preparing solutions was obtained from a Seralpur Pro 90 CN purification system (Seral, Germany). All buffer solutions were filtered before use through a 0.2-μm membrane filter.

2.3. Procedure

For all experiments, injections were made hydrodynamically by pressure for 2 s and the applied voltage was 20 kV. The temperature was kept constant at 25°C. Detection was performed at 214 nm. Before each injection, separating buffer was passed through the capillary for at least 4 min.

2.4. Sample preparation

Twenty tablets were weighed and ground to a fine homogeneous powder. An accurately weighed portion of powder corresponding to one tablet was transferred to a centrifuge tube to which 50.0 ml of the solvent mixture were added. After shaking and sonicating for 15 min and centrifuging until a clear solution was obtained, 25.0 ml of the clear supernatant were transferred into a 50-ml volumetric flask to which 10.0 ml of internal standard solution (propyl hydroxybenzoate, 50 mg per 100 ml) were added. The volume was adjusted to 50.0 ml with the same solvent.

3. Results and discussion

3.1. Separation by capillary zone electrophoresis (CZE)

From some preliminary experiments carried out in the CZE mode using a buffer at pH 9, the following features were observed.

The migration order of the six compounds was as predicted from their acid–base dissociation constants. The two basic compounds Ch and D migrated first because of the combined effect of the electrophoretic mobility and the electroosmotic flow (EOF) in the direction of the cathode. Caffeine, a neutral xanthine derivative, migrated with the velocity of the EOF. The negatively charged acidic compounds, P, ASA and SA, migrated in this order with different velocities lower than that of the EOF. Although all compounds were well separated, D exhibited a distorted peak.

When performing the separation at lower pH values (down to 7), the distortion of the D peak gradually disappeared but two pairs of compounds co-eluted: P together with C and Ch together with D.

Moreover, the presence of the uncharged

compound C as test substance implied the need to add a surfactant to the separating buffer with a view to quantitative analysis.

3.2. Separation by micellar electrokinetic chromatography (MEKC)

Sodium dodecyl sulphate

Adding SDS to the buffer provided the largest effect on the migration behaviour of the two basic compounds Ch and D. In contrast to their migration in CZE, they eluted last among all the solutes but were not separated from each other in the pH range examined (7–9). Some buffer additives that were reported to improve the selectivity in MEKC, such as organic solvents [8], urea [9] and glucose [10], were investigated but none of these attempts was successful. This can be explained by the fact that both Ch and D are completely taken up by the SDS micelles owing to strong electrostatic interactions. As suggested by Nishi et al. [11,12], these basic compounds migrate at the same velocity as the micelles. Consequently, the migration time of Ch or D can be considered as the migration time of the micelles (t_{mc}). Based on this assumption, the apparent capacity factors (k') are calculated from the equation introduced by Terabe et al. [13]:

$$k' = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} \quad (1)$$

and plotted as a function of the concentration of SDS. The results obtained at pH 7 are shown in Fig. 1. As only the k' values of the uncharged compounds C and P showed a linear relationship with the surfactant concentration, their migration can be considered as a purely chromatographic process based on the partitioning between the two phases [13]. However, in the k'-equation mentioned above a possible effect of charge on the migration is not included. Therefore, with an anionic compound it is necessary to correct for the electrophoretic mobility in order to obtain an accurate estimate of capacity factors [14]. Using the effective mobility (u_{eff}) of each of the two solutes ASA and SA calculated from

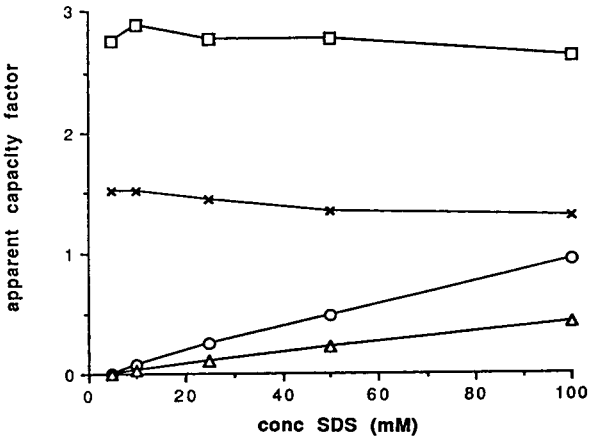

Fig. 1. Apparent capacity factors (calculated from Eq. 1) of (○) caffeine (C), (△) paracetamol (P), (×) acetylsalicylic acid (ASA) and (□) salicylic acid (SA) as a function of SDS concentration. Buffer, SDS in 20 m*M* borate–phosphate (pH 7); capillary, fused silica, 570 mm × 0.075 mm I.D.; injection time, 2 s; voltage, 20 kV, temperature, 25°C.

their migration velocities in CZE, migration times t_{ion} were calculated (at each level of SDS concentration) as would be expected in the absence of micelles [14]:

$$t_{ion} = \frac{1}{u_{eff} + u_{eof}} \cdot \frac{Ll}{V} \quad (2)$$

where u_{eof} is the mobility of the EOF observed at each SDS concentration, V is the applied voltage, and L and l are the total length and the length to the detector of the capillary, respectively. On replacing t_0 by t_{ion} in Eq. 1, the capacity factors obtained show small negative values (Table 1), which means that their migration is not affected by any partitioning with the micelles. This is not surprising, as ASA and SA are fully charged at pH 7 (pK_a = 3.5 and 3.0 for ASA and SA, respectively) and therefore there is no partitioning of these solutes in the micelles owing to the electrostatic repulsion of the anionic solutes from the micelles. The negative values of the capacity factors are the result of some errors according to the t_{ion} values which were calculated and expected in the absence of the surfactant and could be different from the actual t_{ion} values that would occur in the system with surfactant owing to the different factors,

Table 1
Capacity factors (k') calculated from Eq. 1 after correcting for t_0 by t_{ion} ($t_{mc} = t_m$ of Ch or D)

SDS concentration (mM)	t_{mc} (min)	ASA			SA		
		t_r (min)	t_{ion} (min)	k'	t_r (min)	t_{ion} (min)	k'
5	8.76	5.48	5.63	−0.07	6.34	6.43	−0.05
10	9.05	5.52	5.69	−0.08	6.42	6.50	−0.04
25	9.62	5.66	5.90	−0.11	6.62	6.78	−0.08
50	12.28	6.37	6.81	−0.13	7.72	8.01	−0.10
100	15.06	6.95	7.56	−0.15	8.66	9.07	−0.11

i.e., ionic strength, viscosity, charge interaction, etc.

Bile salts

Among several ways of manipulating the selectivity in MEKC, the most effective is to change the type of surfactant [15]. The fact that bile salts possess both hydrophilic and hydrophobic faces [11,12] gives rise to advantages over the conventional use of SDS for the separation of hydrophobic molecules [12,16,17] and basic compounds [11]. In this case SC and SDC were employed as surfactants, especially to separate Ch and D, together with the other solutes. With SC at a concentration of 50 mM in borate buffer (pH 9), all compounds were separated but the peak of D was distorted. Moreover, SA eluted in a region where the baseline was disturbed by some impurity in SC. With SDC used under the same conditions, however, Ch eluted together with SA in addition to ASA with propyl hydroxybenzoate (internal standard). Varying the concentration of SC and SDC from 50 to 100 mM did not improve the selectivity of the separation. Co-elution of some components still occurred. The selectivity could be optimized by mixing SC and SDC in different proportions as follows: (A) 25 mM SC + 25 mM SDC and (B) 25 mM SC + 50 mM SDC. These mixtures of the two bile salts gave a good compromise for the mobility profile of the solutes compared with that obtained from the individual bile salts (Fig. 2). Combination B provided good separation and peak shapes whereas the electropherogram with mixture A still exhibited a distorted peak of D. The reason for the distortion could be partly the effect of acetonitrile in the sample solution on the micelles in the separation buffer [18]. The fact that the distortion occurred only with D (it was also observed with Ch when the concentration of Ch in the sample solution was high) and only in the presence of SC might be due to the higher critical micellar concentration of SC compared with SDS and SDC (SC, $13 \cdot 10^{-3}$–$15 \cdot 10^{-3}$ M; SDS, $8.1 \cdot 10^{-3}$ M; SDC, $4 \cdot 10^{-3}$–$6 \cdot 10^{-3}$ M at 25°C) and also to the lower aggregation number of SC compared with SDS and SDC (SC, 2–4; SDS, 62; SDC, 4–10) [19]. Therefore SC micelles could tolerate high amounts of

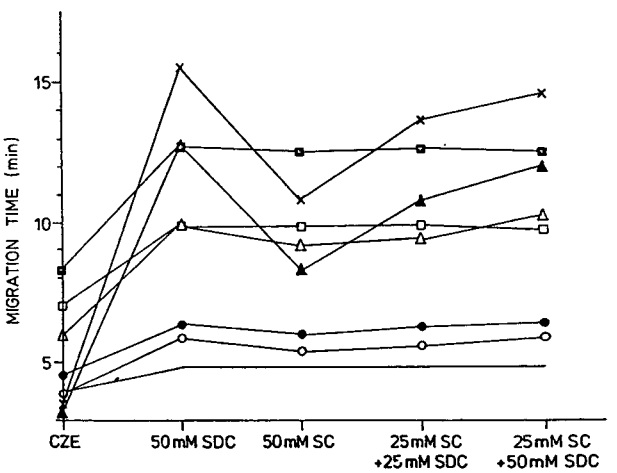

Fig. 2. Migration times of the analytes in 20 mM borate buffer (pH 9) containing different proportions of bile salts (SC and SDC). Other conditions as in Fig. 1. Line with no symbols = EOF; ○ = C; ● = P; □ = ASA; ■ = SA; △ = internal standard; ▲ = CH; × = D.

organic solvent to a lesser extent than SDS or SDC micelles. Further, D and Ch, both of which are positively charged, can be adsorbed on the negatively charged surface of the capillary.

3.3. Analysis of a tablet formulation

An analgesic tablet formulation containing the active ingredients for which the separation had been optimized was analysed by the internal standard method, which was proved to be necessary for quantitative purposes [20], using 20 m*M* borate buffer (pH 9) containing 25 m*M* SC and 50 m*M* SDC.

Calibration

Calibration lines were constructed in the concentration range covering the nominal concentration used for analysis, i.e., 10–40 mg per 100 ml for C, 25–150 mg per 100 ml for P, 100–300 mg per 100 ml for ASA, 0.8–6.5 mg per 100 ml for Ch, 12–40 mg per 100 ml for D and 1–10 mg per 100 ml for SA, together with 10 mg per 100 ml of propyl hydroxybenzoate as an internal standard. Linear regression lines of the ratios of the peak area as a function of the concentration were calculated using the least-squares method. Good linearity ($r = 0.998$–0.999) confirmed by statistical analysis was obtained for all compounds.

Precision and accuracy

The accuracy and repeatability were evaluated from a recovery experiment performed on an artificially prepared powder mixture containing all active ingredients in the concentrations present in a real sample, added to a mixture of tablet excipients composed of corn starch, microcrystalline cellulose, magnesium stearate and talc. Samples were treated as described in the procedure for sample preparation. The final concentrations of each compound expressed per 100 ml were ca. 36 mg for C, 120 mg for P, 30 mg for D, 250 mg for ASA and 1.6 mg for Ch, using 10 mg per 100 ml of propyl hydroxybenzoate as an internal standard. Six replicate samples were analysed by comparison with an external reference solution with approximately the same concentrations as the sample solution. Mean recoveries are given in Table 2 together with the relative standard deviations (R.S.D.) and 95% confidence intervals of the mean (95% C.I.). Good accuracy and acceptable precision were obtained, except for Ch, which showed a high R.S.D. owing to its very low dose.

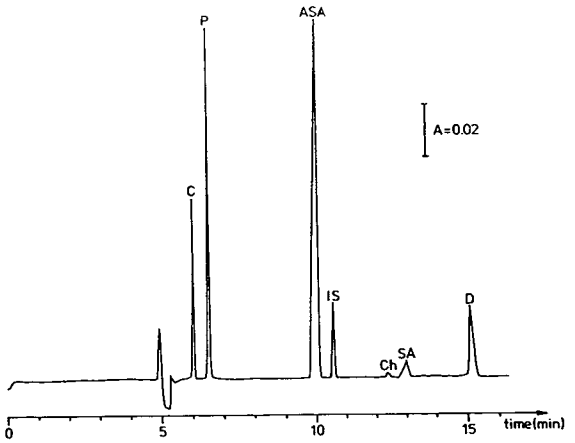

Fig. 3. Typical electropherogram for a real tablet. Buffer, 25 m*M* SC + 50 m*M* SDC in 20 m*M* borate (pH 9). Other conditions as in Fig. 1.

Table 2
Results of mean recovery ($\bar{X}$), repeatability (R.S.D.) and accuracy [95% confidence interval (95% C.I.)] from spiked powder mixture ($n = 6$)

Component	$\bar{X}$ (%)	R.S.D. (%)	95% C.I.
Caffeine	98.97	1.54	97.6–100.4
Paracetamol	100.54	0.94	99.7–101.4
Acetylsalicylic acid	99.28	0.98	98.3–100.2
Chlorpheniramine maleate	98.69	6.19	93.0–104.3
Dextropropoxyphene hydrochloride	100.43	3.92	97.3–104.6

Table 3
Results of the analysis of a real tablet formulation

Component	Label claim (mg)	Amount found (mean ±S.D.)[a]	% of label claim (mean ± S.D.)[a]
Caffeine	36	36.7 ± 0.34	101.9 ± 0.88
Paracetamol	120	121.7 ± 2.32	101.4 ± 1.79
Acetylsalicylic acid	250	251.7 ± 6.62	100.7 ± 2.65
Chlorpheniramine maleate	1.6	1.65 ± 0.09	103.2 ± 5.75
Dextropropoxyphene hydrochloride	30	29.6 ± 0.62	98.4 ± 2.08
Salicylic acid	–	4.8 ± 0.25	

[a] Means of three samples ± standard deviation.

Analysis of real samples

Determination of the active ingredients including salicylic acid, the degradation product of ASA, was performed on a real tablet with the method described above (Fig. 3). As can be seen from Table 3, the results are in agreement with the 95–105% limits during the shelf-life.

4. Conclusions

In this CE application for the determination of the active ingredients of an analgesic formulation using MEKC, it has been demonstrated that the use of bile salts is more advantageous than SDS micelles, especially for the separation of basic compounds. Optimization of the separation could be achieved by mixing sodium cholate and sodium deoxycholate in different proportions. The simultaneous determination of five active components and one degradation product in an analgesic tablet formulation could be performed accurately, although there was a large difference in the dose of the ingredients.

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JOURNAL OF CHROMATOGRAPHY A

Simultaneous determination of Cr(III), Fe(III), Cu(II) and Pb(II) as UV-absorbing EDTA complexes by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) with on-column UV detection was used to separate and determine Cr(III), Fe(III), Cu(II) and Pb(II) complexes with EDTA. Distribution species–pH diagrams showed that, under the experimental conditions chosen (pH 5.5), $[Me–EDTA]^-$ and $[Me–EDTA]^{2-}$ species for trivalent and divalent metals, respectively, were present. By adding a cationic surfactant, such as TTAB (tetradecyltrimethylammonium bromide), to the background electrolyte, an improvement in the peak shapes and shorter migration times were achieved. The chelating complexes showed the following order of mobility in electropherograms: $[EDTA]^{2-} > [Cu–EDTA]^{2-} > [Pb–EDTA]^{2-} > [Cr–EDTA]^- > [Fe–EDTA]^-$. At 225 nm under a negative applied voltage of 30 kV, using a capillary of 30.5 cm effective length, in 0.1 *M* acetate buffer and 0.1 m*M* TTAB as carrier solution, the complexes were determined within 6 min, but the resolution of the Cu(II) and Pb(II) chelates was poor. However, by using a capillary of 60 cm effective length, simultaneous separation of these chelates the EDTA was achieved. Factorial design was used to investigate the effects of chromium, excess of EDTA and boiling time of the solution on the formation of the $[Cr–EDTA]^-$ complex. A fitting model was found in which the EDTA concentration was a significant factor. The detection limits of all chelates were in the range 6–27 μM.

1. Introduction

Many methods for the determination of heavy metals have been reported, but often they are tedious and time consuming since they involve preliminary separation steps based on solved extraction, coprecipitation or ion exchange followed by inductively coupled plasma or atomic absorption spectrometric determination [1,2].

In recent years, capillary zone electrophoresis (CZE) has been shown to be a highly efficient technique for the separation and determination of both organic and inorganic cations and anions [3–7]. The determination and speciation of some metals without using chelating agent have been reported [8,9]. By coupling the electrophoresis

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with metal complex formation, the separation and detection limit of metals have been enhanced. Weston et al. [10] described indirect UV detection for the separation of Group IA, IIA, Mn, Cd, Fe(II), Co, Pb, Ni, Cu, Zn and lanthanide mixtures by CE. In this method, α-hydroxyisobutyric acid was utilized as a complexing agent to aid the separation. On the other hand, Swaile and Sepianak [11] used 8-hydroxyquinoline-5-sulfonic acid in the mobile phase, so complexes of Ca, Mg and Zn metals were formed within the capillary. In this method, an on-column laser-based fluorescence detection scheme was employed. Neither of the above organic compounds [10,11] forms complexes with Cr(III) [12].

The use of EDTA as a chelating agent in CZE has been reported for the determination of alkaline earth metal ions [13]. EDTA is by far the most widely used chelating agent in analytical chemistry. It forms strong 1 : 1 complexes with many metal ions regardless of the charge of the metal. Several ions of complex nature or high charge react slowly with EDTA. Thus, chromium (III) ions bind water molecules so strongly that these are displaced by EDTA only very slowly, during the course of several days [14].

There are many contradictory reports about the reaction of Cr(III) with EDTA [15–18]. Hamm [19] reported that the reaction rate is independent of the EDTA concentration. Later, it was reported that the reaction could be described as second-order, depending on the EDTA concentration [20]. Various compounds have been reported to have a catalytic effect on this reaction [20,21].

Our aim in this work was to develop a procedure for the simultaneous determination of Cr(III), Fe(III), Cu(II) and Pb(II) by CZE as UV-absorbing EDTA chelates. The experimental conditions of the reaction between chromium and EDTA were investigated by using a factorial experimental design. Based on the distribution species–pH diagrams [22–24], the optimum pH range for the determination of Cr(III), Fe(III), Cu(II) and Pb(II) chelates was found. The method was applied to the determination of heavy metals in waste waters from tanning processes.

2. Experimental

2.1. Instrumentation

An ISCO (Lincoln, NE, USA) Model 3850 integrated capillary electrophoresis system equipped with high voltage up to 30 kV and reversible polarity was used. Samples were introduced by applying a 3.4-kPa vacuum at the detector end of the capillary. Separations were performed with unmodified fused-silica capillary columns of length 46.5 cm (30.5 cm to the UV detector) and 80 cm (60 cm to the UV detector) with 0.05 mm I.D. A Model 4270 integrator (Spectra-Physics, San José CA, USA) was used to record all data.

A Shimadzu UV-240 UV–Vis recording spectrophotometer was used to record the absorption spectra of metal–EDTA complexes. The pH of solutions was monitored with a Crison Digilab 517 pH meter.

2.2. Reagents and solutions

Stock standard solutions of 1000 mg/l $CrCl_3$, $FeCl_3$, $Pb(NO_3)_2$, $CuCl_2$, $ZnCl_2$ and $AlCl_3$ and EDTA (solid), purchased from Merck, were used to prepare dilute metal–chelate solutions. Stock standard solutions of 0.2 *M* sodium acetate and acetic acid (Merck), prepared separately, were used to prepare dilute buffer solutions [25]. HPLC-grade TTAB (tetradecyltrimethylammonium bromide), purchased from Scharlau, was used. Super-pure methanol from Romil Chemicals was used as a marker to measure the rate of electroosmotic flow. Purified (18 MΩ) water, obtained using a Millipore Milli-Q water-purification system, was used for all solutions. All solutions were filtered through a 0.45-μm membrane filter and were degassed by ultrasound.

2.3. Procedure for electrophoresis

The capillary tube was rinsed with deionized water for several hours, then equilibrated with carrier solution for 40 min before the first run. Between each injection the capillary was filled with carrier solution using a syringe purge that flushed the entire capillary in a few seconds. Both ends were dipped into two separate beakers filled with the same carrier solution. The sample solution was introduced into the anodic or cathodic end of the capillary by vacuum injection. Lastly, a high voltage was applied.

2.4. Metal complex preparation

The chelates of Fe(III), Cu(II) and Pb(II) with EDTA were prepared from a mixture of the corresponding metal standard and EDTA solution in 0.05 *M* acetate–acetic acid and buffer (pH 5.5) at room temperature. An examination of the effects of Cr concentration, excess EDTA concentration and boiling time on the absorbance (A) of [Cr–EDTA] complex formed was carried out by a classical univariate method and by factorial design [26,27]. The fitting model

$$A = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

allowed us to estimate the effects in which we were interested, and also their possible interactions (b_{12}, b_{13}, b_{23} and b_{123}).

3. Results and discussion

3.1. Effects of EDTA and boiling time on the formation of $[Cr–EDTA]^-$ complex

Three factors were taken into account, each at two levels: chromium concentration (x_1), EDTA concentration (x_2) and boiling time (x_3). The concentration levels were 0.38 and 0.77 m*M* for Cr(III) and 1.07 and 5.37 m*M* for EDTA. The boiling time of the solution was 10 and 20 min. The heights of the peaks (H) obtained by CZE were the responses studied. Samples were analysed in triplicate. The experimental design and the results obtained are shown in Table 1. Calculations were made using Statgrafics Software Version 5.0, which yields the following expression:

$$H = 48.89 + 14.66x_1 + 2.56x_2 + 0.988x_1x_2 - 1.388x_1x_2x_3 \quad (2)$$

It was verified that this function was adequate [26]. The third factor b_3, and also the interactions b_{13} (Cr and t) and b_{23} (EDTA and t),

Table 1
The 2^3 factorial experimental design and results of experiments

Expt. No.	x_1	x_2	x_3	H_1	H_2	H_3	H_{mean}	S.D.	H^*
1	−1	−1	−1	3.40	3.20	3.30	3.30	0.10	3.30
2	+1	−1	−1	5.80	5.70	5.80	5.77	0.058	5.76
3	−1	+1	−1	3.35	3.40	3.55	3.43	0.10	3.34
4	+1	+1	−1	6.85	6.90	6.80	6.85	0.05	6.74
5	−1	−1	+1	3.00	3.10	3.00	3.03	0.058	3.02
6	+1	−1	+1	6.00	6.10	6.00	6.03	0.058	6.03
7	−1	+1	+1	3.70	3.40	3.50	3.53	0.15	3.61
8	+1	+1	+1	6.60	6.30	6.20	6.37	0.208	6.46

x_1, x_2, x_3 are chromium concentration, EDTA concentration and boiling time, respectively. H (cm) is the experimental peak height and H^* is the calculated value from the fitted model. Experimental conditions: −20 kV, 225 nm, 0.05 *M* acetate and 0.5 m*M* TTAB carrier solution (pH 5.5). A fused-silica capillary of 46.5 cm × 0.05 mm I.D. was used.

proved insignificant. On replacing the coded variables with real variables, the fitted model was

$$H = 23.58 + 35.62[\mathrm{Cr}] - 5.87[\mathrm{E}] - 1.23t + 12.29[\mathrm{Cr}][\mathrm{E}] + 0.38[\mathrm{E}]t + 2.13[\mathrm{Cr}]t - 0.66[\mathrm{Cr}][\mathrm{E}]t \quad (3)$$

where [E] = EDTA concentration, [Cr] = chromium concentration and t = boiling time.

From the factorial experiment investigation, we concluded that the excess EDTA for low concentrations of Cr(III) has a significant effect on promoting the attainment of equilibrium. It seems that this complex will dissociate into chromium cation and EDTA at low excess concentrations of EDTA. Boiling for 10 min was sufficient for completion of the reaction. Good agreement between the experimental design conclusions and the univariate method was obtained. For an EDTA to metal molar ratio of 5 and higher and with boiling for 5–10 min the chelate was formed quantitatively. It was also

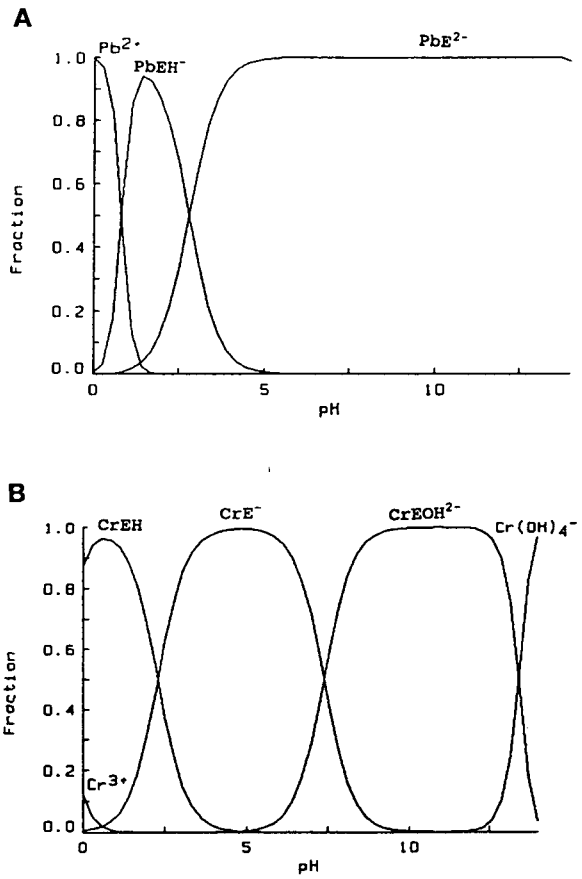

Fig. 1. Distribution species–pH diagrams. Total metal concentration, 20 μg/ml. (B) For Cr–EDTA complex; similar diagrams were obtained for Fe–EDTA and Al–EDTA. (A) For Pb–EDTA; similar diagrams were obtained for Cu–EDTA and Zn–EDTA. E = EDTA.

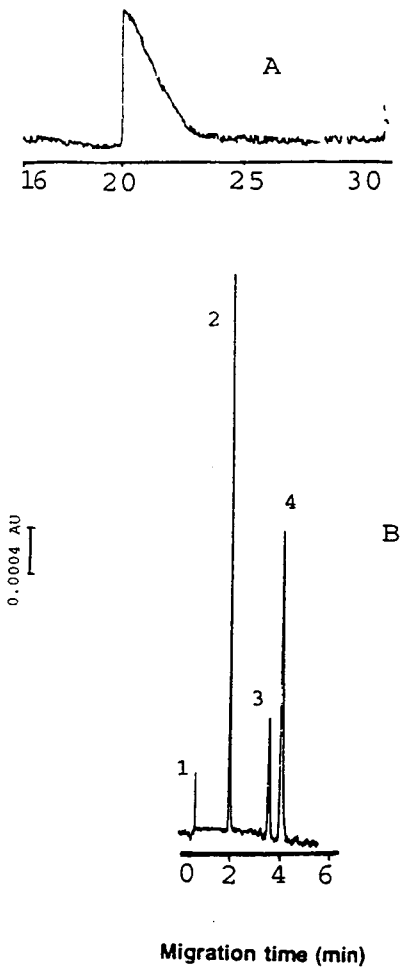

Fig. 2. Electropherogram obtained at 20 kV, negative voltage applied. Detection wavelength, 225 nm; fused-silica capillary (46.5 cm × 50 μm I.D.); 0.05 *M* acetate buffer (pH 5.5); sampling time, 2 s (A) No TTAB, 40 μg/ml Cr(III); (B) 0.5 m*M* TTAB in carrier solution, 20 μg/ml of each metal. Peaks: 1 = NO_3^-; 2 = Cu–EDTA, Pb–EDTA, EDTA; 3 = Cr–EDTA; 4 = Fe–EDTA.

observed that the formation of the $[Cr–EDTA]^-$ complex at room temperature lasted several days, which has also been reported on the literature [14]. We observed that, after 3 h at 60°C, the peak height on the electropherogram was smaller than that obtained for the solution boiled for 10 min.

3.2. Detection of metal complexes

The distribution species–pH diagrams (Fig. 1) showed that in the pH range 4.0–6.5, the following species were present: $[Cr–EDTA]^-$, $[Fe–EDTA]^-$, $[Al–EDTA]^-$, $[Zn–EDTA]^{2-}$, $[Cu–EDTA]^{2-}$ and $[Pb–EDTA]^{2-}$. This information was in good agreement with the peaks obtained in the electropherograms. The maxima of the absorption spectra of these species were at the wavelengths 225 nm for $[Cr–EDTA]^-$, 260 nm for $[Fe–EDTA]^-$ and $[Cu–EDTA]^{2-}$ (both broad shaped) and 245 nm for $[Pb–EDTA]^{2-}$, while $[Zn–EDTA]^{2-}$ and $[Al–EDTA]^-$ absorb only negligibly in the range 190–215 nm. The molar absorptivities of $[Cr–EDTA]^-$, $[Cu–EDTA]^{2-}$, $[Pb–EDTA]^{2-}$ and $[Fe–EDTA]^-$ were 2000–4500 $dm^3\ mol^{-1}\ cm^{-1}$. The highest value was found for iron. EDTA also absorbed in the aforementioned range, having a maximum at 202 nm. In our experiments, detection was generally carried out at 225 nm, as the complexes studied can be determined simultaneously. Nevertheless, depending on the solute, a suitable wavelength can be chosen in order to achieve the highest sensitivity.

3.3. Modification of electroosmotic flow

Table 2 shows the migration times of the chelates and of methanol for different buffer and TTAB concentrations. The electrophoretic mobilities of $[M–EDTA]^-$ (trivalent metals) or $[M–EDTA]^{2-}$ (divalent metals) are higher than and in the opposite direction to those for electroosmotic flow. Without TTAB in the carrier solution, the peak shapes obtained were broad and the migration times were long (Fig. 2A). To control the electroosmotic flow-rate, a cationic surfactant (TTAB) was used, which was found to provide sharp peaks for the organic mixture [28]. From the Table 2, it can be seen that without a modifier the electroosmotic mobility decreases when the buffer concentration increases, as the zeta potential is directly proportional to the

Table 2
Comparison of migration times (min) of metal–EDTA complexes and methanol at different buffer and surfactant concentrations

Buffer (*M*)	Complex	TTAB (m*M*)				
		0	0.1	0.2	0.4	0.5
0.025	M–EDTA[a]	7.01	4.35	3.78	3.27	2.84
	Cr–EDTA	46.0	7.82	6.21	4.89	4.03
	Fe–EDTA	>50	9.05	6.99	5.49	4.34
	CH_3OH	6.7	19.63		23.0	11.6
0.05	M–EDTA[a]	6.45	4.06	3.73	2.99	2.64
	Cr–EDTA	20.6	6.9	6.04	4.31	3.83
	Fe–EDTA	34.0	8.09	6.86	4.71	4.04
	CH_3OH	8.4	32.8		14.9	10.60
0.1	Cu–EDTA[a]	5.68	3.82	3.52	2.77	2.57
	Cr–EDTA	13.08	6.27	5.54	3.89	3.50
	Fe–EDTA	17.09	7.93	6.32	4.26	3.80
	CH_3OH	8.50	>35		13.3	9.50

Experimental conditions: −20 kV, except for methanol at TTAB concentrations of 0 and 0.1 m*M* a positive voltage was applied; sampling time, 2 s; 40 μg/ml of each metal. Other conditions as in Table 1.
[a] M is Cu or Pb.

thickness of the double layer, which decreases in concentrated solutions. For up to 0.1 m*M* TTAB, the direction of the electroosmotic flow is still towards the cathode, whereas between 0.2 and 0.3 m*M* the electroosmotic flow-rate is too small as the negative peak of methanol was not obtained even after 40 min. At 0.4 m*M* TTAB the electroosmotic flow is reversed (towards the anode) as the cationic surfactant is absorbed in the inner wall of the capillary, becoming the dominant factor in determining the interfacial zeta potential. It was verified that at 0.5 m*M* TTAB the micelles were not formed. Hence TTAB offered an improvement in the peak shape (Fig. 2B) and made the analysis faster.

3.4. Separation of metal complexes

At 0.05 *M* buffer and 0.5 m*M* TTAB as the carrier solution, the peaks obtained were sharp, the migration times short and the results reproducible. However, under such conditions the separation of EDTA from $[M–EDTA]^{2-}$ (M = Pb, Cu) was not possible, even when a negative voltage of 30 kV was applied. However, at 0.1 *M* of buffer and 0.1 m*M* TTAB as the carrier solution, the separation was achieved when the voltage applied was ≥23 kV (Fig. 3). We believe that two factors contributed to making the peaks sharper (increasing the resolution) at higher concentrations of carrier solution: (1) the stacking effect and (2) a lower electroosmotic flow-rate. However, under the conditions studied it was not possible to separate $[Cu–EDTA]^{2-}$ and $[Pb–EDTA]^{2-}$ species. The difference in migration times in the best case was 0.06 s. The determination of these species can be achieved by changing the wavelength. At 270 nm only $[Cu–EDTA]^{2-}$ absorbs, while the sum of $[Pb–EDTA]^{2-}$ and $[Cu–EDTA]^{2-}$ can be determined at 245 nm, at which $[Pb–EDTA]^{2-}$ has the highest sensitivity. However, by using a greater capillary length (80 cm), the separation of $[Cu–EDTA]^{2-}$ and $[Pb–EDTA]^{2-}$ was achieved (Fig. 4) and the resolution was 2.32 for both species. The resolutions was calculated as $R_S = \Delta t/w$, where Δt is the difference in migration times

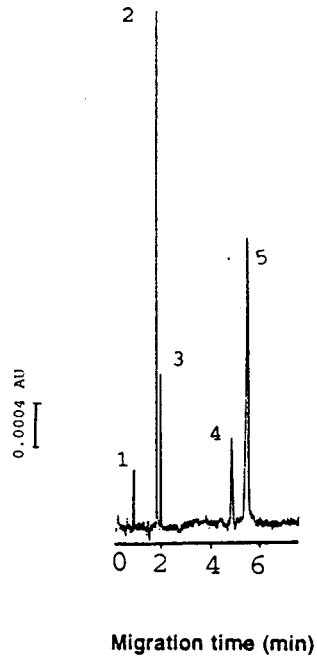

Fig. 3. Electropherogram obtained for a mixture of standard metal solutions in excess of EDTA at −30 kV; 0.1 *M* acetate and 0.1 m*M* TTAB in carrier solution; 20 μg/ml of each metal ion. Other conditions as in Fig. 2. Peaks: 1 = NO_3^-; 2 = EDTA; 3 = Cu–EDTA, Pb–EDTA; 4 = Cr–EDTA; 5 = Fe–EDTA.

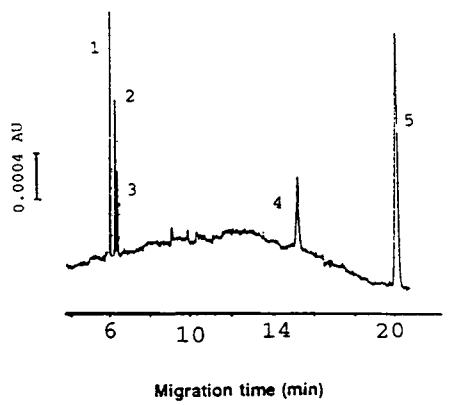

Fig. 4. Electropherogram obtained for a mixture of standard metal solutions in excess of EDTA. Fused-silica capillary (80 cm × 50 μm I.D.); 40 μg/ml Cu and Pb, 20 μg/ml Cr and Fe. Other conditions as in Fig. 3. Peaks: 1 = EDTA; 2 = Cu–EDTA; 3 = Pb–EDTA; 4 = Cr–EDTA; 5 = Fe–EDTA.

between two peaks and w is the width of each peak [29].

3.5. Calibration graphs, detection limit and reproducibility

The calibration graphs obtained for metal complexes at their respective maximum wavelengths were linear up to 60 mg/l, using peak areas (PA) or peak heights (PH), when the sampling time was 2 s. The correlation coefficients were 0.997–0.999 ($n = 6$). The detection limits calculated [30] were 16 μM for Cr, 6.4 μM for Fe, 15 μM for Cu and 27 μM for Pb. At 225 nm the sensitivity of Fe, and Pb determination decreased by 30%, whereas for Cu it decreased by 20%.

The relative standard deviations of peak heights and peak areas for successive injections were found to be less than 6%, and for the migration time it was below 1% ($n = 8$). The mean values obtained for PH, PA and migration times (t_r) with their standard deviations (S.D.) and electrophoretic mobilities (μ_{ep}) are given in Table 3.

In a CZE system, the main factors affecting the separation are the buffer composition, pH and applied voltage. Different voltages in the range 10–30 kV (negative power supply) were applied. The slopes increased with decreasing voltage, but the peaks were broader and the resolution worse. On increasing the voltage, the peaks obtained became sharper and the resolution much better. For all applied voltages, the calibration graphs obtained by regression analysis were linear with correlation coefficients of 0.994–0.999.

The sampling time when a 20 μg/ml [Cr–EDTA]$^-$ standard solution was injected was varied from 1 to 65 s. The PH increased linearly over the sampling time from 1 to 10 s ($r = 0.992$), whereas the PA increased from 1 to 65 s ($r = 0.996$). On increasing the injection interval, the peaks became broader and the separation efficiency was poorer, at least for the peaks that have comparable migration times, as is the case with EDTA, Cu and Pb complexes. The sample and carrier solution had similar conductivities (0.05 M buffer concentration of sample and carrier solution), so the stacking effect was not present to improve the peak shapes. On the other hand, it appears that the injected sample volume can be increased when the buffer is 0.1 M and the high voltage applied is around −30 kV. In these cases, two factors with opposite effects affect the sensitivity, and these parameters could be optimized to achieve the optimum conditions.

3.6. Application to waste waters from tanning industries

A suitable volume of the unknown sample was transferred into a 100-ml erlenmeyer flask and the pH was adjusted to 5.5 by adding ca. 15 ml of 0.1 M acetate buffer. Then 0.2 g of EDTA was added and the mixture boiled for 10 min. The violet colour of [Cr–EDTA]$^-$ formed very rapidly. After allowing the solution to cool it was transferred quantitatively into a 50-ml measuring

Table 3
Results obtained for migration time (t_R), peak heights (PH), peak areas (PA) (mean ± S.D.) and electrophoretic mobility (μ_{ep}) with successive injections ($n = 8$)

Complex	t_R (min)	PH (cm)	PA	μ_{ep} (10^{-4} cm^2/v·s)
Cu–EDTA[a]	2.64 ± 0.011	4.8 ± 0.22	9987 ± 594	−3.4
Cr–EDTA	3.83 ± 0.013	4.5 ± 0.21	9789 ± 520	−2.0
Fe–EDTA	4.04 ± 0.014	11.5 ± 0.43	27178 ± 1426	−1.8

Experimental conditions: 40 μg/ml of each metal in excess EDTA solution (1 : 5); 0.05 M acetate and 0.5 mM TTAB in carrier solution. Other conditions as in Table 2

[a] Under such conditions only one peak was obtained for Pb and Cu chelates.

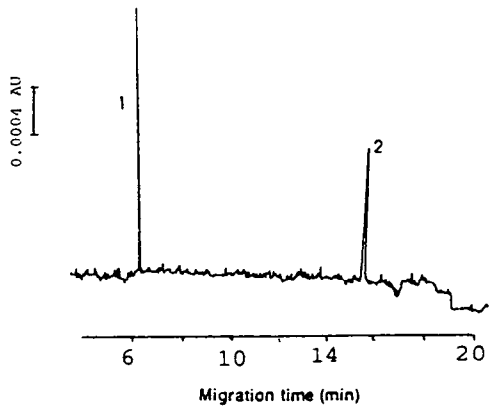

Fig. 5. Electropherogram of a tannery sample obtained at −30 kV; 0.1 *M* acetate and 0.1 m*M* TTAB in carrier solution (pH 5.5); Fused-silica capillary (80 cm × 50 μm I.D.). Sample preparation: diluted, pH adjusted to 5.5, Me–EDTA formed by boiling for 10 min in excess EDTA, filtered, degassed and injected. Peaks: 1 = EDTA; 2 = Cr–EDTA, 26.14 μg/ml Cr(III).

flask and diluted to volume with 0.1 *M* acetate buffer. After homogenization the solution was filtered through a 0.45-μm filter, degassed and injected directly into the CZE system. The final concentration of Cr(III) in the prepared solution has to be 3–60 μg/ml. Depending on the particular application, this procedure can be modified.

Waste waters from a tanning process (Colomer y Munmany, Barcelona, Spain) were analysed. After the appropriate dilution and complexation according to the above procedure, only Cr(III) was found (Fig. 5). The chromium content in the sample was 653.5 μg/ml.

4. Conclusions

The absorptivity curves of $[Cr–EDTA]^-$, $[Fe–EDTA]^-$, $[Cu–EDTA]^{2-}$, $[Pb–EDTA]^{2-}$ and $[EDTA]^{2-}$ showed maxima in the wavelength range 200–270 nm and in the pH range 5–6, which are suitable for analytical purposes. This work demonstrated that CZE provides fast and highly efficient separations of these cations as UV-absorbing chelates with EDTA. Excellent agreement between data taken from distribution diagrams and peaks of the electropherograms were observed. The separation was improved by increasing the capillary length and voltage.

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Ampholytic dyes for spectroscopic determination of pH in electrofocusing

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Abstract

Ampholytic dyes were studied for the purpose of spectroscopic monitoring of the local pH of solutions within capillary systems used for the focusing and separation of ampholytes. The dyes are compounds selected from previously described aminomethylnitrophenols and also a group of compounds based on aminomethylated sulfonphthaleins. The suggested ampholytic dyes are good ampholytes and are also pH indicators with colour transition around the p*I* value. The possibility of the spectroscopic monitoring of actual pH was verified in a system involving pH gradient ion chromatography with diode-array detection and on-line flow-through pH detection.

1. Introduction

In electrophoretic ampholyte focusing, the pH of the analyte environment for a series of compounds to be separated should be known for the experimental determination of the pH gradient and local pH. In ampholyte focusing, this can be achieved, e.g., with help of suitable internal standards or pH (p*I*) markers. Apart from their detectability, the p*I* markers should behave as "good" ampholytes. The term "good" ampholytes means that their effective charge vs. pH dependence should be sufficiently steep close to the marker p*I*. For flat-bed focusing formats, markers based on natural compounds are widely used. These include purified proteins [1–3], carbamylated proteins [4] and proteins stained by dyes [5]. In capillary focusing, it has been suggested that the p*I* be determined via gradient mobilization [6]. In addition to electrophoretic methods, ampholytes can be focused in a pH gradient in an ion-exchange column and by chromatofocusing. Also, focusing of ampholytes in isoelectric focusing field-flow fraction (IEF-FFF) has been described [7]. When performing such experiments in miniaturized format, on-line pH monitoring entails technical difficulties.

In the above free-flow focusing methods and, generally, in capillary analytical methods, on-line spectrophotometric multi-channel optical detection is advantageously used for the solute monitoring. Such detection offers the ability to determine the extent of compound protonation when the spectra of particular forms are sufficiently different from one another. For example, this information was used to calculate the equilibrium constants, pK_a, of pH indicators in proton–ligand systems by flow-injection analysis (FIA) with diode-array multi-dimensional spectral detection [8].

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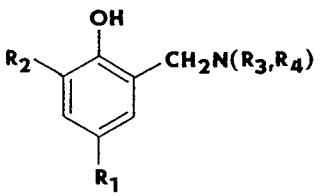
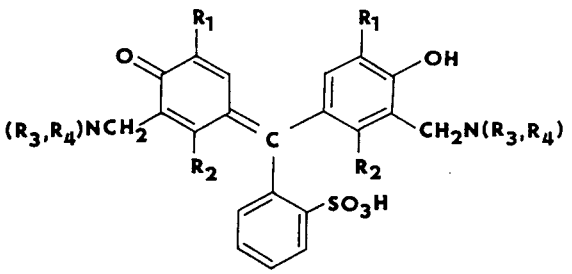
In principle the calculation can be reversed, i.e., the local pH and pH gradient steepness should be available from the monitored spectra provided that the spectral and acid–base characteristics of the compound are known. When applied to ampholyte focusing, the suitable compound should simultaneously be a good ampholyte [9] and a pH indicator with its colour transition close to its p*I*. For spectroscopic pH monitoring near the component p*I*, it is advantageous when the spectra of the isoelectric form and those of the negatively and positively charged forms are very different. In this way, ampholytic dyes that behave also as pH indicators with a colour transition close to the p*I* value may yield a deeper insight into the focusing of ampholytes in free-flow systems.



I

Compounds with selected spectroscopic properties were sought among low-molecular-mass p*I* markers, based on aminomethylated nitrophenols (**I**) [9], which are characterized by sufficient purity, stability and low hydrophobicity. These compounds have been successfully focused both in LC with a pH gradient [9] and isoelectric capillary and preparative free-flow separations [10–13]. Also, the p*I* values of these compounds have been determined independently of IEF [9]. The nitrophenol-based p*I* markers [9] investigated in this work were 4-methyl-6-nitro-2-(4-morpholinomethyl)phenol, 4-nitro-2-(4-morpholinomethyl)phenol and 2-chloro-6-nitro-4-(4-morpholinomethyl)phenol (see Table 1).

In this paper, a group of low-molecular-mass dyes was developed and investigated, namely aminomethylated sulfonphthaleins of general formula **II**, where groups R_1–R_4 are explained in Table 1.



II

The structures of these compounds can be regarded as modified known acid–base and complexometric indicators. Thus, the design of the aminomethylated sulfonphthaleins was based on well known indicators such as phthalein complexone [14], fluorexone [15], xylenol orange and other sulfonphthaleins [16,17], the acid–base properties of which have been reviewed [18,19].

Table 1
Structures of the studied ampholytic dyes of general formulae **I** and **II**

Formula	No.	R_1	R_2	$N(R_3R_4)$[a]	M_r
I	**1**	CH_3	NO_2	MOR	289
	2	NO_2	H	MOR	275
	3	4-$CH_2N(R_3R_4)$	2-Cl-6-NO_2	MOR	309
II	**4**	CH_3	H	MOR	561
	5	$CH(CH_3)_2$	CH_3	MOR	665
	6	$CH_2N(R_3R_4)$	H	MOR	679
	7	$CH(CH_3)_2$	CH_3	PIP	661
	8	$CH_2N(R_3R_4)$	H	PIP	671

[a] MOR = 4-Morpholinyl; PIP = 1-piperidyl.

Spectroscopic and acid–base studies [20–22] indicated several colour transitions with marked changes in the spectra over a broad pH range. Such spectral properties were also expected for the compounds developed here.

Additionally, the investigated ampholytic dyes included methyl red, which is a common pH indicator and p*I* marker, and 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine, used previously in IEF-FFF experiments [7]. The possibility of the spectroscopic monitoring of actual pH profiles was verified using a system involving pH gradient ion chromatography with diode-array detection (DAD) and on-line flow-through pH detection.

2. Experimental

2.1. Materials

The preparation and characterization of 4-methyl - 6 - nitro - 2 - (4 - morpholinomethyl)-phenol (**1**), 4-nitro-2-(4-morpholinomethyl)-phenol (**2**) and 2-chloro-6-nitro-4-(4-morpholinomethyl)phenol (**3**) were described previously [9]. The new compounds (Table 1) were 3′,3″ - bis(4-morpholinomethyl) - *o* - cresolsulfonphthalein (**4**), 3′,3″ - bis(4 - morpholinomethyl)-thymolsulfonphthalein (**5**), 3′,3″,5′,5″ - tetrakis(4 - morpholinomethyl)phenolsulfonphthalein (**6**), 3′,3″ - bis(1 - piperidinomethyl)thymolsulfonphthalein (**7**) and 3′,3″,5′,5″ - tetrakis(1 - piperidinomethyl)phenolsulfonphthalein (**8**). They were prepared from the commercially available sulfonphthaleins phenol red, *o*-cresol red and thymol blue (Fluka, Buchs, Switzerland) by means of the Mannich reaction [17,23]. The appropriate amine (50 mmol) was added portionwise, with cooling, to 37% aqueous formaldehyde (60 mmol) in 25 ml of ethanol. After addition of sulfonphthaleins (50 mmol), the reaction mixture was heated under reflux for 10 h. The aqueous ethanol was removed under reduced pressure, the residue was dissolved in 25 ml of methanol and 5 ml of concentrated hydrochloric acid were added portionwise to the resulting solution. After cooling, the products were separated by filtration or removal of the solvent under reduced pressure. The isolated hydrochlorides were recrystallized from methanol or aqueous ethanol. The purity of all compounds was checked by TLC and ion-exchange liquid chromatography with a pH gradient and diode-array UV–Vis detection [24].

The azo dyes methyl red (**9**) and 4-(4′-hydroxyphenylazo) - 1 - naphthylethylenediamine (**10**), both from Lachema (Brno, Czech Republic) were used as received.

2.2. Liquid chromatography

The conditions for ion-exchange chromatography with a wide-range pH gradient were described previously [9,24]. A PU 4100M liquid chromatograph (Philips, Cambridge, UK) equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) and a PU 4021 multi-channel detector (Philips) were used. Data collection and post-run evaluation were controlled by PU 6003 v.3.0 diode-array detector software (Philips). The actual pH profile of the column effluent was monitored with an OP-0745P pH capillary flow-through electrode connected to an OP-208/1 pH meter (Radelkis, Budapest, Hungary) and a line recorder. A 150×2 mm I.D. Separon HEMA-BIO 1000 Q ion-exchange column (Tessek, Prague, Czech Republic) was used as received. The alkaline buffer (A) was an aqueous solution of 10 m*M* each of piperazine, L-histidine, ethylenediamine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 20 m*M* ammonia solution. The pH of buffer A was adjusted to 10.1 with 2 *M* potassium hydroxide solution. The acidic buffer (B) was 1.0 *M* formic acid. Chemicals used for the buffer preparation were obtained from Fluka and Merck (Darmstadt, Germany). The dyes were sampled in the alkaline buffer in a sample volume of 10 μl.

2.3. Determination of pK_a, *pI* and $[-dz/d(pH)]$

The acid–base properties of the prepared compounds **4–8** were evaluated by potentiomet-

ric titration using an MS 22 pH meter (Laboratory Instruments, Prague, Czech Republic), equipped with a Model 01-29 combined glass pH electrode (Crytur, Turnov, Czech Republic). The instrument was calibrated by means of commercial standard buffer solutions (Institute of Sera and Vaccines, SEVAC, Prague, Czech Republic). The temperature during titration was kept at 23°C. The titration curves obtained were evaluated both graphically and numerically to obtain pK_a, pI and $-dz/dpH$ values for each compound. The $-dz/dpH$ values reported in Table 2 were the tangents of the found pH dependence of the effective charge of the compound at the isoelectric point. Curve fitting was carried out with the program Eureka V. 1.0 (Borland, Scotts Valley, CA, USA). The whole procedure for pK_a determination was verified by determination of the pK_a of L-histidine monohydrochloride (Reanal, Budapest, Hungary) as a standard. The differences between the determined and tabulated [25] pK_a values of L-histidine were less than 0.1 pH unit.

2.4. Spectroscopy

The absorptivity in aqueous buffer solutions with pH corresponding to the compound pI value was determined with a Series 634 UV–Vis spectrophotometer (Varian Techtron, North Springvale, Australia). Spectra of some compounds were treated using LETAGROP PC software [26,27] to obtain pK_a values and the spectra of the individual protonated forms. Examples of the spectra obtained are shown in Figs. 1 and 2.

2.5. Determination of log P_{ow}

The partition coefficient between 1-octanol and water, P_{ow}, was determined spectroscopically by the shake-flask method as described [28]. The log P_{ow} values presented in Table 2 correspond to the pH of the water-rich phase equal to the pI value of the respective dye. The pH of the water-rich phase was adjusted with 0.1 M phosphate buffer. The absorptivity of the water-rich phase was determined at its λ_{max} in the visible spectrum; the solution was equilibrated for 3 h at 25°C with a known amount of water-saturated 1-octanol and the absorptivity of the aqueous phase was measured again.

3. Results and discussion

3.1. Characteristics of dyes

Nitrophenols

From the set of nitrophenol-based pI markers [9], compounds **1–3** (see Tables 1 and 2), were chosen owing to the variation of their spectra over a broad pH range; see Fig. 1a, where spectra of compound **1** are shown for several pH values in the range 4–8.6. The spectra of particular protonated forms of compounds **1–3** (see Fig. 2a–c) were extracted from composite spectra vs. pH dependence. The cross-over points of the spectra of positively charged and neutral forms (curves 1 and 2 in Fig. 2) and the spectra of neutral and negatively charged forms (curves 2 and 3 in Fig. 2) correspond to the isosbectic points. Although the difference between the spectra of the anionic and neutral forms of compound **3** is small, the spectra of compounds **1–3** support their applicability for the spectroscopic determination of pH close to the compound pI. In Fig. 2d, the spectra obtained for methyl red forms are depicted for comparison.

The acid–base characteristics summarized in Table 2 are, except where indicated otherwise in the footnotes, those determined by evaluation of the titration curves. Additionally, some dissociation steps can also be reliably evaluated spectroscopically. A comparison of the values obtained by the different methods can be made from the data summarized in Table 3, which includes the acid–base characteristics of nitrophenol compounds obtained both by titration and spectroscopically. Comparison of the pK_a values determined by different methods shows the need for further work if the pK_a value is to be known reliably with an accuracy of better than 0.1 pH unit. Despite the differences between values obtained by the different methods, the pI values in Table 2 are mostly those derived from the pH titration curves, which make the data more consistent. Moreover, the order of compound

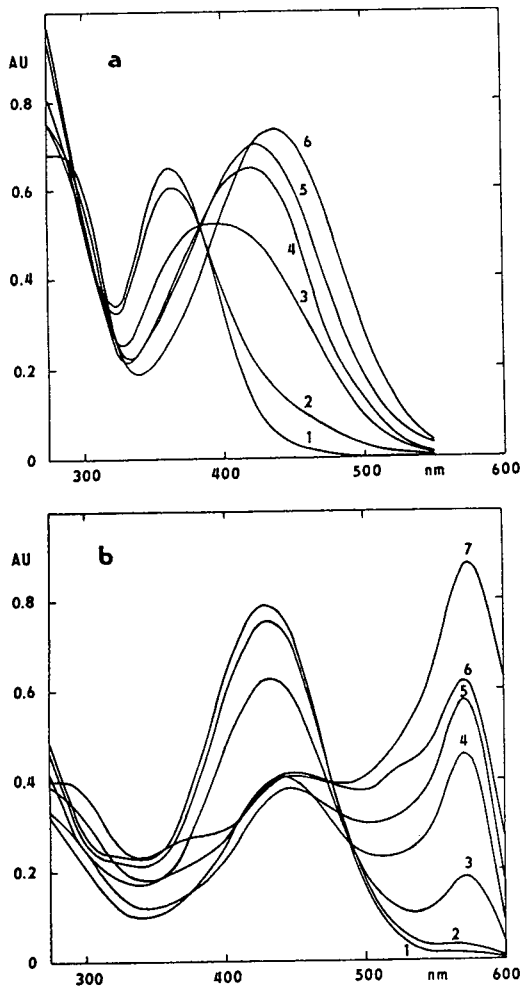


Fig. 1. Absorption curves of ampholytic dyes at various pH values. (a) Compound **1**: pH = (1) 4.03, (2) 5.14, (3) 6.08, (4) 7.08, (5) 7.88, (6) 8.60, Concentration of dye, $1.73 \cdot 10^{-4}$ *M*. (b) Compound **4**: pH = (1) 3.05, (2) 4.04, (3) 5.13, (4) 6.13, (5) 7.08, (6) 7.86, (7) 9.08. Concentration of dye, $6.3 \cdot 10^{-5}$ *M*.

zones focused in capillary IEF corresponds to the order of titration-based values even for p*I* differences of the separated compounds down to 0.1 pH unit [13].

Triphenylmethane dyes

The isoelectric point of compound **4** was determined both by titration (p*I* = 6.15, see Table 2) and by zone electrophoresis [30], which gave a p*I* value of 6.17 together with a $-d\mu/dpH$ value of $9.0 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} pH^{-1} around the compound p*I* value. The dependence of spectra on pH is shown in Fig. 1b. As expected, the complexity of the molecule leads to the several colour transitions. The cationic form is yellow, the anionic form is violet and the isoelectrically focused neutral form appears in the red zone. However, the calculation of the properties of particular protonated forms would probably need a much larger number of measurements than summarized in Fig. 1b. Nevertheless, the lack of acid–base and spectroscopic data for a particular dye form does not hinder its use as a p*I* marker with sought spectroscopic properties. The dye focusing ability, good water solubility, visual detectability and colour vs. pH dependence was appreciated, e.g., in the development of a new capillary electrofocusing method [31].

The phenol red structure enables one to introduce up to four aminomethyl groups, leading to compounds **6** and **8** (see Table 1). The pK_a of the colour contrast yellow to violet transition was determined spectroscopically to be 3.0 in both instances. Throughout the entire pH range from 4 to 12, the spectrum showed no marked changes. Apparently, the dissociation of amino groups is accompanied only by marginal changes in the spectra; the respective pK_a values are in the region where the phenolic group is fully dissociated. On the other hand, the presence of four equivalent amino groups in the compound moiety generates a favourably large $-dz/dpH$ value close to the compound p*I*.

The more basic thymol blue-based indicators (**5** and **7**, Table 1) have their p*I* values in the region where the phenolic group is almost undissociated. This may be the origin of the low absorption coefficients at pH around the dye p*I*. The colour contrast transition is in a more alkaline region than the compound p*I*. The presence of aliphatic substituents causes an increase in log P_{ow} of these derivatives (see Table 2).

Although the number of ampholytic sulfonphthaleins characterized in Table 2 is limited, the values presented show that the suggested ampholytic sulfonphthaleins can have their p*I* in both acidic and alkaline regions. The effective charge vs. pH dependence at the compound p*I*,

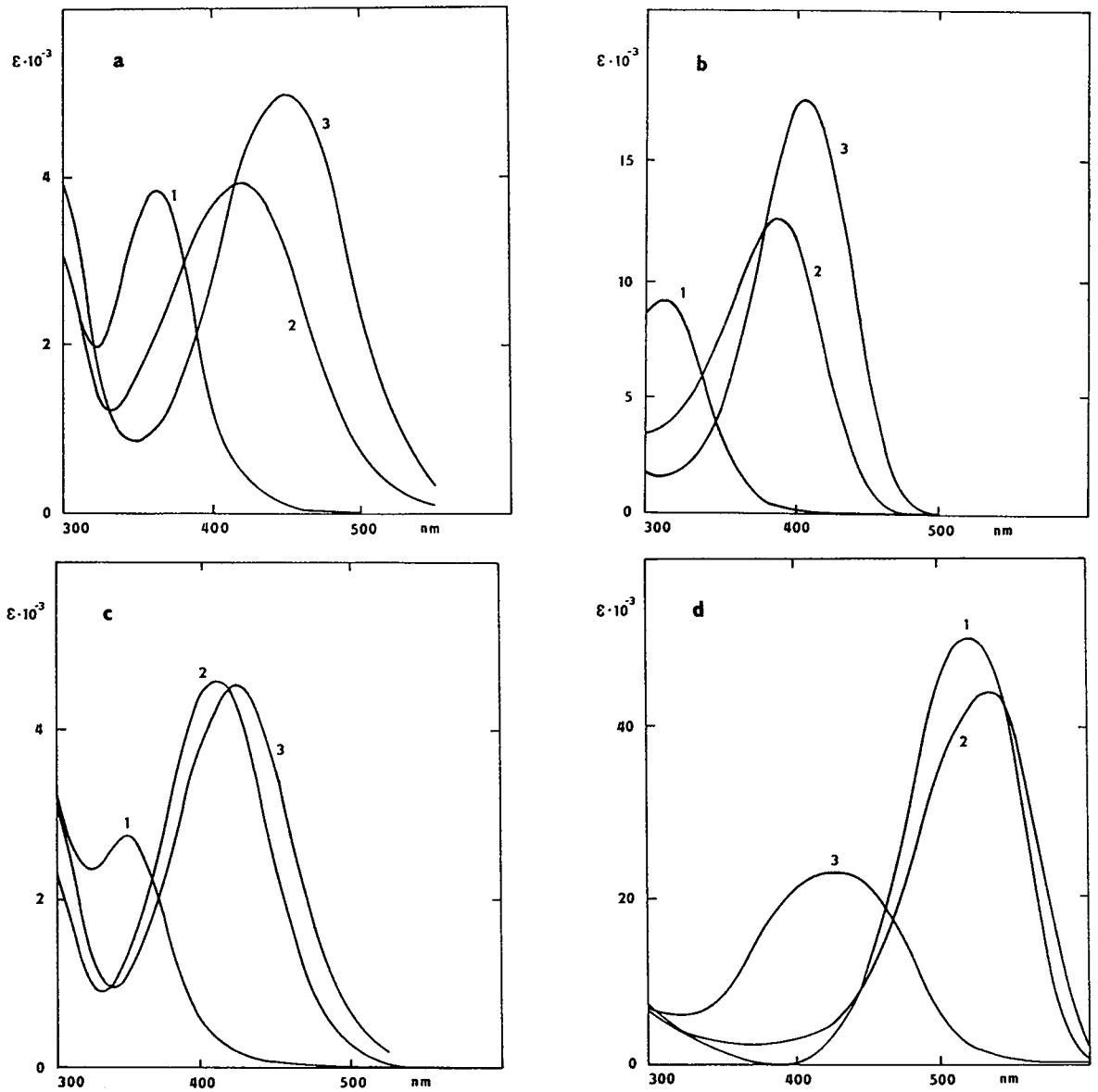


Fig. 2. Absorption curves of particular protonated forms. 1 = Cationic form; 2 = neutral form; 3 = anionic form. Compounds: (a) **1**; (b) **2**; (c) **3**; (d) **9** (methyl red).

$-dz/dpH$, is high enough for all compounds, which gives a good possibility of using them as p*I* markers in electrofocusing experiments. The log P_{ow} values indicate that a low component hydrophobicity can be achieved by a suitable choice of both the starting substrate and introduced amino groups. The low colour intensity of the thymolsulfonphthalein derivatives close to the compound p*I* is surprising.

In comparison with nitrophenol dyes, the spectral transitions of sulfonphthalein dyes are more complicated (see, e.g., Fig. 1b) and close together so that the reliable evaluation of the spectra of different protonated forms would need

Table 2
IEF, spectral and lipophilic characteristics of the studied ampholytic dyes

No.	p*I*	$(-dz/dpH)_{pI}$	λ_{max}(nm)[a]	$A^{1\%}$ [b]	log P_{ow} [c]
1[d]	7.2	0.15	416	162	1.05
2[d]	6.6	0.15	400	562	0.49
3[d]	5.3	0.12	409	142	−0.16
4	6.2	0.48	576	190	0.48
5	7.1	0.16	450	43	1.52
6	7.4	1.01	569	285	−0.01
7	8.1	0.24	582	33	1.09
8	10.3	1.05	570	330	0.88
9[e]	3.9[g]	0.20	523	1440	3.5
10[f]	9.5[g]	0.90	482	640	2.72

[a] Wavelength of absorption maximum in the visible spectrum of aqueous buffer solution at pH equal to the p*I* value.
[b] Absorptivity of a 1% aqueous buffer solution at pH equal to the p*I* value.
[c] Partition coefficient between 1-octanol and water at 25°C.
[d] Values from Ref. [9].
[e] Methyl red.
[f] Azo dye **10** (Ref. [7]).
[g] Determined spectroscopically.

more detailed study. Therefore, the development of a method of characterization similar to that previously described [8] is in progress.

An attempt was made to synthesize coloured ampholytes based on other aminomethylated triphenylmethane indicators. However, they were found to be unsuitable as soluble ampholytic testing dyes or p*I* markers. They included derivatives of phenolphthalein, which form white precipitates close to the pH of the expected p*I* value. The precipitate is soluble to give a colourless solution in acidic medium and a violet solution in alkaline medium. The products of aminomethylation of fluorescein and aurin were composed of a number (up to eight) of components distinguishable by gradient LC. Although they behaved well in electrophoretic focusing experiments, their purification to defined products was unsuccessful.

Table 3
Comparison of potentiometrically determined pK_a and spectroscopically determined pK_a (s) values of the selected nitrophenol dyes

No.	pK_{a_1}	pK_{a_1}(s)	pK_{a_2}	pK_{a_2}(s)
1	5.70	5.66	8.66	8.52
2	5.16	5.42[a]	8.07	8.13[b]
3	3.76	3.94	6.91	−[c]

[a] Ref. [29]: 5.53.
[b] Ref. [29]: 8.02.
[c] Not determined.

Azo dyes

The spectroscopically determined pK_a values of methyl red (compound **9**, see Table 2) are 2.61 and 5.17 ± 0.13 (3σ), which can be compared with the literature values of p$K_{a_1} = 2.6$ [32] and p$K_{a_2} = 5.0$ [18,32]. This indicator is known to be suitable for spectroscopic pH determination [18]. However, the p*I* value of methyl red lies relatively far into the acidic region so that its use as an internal standard in electrofocusing experiments is limited.

Azo dye **10** (see Table 2) has a satisfactory $-dz/dpH$ and its p*I* value of 9.5 fills the gap (8.6–10.1) in the pH range of nitrophenol dyes [9]. The spectroscopically determined pK_a of the colour contrast transition (yellow to violet) is 2.49 ± 0.21, which is far from the compound p*I*. The pK_a of dissociation of the phenolic group, 8.92 ± 0.10, is accompanied by a transition from yellow to orange.

The high hydrophobicity of compounds **9** and

10 can cause some complications in their use, e.g., they may adsorb on the parts of equipment made of plastics.

3.2. On-line spectroscopic pH monitoring

The properties specified above indicate that some of the dyes studied have potential for spectroscopic monitoring of pH by DAD close to the compound p*I*. The relationship between the observed actual spectrum of the compound in the capillary and the local pH can be reliably calculated from sufficiently different spectra and pK_a values of the ionized forms of the indicator. However, the calculations based on spectral and acid–base characteristics of different protonated forms of the indicator are complicated. Tautomeric forms can be expected close to the compound p*I* [29,33]. Further, the specification of individual forms of the sulfonphthalein ampholytes is not available at present. The use of the pH dependence of composite spectra is more straightforward and applicable for all the compounds studied. As the diode-array detector enables one to monitor the absorbance ratio, the pH dependence of the absorbance ratio at wavelengths suitably chosen from the composite spectrum should give sufficient information for continuous pH monitoring. Examples of the pH dependences of the absorbance ratio for selected ampholytic dyes are shown in Fig. 3. Based on the highest steepness obtained for the compounds **1** and **4**, suitable wavelength ratios were chosen, i.e. for **1** as 425/390 nm and for **4** as 475/400 nm (see Fig. 3). The wavelengths for the other two compounds, **2** and **3**, were selected so that the absorbance ratio was almost constant within the pH range of the compound elution.

The applicability of the above compounds in on-line spectroscopic monitoring of the local pH was verified in ion-exchange LC with DAD and a flow-through pH electrode. Indeed, the monitored absorbance ratios for compounds **2** and **3** are almost constant (see Fig. 4). On the other hand, the absorbance ratio within the peaks of compounds **1** and **4** are functions of time and, consequently, of the elution volume. It allows the spectroscopic determination the pH of compound elution and the local slope of the pH gradient. The profile of the local pH obtained graphically with the help of the curves in Fig. 3 is included in Fig. 4 (broken bold lines) together with the pH gradient monitored by the flow-through capillary glass pH electrode (solid bold lines). Corrections were made for the volume difference between the diode-array and on-line pH detectors and for the correlation between the output of the on-line pH detector and that of the pH meter used for off-line pH measurement of solutions used for recording spectra. In both figures, the differences between the spectroscopically and potentiometrically determined local pHs are within a few tenths of a pH unit. Such differences may probably be explained by the complicated comparison of both approaches. On the other hand, the results support the statement that the suggested dyes have potential for on-line spectroscopic pH monitoring close to the dye p*I*. This may be particularly important in miniaturized formats of focusing where changes in pH profiles with time are of interest. The potentiometric measurements of changes in pH profiles may suffer from a high time constant of the electrode response. It should be noted that in

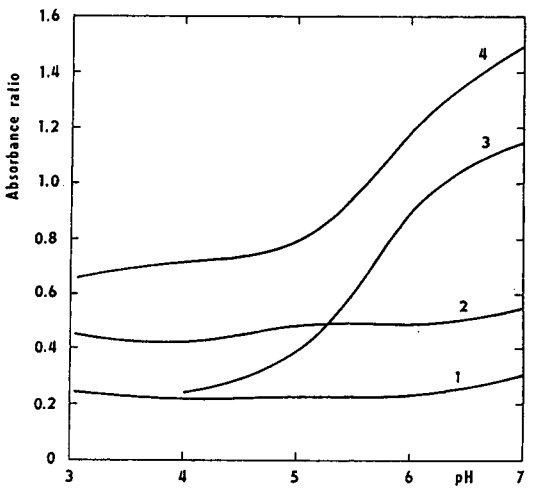


Fig. 3. pH dependences of some absorption ratios for selected dyes. $1 = A_{475}/A_{425}$ for compound **3**; $2 = A_{425}/A_{390}$ for compound **2**; $3 = A_{425}/A_{390}$ for compound **1**; $4 = A_{475}/A_{400}$ for compound **4**.

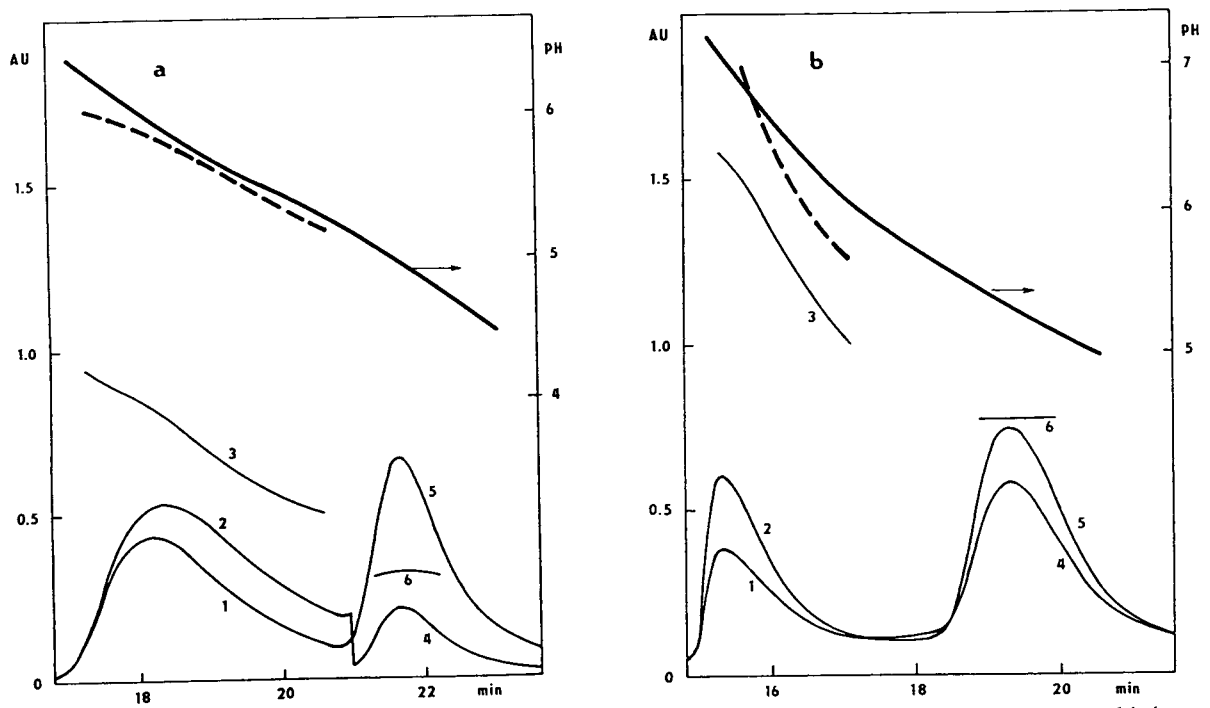


Fig. 4. Chromatograms of selected ampholytic dyes obtained by anion-exchange chromatography with pH gradient, multi-channel spectrophotometric detection and on-line pH detection. Column, 150 × 2 mm I.D. HEMA-BIO 1000 Q, 10 μm; flow-rate, 0.2 ml min^{-1}. Solutions (concentrations in water, m*M*): (A) piperazine 10, L-histidine 10, ethylenediamine 10, Tris 10, ammonia 20 (pH 10.1); (B) formic acid 1000. Gradient steepness: from 0 to 13% (v/v) B in A in 30 min. Spectrophotometric detector: PU 4021 multi-channel diode-array detector. pH detector: OP-0745P flow-through glass capillary electrode (15 μl) with OP-208/1 pH meter. Details of chromatogram of (a) compounds **1** and **3** and (b) compounds **4** and **2**. Curve numbers and corresponding wavelengths and wavelength ratios used for eluent monitoring (nm): (a) 1 = 400; 2 = 475; 3 = 475/400; 4 = 425; 5 = 390; 6 = 425/390. (b) 1 = 425; 2 = 390; 3 = 425/390; 4 = 475; 5 = 425; 6 = 475/425. Full bold lines, pH course monitored by on-line pH detector; Broken bold lines, pH course calculated from absorption ratios and curves 3 and 4 in Fig. 3, respectively.

LC, the pH of the compound elution is generally different from the compound p*I*.

4. Conclusions

The described sulfonphthalein ampholytic dyes have properties suitable for their use as low-molecular-mass p*I* markers. The hydrophilic ampholytic dyes selected from aminomethylnitrophenols and aminomethylsulfophthaleins have been shown to be able to monitor the local pH profile close to a compound p*I* by means of evaluation of collected multi-wavelength spectra. Although a more detailed characterization of the compounds is necessary to enhance the accuracy of the values obtained, the use of the pH dependence of dye composite spectra and the monitoring of selected two-wavelength ratios has the potential for on-line elucidation of the time dependence of actual pH profiles. Application of the selected compounds in capillary electrofocusing is in progress.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Mixed ion-pair high-performance liquid chromatography of uridine 5′-diphospho-α-D-glucuronic acid and its hydrolysis products

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Abstract

Although conventional ion-exchange HPLC and ion-pair reversed-phase HPLC using tetrabutylammonium hydroxide allowed ready determination of uridine 5′-diphospho-α-D-glucose and its hydrolysis products, neither method was suitable for the determination of uridine 5′-diphospho-α-D-glucuronic acid (α-UDPGA). However, mixed ion-pair reversed-phase HPLC, using a combination of tetrabutylammonium hydroxide and tetraethylammonium hydroxide (1:1), has been found to offer a convenient and effective means of simultaneously determining α-UDPGA and its hydrolysis products, UDP, UMP and cUMP.

1. Introduction

During our structure activity studies of glucose transfer [1], it became necessary to develop a method of analysing the glycosylating coenzymes, uridine 5′-diphospho-α-D-glucose (α-UDPG) and uridine 5′-diphospho-α-D-glucuronic acid (α-UDPGA) in the presence of their hydrolysis products, uridine 5′-diphosphate (UDP) and uridine 5′-phosphate (UMP) (Fig. 1). At first, we investigated ion-exchange HPLC [2,3] and ion-pair reversed-phase HPLC [4,5]. As this was only partly successful, mixed ion-pair HPLC [6] was studied.

2. Experimental

2.1. Materials

α-UDPG, α-UDPGA, UDP, uridine 3′,5′-cyclic phosphate (cUMP) and UMP were obtained as sodium salts from Sigma. Tetrabutylammonium hydroxide and tetraethylammonium hydroxide were obtained as aqueous solutions from BDH. All other reagents were of analytical-reagent grade.

2.2. HPLC

The analytical HPLC system was composed of a Laboratory Data Control (LDC) gradient solvent-delivery system, a Rheodyne injection valve fitted with a 20-μl loop, a Pye LC3 spectrophotometric detector operating at 262 nm and a

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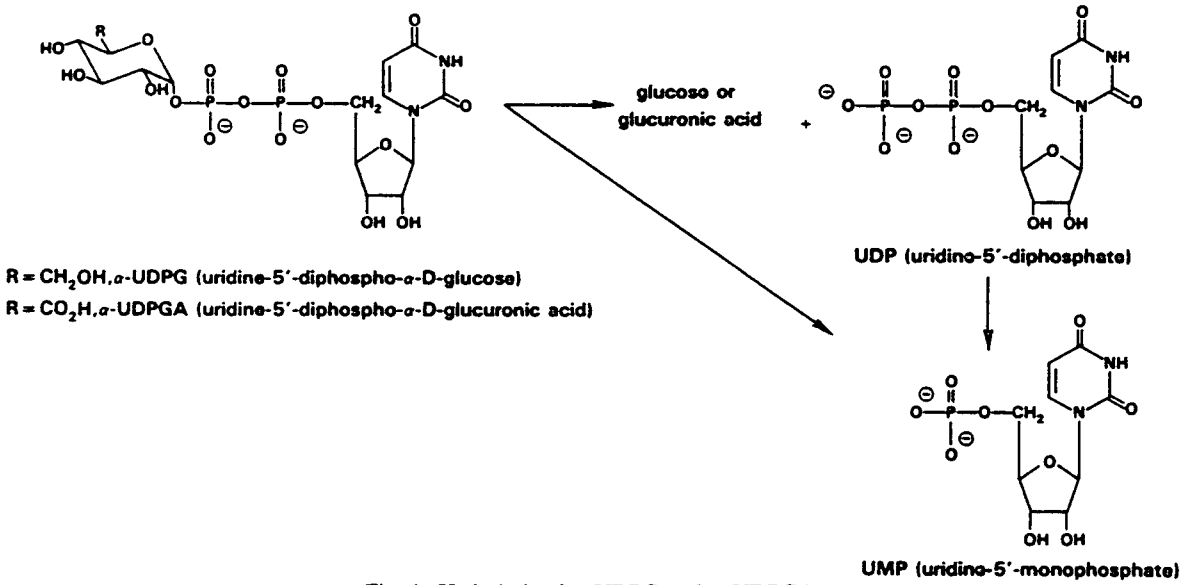


Fig. 1. Hydrolysis of α-UDPG and α-UDPGA.

Hewlett-Packard 338S integrator. The columns (200 × 4.5 mm) were either Whatman Partisil 10 SAX for ion-exchange or Partisil 10 ODS2 for ion-pair reversed-phase chromatography. Ion-exchange analyses were run at room temperature, but ion-pair analyses were run with the column maintained at 30°C by means of a Magnus Scientific water jacket and a Churchill water pump and heater. The flow-rate was 2 ml/min.

3. Results

3.1. Ion-exchange chromatography

Ion-exchange HPLC using a strong anion exchanger was found to give good separation of all the compounds when the sample solution was close to neutrality, as shown in Fig. 2. However, acidic samples (pH > 3) gave poor, and often merged peaks. This, coupled with the degradation of the silica in the columns by the mobile phase, led us to consider ion-pair reversed-phase HPLC.

3.2. Ion-pair reversed-phase chromatography

Employing a C_{18} column and using tetra-

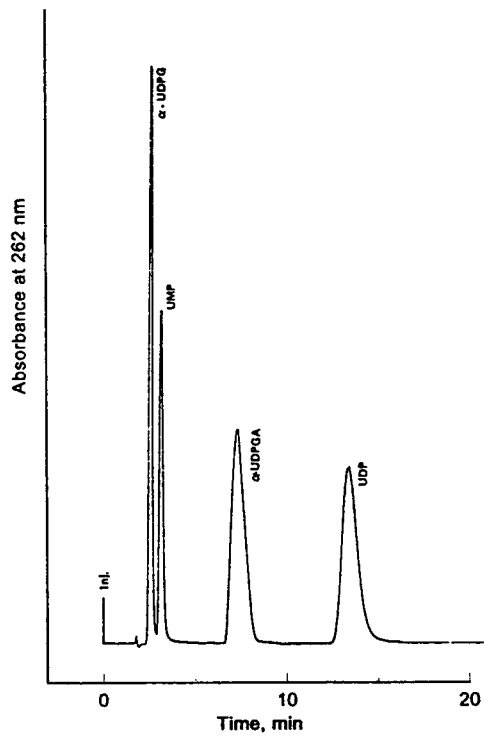


Fig. 2. Ion-exchange HPLC trace of a mixture of α-UDPG, UMP, α-UDPGA and UDP. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 SAX. The mobile phase was 0.05 *M* potassium dihydrogenorthophosphate, 0.05 *M* potassium chloride, adjusted to pH 6 with KOH.

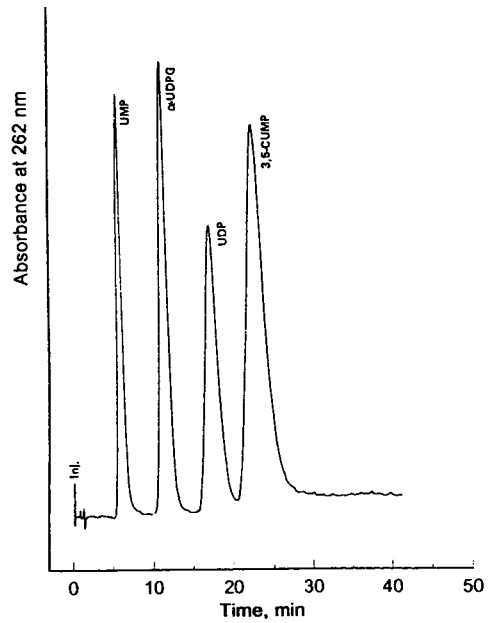


Fig. 3. Ion-pair reversed-phase HPLC trace of a mixture of UMP, α-UDPG, UDP and cUMP. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.0475 *M* tetrabutylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 5% aqueous methanol.

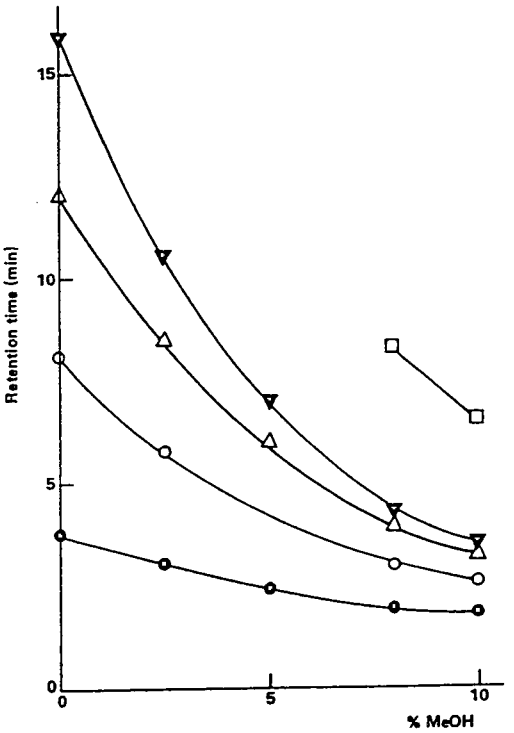


Fig. 4. Effect upon retention time of increasing methanol content of eluent buffer in ion-pair reversed-phase HPLC of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phases were 0.0475 *M* tetrabutylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in water, or in 2.5, 5, 8 and 10% aqueous methanol. □ = α-UDPGA; ▼ = cUMP; △ = UDP; ○ = α-UDPG; ● = UMP.

butylammonium hydroxide as the ion-pairing reagent in a phosphate buffer at pH 6 [4,5], a good separation under isocratic conditions of α-UDPG, UDP and UMP —and of the possible hydrolysis product cUMP— was achieved, as shown in Fig. 3. It was necessary to use methanol (5%, v/v) in the eluent to decrease the retention times to below 30 min. However, α-UDPGA could not be analysed under these conditions, as it was indefinitely retained on the column. In order to reduce the retention time of α-UDPGA the proportion of methanol was increased to 10%. However, since this led to the merging of the peaks due to UDP and cUMP, as shown in Fig. 4, an alternative method was sought. When tetrabutylammonium hydroxide was replaced with tetraethylammonium hydroxide the retention time of α-UDPGA was lowered to 6 min with no methanol added. Unfortunately, this caused UMP and UDP to merge together at a retention time of 2.5 min. This led to the development of a system using a mixed ion pair, similar to that used by Au et al. [6] for the analysis of fluorinated nucleotides.

Using various ratios of tetrabutylammonium hydroxide and tetraethylammonium hydroxide in the eluent allowed the retention times of all the phosphates to be varied to different degrees, as shown in Fig. 5. The best mobile phase for an isocratic system, which utilised the two reagents in equal proportions, effected a relatively rapid and efficient separation of all five phosphates, as shown in Fig. 6.

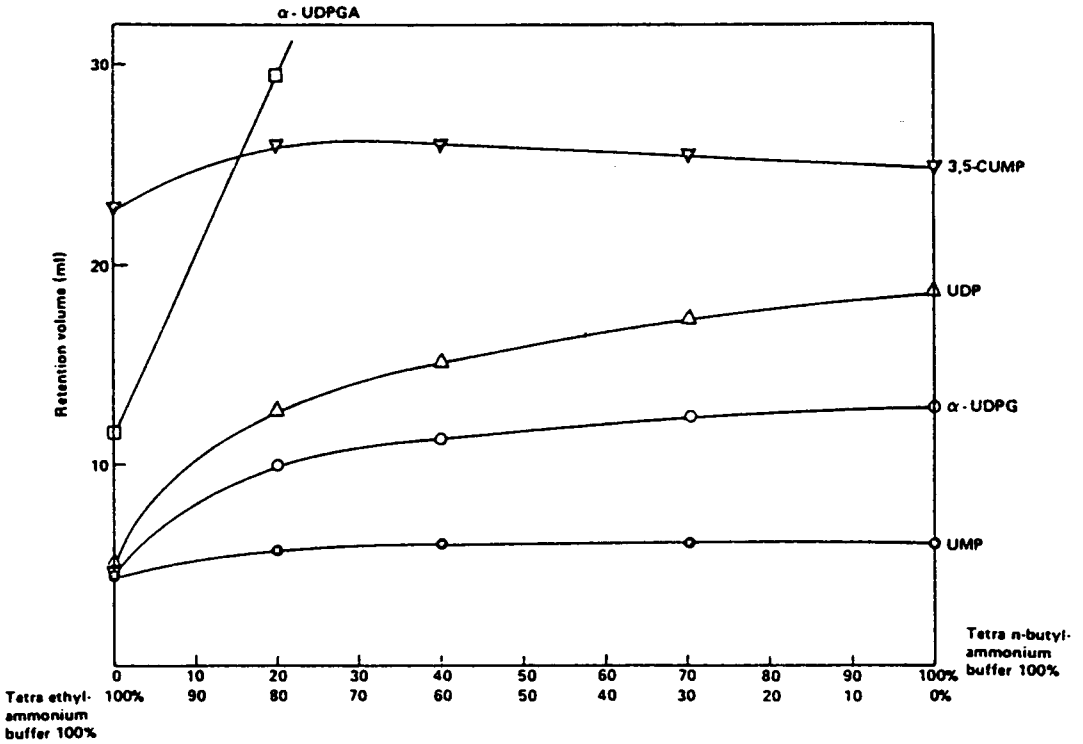
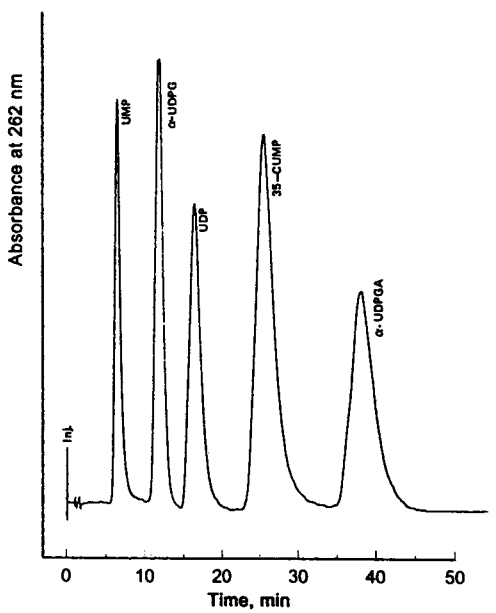


Fig. 5. Effect of the composition of mixtures of tetrabutylammonium hydroxide and tetraethylammonium hydroxide upon the ion-pair reversed-phase HPLC retention volumes of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 20 μl from a solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. Mobile phase: various ratios (0:100, 20:80, 40:60, 70:30 and 100:0) of 0.002375 *M* tetrabutylammonium hydroxide and 0.002375 *M* tetraethylammonium hydroxide, in 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 10% aqueous methanol.



4. Discussion

Three methods of separating α-UDPG and α-UDPGA and their hydrolysis products have been developed. The use of ion-pair reversed-phase HPLC proved to be most versatile, although not as rapid as ion-exchange HPLC. It was found that varying the ratio of two ion-pairing reagents, tetrabutylammonium hydroxide

Fig. 6. Mixed ion-pair reversed-phase HPLC trace of a mixture of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 10 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.002375 *M* tetrabutylammonium hydroxide, 0.002375 *M* tetraethylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 10% aqueous methanol.

and tetraethylammonium hydroxide, from 0 to 100% (Fig. 5) allowed the relative retention times of all the phosphates to be varied to differing degrees. It is particularly interesting to note the very high sensitivity for α-UDPGA, the only carboxylic acid, to the ratio of the ion-pairing reagents compared to the relatively low sensitivity of all of the other phosphate compounds. At pH 6 this carboxylic group will exist as anionic carboxylate. The coupling of this extra anionic group of UDPGA with tetrabutylammonium clearly, as could be expected, provides highly efficient binding to the C_{18} stationary phase and thereby dramatically lengthens the retention time.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Preparative high-performance liquid chromatographic separation of fluorodeoxy sugars☆

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Abstract

Normal- and reversed-phase preparative chromatography methods were developed to isolate gram quantities of analytically pure 6-amino-2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-9H-purine (arafluoro-2-CdA; RWJ 29727) and its α-anomer (RWJ 48667). The complex reaction mixture (ca. 171 g), from a Parr Bomb synthesis, was prepurified by normal-phase chromatography to yield ca. 40 g. Twelve reversed-phase preparative isolations were run on a custom-packed YMC column to yield ca. 12 g of arafluoro-2-CdA (99.7%) and ca. 3 g of the α-anomer (99.2%).

1. Introduction

Replacement of hydroxyl groups or hydrogen atoms with fluorine atoms provides useful derivatives for blocking or retarding metabolic processes [1–3]. Unfortunately synthesis of these compounds requires vigorous conditions such as the use of hydrofluoric acid at high temperatures [4] which results in complex mixtures. Recently 6-amino-2-chloro-9-(2-deoxy-β-D-arabinofuranosyl)-9H-purine [2-CdA (Cladribine); RWJ 26251; **1**] was approved for the treatment of hairy cell leukemia (Fig. 1). In the early development of 2-CdA, it became of interest to study the pharmacological profile of 6-amino-2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-9H-purine (arafluoro-2-CdA; RWJ 29727; **2**). Normal- and reversed-phase preparative chromatography methods were developed to isolate gram quantities of analytically pure arafluoro-2-CdA and its α-anomer (RWJ 48667; **3**).

2. Experimental

2.1. Synthesis and HPLC chemicals

Arafluoro-2-CdA was synthesized by the following procedure. 2-O-(Imidazolylsulfonyl)-1,3,5-tri-O-benzoyl-α-D-ribofuranose (Pfanstiehl, Waukegan, IL, USA) was treated with 48% hydrofluoric acid in a Monel Parr Bomb at 170°C to yield 2-deoxy-2-fluoro-1,3,5-tri-O-benzoyl-α-D-arabinofuranose. This fluoro sugar was

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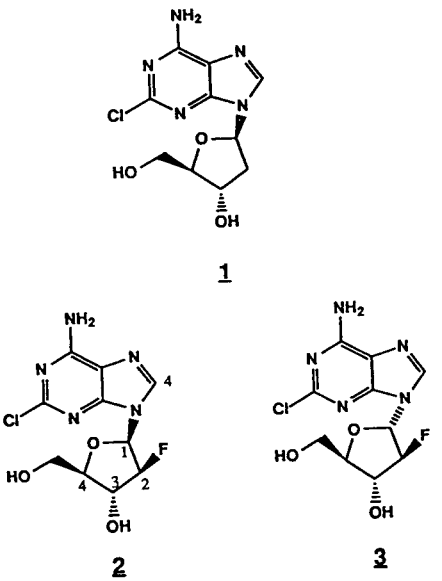

Fig. 1. Structures of 2-CdA (RWJ 26251, **1**), arafluoro-2-CdA (RWJ 29727, **2**) and the α-anomer of 2-CdA (RWJ 48667, **3**).

treated in CH_2Cl_2 with 30% HBr in acetic acid dropwise over 20 min and stirred at room temperature for 16 h. The reaction mixture was cooled and adjusted to pH 8 with saturated $NaHCO_3$ solution. The organic phase was separated, washed with water, brine and dried with Na_2SO_4 to give 2-deoxy-2-fluoro-3,5-di-O-benzoyl-α-D-arabinofuranosyl bromide. 2,6-Dichloropurine was dissolved in CH_2Cl_2 and treated with 60% NaH under a nitrogen atmosphere at room temperature. Dimethylformamide was added and after 15 min the previously prepared bromide compound was added and allowed to stir for 48 h. The reaction mixture was filtered through a bed of Celite and concentrated to give a crude oil. This oil was suspended in CH_3OH and treated with concentrated NH_4OH in a Parr Bomb at 80°C for 16 h. The resulting reaction produced a highly impure crude oil containing **2** and **3**. Reactants were obtained from Aldrich, Milwaukee, WI, USA.

HPLC-grade water, CH_2Cl_2, CH_3OH and CH_3CN were obtained from Fisher Scientific (Springfield, NJ, USA).

2.2. Chromatography

A complex mixture (171 g) containing **2** was prepurified via normal-phase chromatography. The sample was dissolved in 1 l of CH_2Cl_2–CH_3OH (95:5) with heat and stirring. The sample was divided into three parts and each aliquot was separated on a Waters Prep 500A (Milford, MA, USA) using two new Waters PrepPak-500 silica gel 60 cartridge columns equilibrated with CH_2Cl_2–CH_3OH (95:5). All fractions were checked by TLC (Merck silica gel 60 F_{254}, 10 × 5 cm, precoated plates, layer thickness 250 μm, Gibbstown, NJ, USA) in CH_2Cl_2–CH_3OH (90:10). Fractions analyzing as a single spot on the TLC plates were combined and concentrated in vacuo. Subsequent analytical reversed-phase HPLC analysis of the sample showing one spot by TLC was carried out on a YMC ODS column (Wilmington, NC, USA) (250 × 4.6 mm, s-15, 120 Å, C_{18}) with UV detection at 254 nm using an isocratic mobile phase of CH_3CN–water (10:90) at a flow-rate of 1.0 ml/min (Fig. 2). The material was determined to be a mixture of 52.2% **2** and 43.0% **3** rather than a single component as indicated by TLC. The normal-phase prepurification chromatography yielded approximately 40 g of material to be further purified by reversed-phase chromatography.

Reversed-phase preparative chromatography was performed using a Waters Prep 500A instrument connected to a 500 × 50 mm stainless-steel column custom packed with 500 g of YMC ODS (s-15/30, spherical, 120 Å, C_{18}). The flow-rate was maintained at 50 ml/min. Samples, ranging from 3 to 5 g, were dissolved in CH_3CN–water (10:90), warmed, sonicated and filtered. The sample was then loaded onto the YMC column equilibrated with CH_3CN–water (10:90). Fraction collection was monitored by a Waters Lambda Max Model 481 LC spectrophotometer at 254 nm. A step gradient to CH_3CN–water (50:50) was utilized to collect fractions determined to be 99.7% pure arafluoro-2-CdA and 99.2% pure α-anomer (Figs. 3 and 4).

Analytical HPLC was recorded using a Waters 486 tunable absorbance detector, and a Waters 740 data module. A Beckman 110 solvent-deliv-

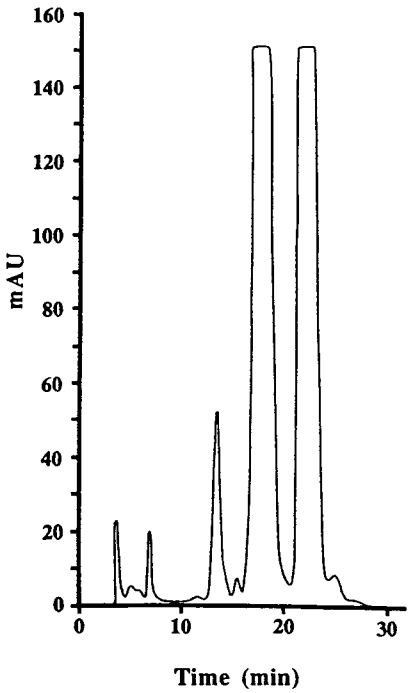

Fig. 2. HPLC of mixture of arafluoro-2-CdA (retention time 18.9 min, area percent 52.2) and the α-anomer (retention time 23.0 min, area percent 43.0) after normal-phase prepurification. Other peaks were not identified.

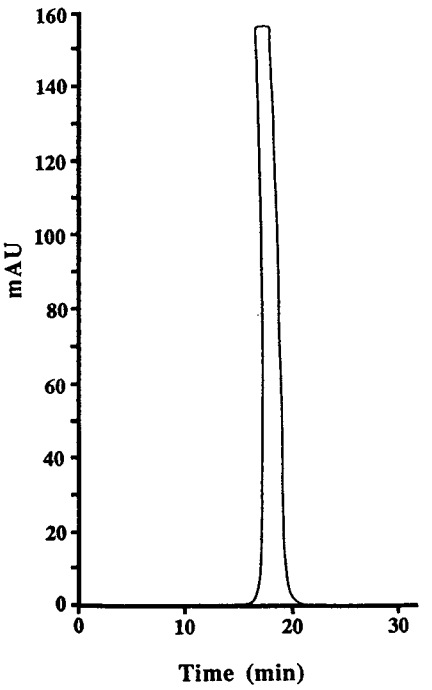

Fig. 3. HPLC of arafluoro-2-CdA (retention time 18.5 min, area percent 99.7) after reversed-phase purification.

ery module (Fullerton, CA, USA) was employed as a pump.

2.3. Spectroscopy

Chemical ionization (DCI-CH_4) mass spectra were obtained on a Finnigan Incos 50 mass spectrometer (San Jose, CA, USA).

The ^1H NMR experiments were performed on a Bruker AM-400 (400.13 MHz) Fourier transform spectrometer (Villerica, MA, USA) equipped with a 5-mm inverse broad band probe. The samples were dissolved in [$^2H_6$]dimethyl sulfoxide (DMSO) at ca. 10 m*M* in 5-mm tubes at 298 K. The chemical shifts were referenced to internal tetramethylsilane (TMS). Nuclear Overhauser Enhancement (NOE) difference experiments were performed and processed using standard Bruker software. Typical operating conditions have been previously reported [5].

3. Results and discussion

The reversed-phase chromatogram of the reaction mixture is shown in Fig. 2. It is noted that the majority of the impurities were removed by the normal-phase chromatography prepurification step. Reversed-phase preparative isolation of each component was accomplished and the results are shown in Figs. 3 and 4. The two major peaks at 18.9 and 23.0 min were identified as **2** and **3**, respectively. Twelve reversed-phase preparative isolations were necessary to produce ca. 12 g of **2** (99.7%) and ca. 3 g of **3** (99.2%).

Both compounds were fully characterized by mass spectral and NMR analysis. The mass spectra of **2** and **3** were almost identical with each producing an $[M+H]^+$, $[M+C_2H_5]^+$, $[M+C_3H_5]^+$, $[MH-Cl]^+$, $[MH-C_4H_7O_2F]^+$,

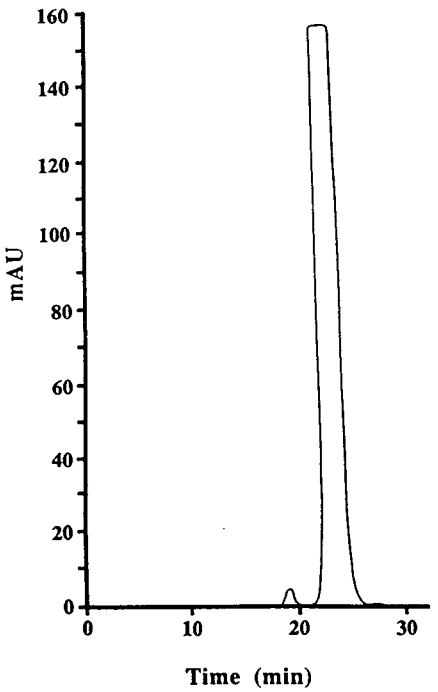

Fig. 4. HPLC of α-anomer (retention time 24.0 min, area percent 99.2) after reversed-phase purification. Arafluoro-2-CdA (retention time 19.2 min, area percent 0.6) is present in this sample.

$[MH - C_5H_7O_3F]^+$ and $[MH - C_5H_8O_3F]^+$ cation. It was interesting to note that **3** showed a significantly greater yield of the $[MH - F]^+$ cation than **2**. The ^1H NMR spectra of **2** and **3** showed only one anomer which was unambiguously assigned. NOE difference experiments were used to assign the configurational preference about the C-1 carbon of the fluoro sugar (Fig. 1). Consideration of Dreiding model distances suggested that the H-4 proton of the 2-chloroadenine moiety could be used as a probe in an NOE difference experiment to assign **2** and **3**. When the H-4 proton of the 2-chloroadenine moiety of **2** was irradiated, the H-1 and the H-3 protons of the fluoro sugar showed a strong NOE. These results are consistent with the 2-chloroadenine group being on the same face as the H-3 proton of the fluoro sugar. When the H-4 proton of the 2-chloroadenine moiety of **3** proton was irradiated, the H-1, H-2 and H-4 protons showed an NOE. These results are consistent with the 2-chloroadenine group being on the same face as the H-2 and H-4 protons of the fluoro sugar. The coupling constant between C_1-H and C_2-H for **3** was consistent with an α-anomer, that is, all the couplings were large. The C_2-H proton for **3** appeared at approximately 5.62 ppm as a doublet of triplets with a geminal fluorine coupling of ca. 52 Hz.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

High-performance liquid chromatographic separation and determination of cobalt(II), cobalt(III) and iron(II) using bis(salicylaldehyde)tetramethylethylenediimine

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Abstract

The reactions of bis(salicyclaldehyde)tetramethylethylenediimine (H_2SA_2Ten) with cobalt(II), cobalt(III), iron(II) and iron(III) were studied and conditions for their extraction as metal chelates from aqueous solution into chloroform were optimized. Cobalt(II), cobalt(III) and iron(II) were completely separated on a 3-μm Microsorb ODS column when eluted isocratically with methanol–water–acetonitrile (60:39:1, v/v/v), with spectrophotometric detection at 270 nm. Linear calibrations were obtained over the range 0–125 μg per 2 ml of extract. The detection limits were in the range 0.25–1.0 μg/ml. The oxidation of cobalt(II) to cobalt(III) with air and nitric acid was examined and the method was applied to the determination of cobalt and iron in pharmaceutical preparations. The results obtained were compared with those given by atomic absorption spectrometry.

1. Introduction

A number of complexing reagents have been reported for the high-performance liquid chromatographic (HPLC) determination of cobalt and iron, the main ones being 8-hydroxyquinoline [1], 6-hydroxy-5-nitrosonaphthalene-2-sulphonic acid [2], acetylacetone [3], diethyldithiocarbonate [4], picolinaldehyde-4-phenyl-3-thiosemicarbazone [5], 8-quinolinethiol [6] and various azo derivatives [7–11].

Zhao and Fu [12] separated iron(II), cobalt(II) and iron(III) chelates with 4-(5-chloro-2-pyridylazo)-1,3-diaminobenzene on a C_{18} bonded stationary phase with methanol–water as the mobile phase. Fernandez et al. [13] separated and determined iron(II) and iron(III) by reversed-phase HPLC using in situ complexation with *o*-phenanthroline.

The tetradentate Schiff bases are interesting complexing reagents owing to their reactions towards a limited number of metal ions [copper(II), nickel(II), palladium(II), platinum (II), cobalt, iron, vanadium(IV) and zinc(II)]. Their cobalt and iron complexes have attracted attention because of their possible use as biological models as oxygen carriers [14–16]. Averill and Broman [17] investigated the electroanalytical properties of cobalt chelates of bis(salicylaldehyde)tetramethylethylenediimine [2,3-dimethyl-2,3-N,N′-butanebis(salicylaldiimine); (H_2SA_2Ten]. H_2SA_2Ten has been reported as a

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complexing reagent for the gas chromatographic (GC) and normal-phase HPLC determination of copper(II) and nickel(II) using precolumn derivatization and solvent extraction with toluene [17]. Recently, H_2SA_2Ten has been examined for the simultaneous extraction and separation of copper(II), nickel(II) and oxovanadium(IV) using normal-phase HPLC [19]. In this work, cobalt(II), cobalt(III) and iron(II) were separated and determined using reversed-phase HPLC. The method was applied to the determination of cobalt and iron in pharmaceutical preparations.

2. Experimental

H_2SA_2Ten was prepared by heating freshly distilled salicylaldehyde and 2,3-dimethyl-2,3-diaminobutane in a 2:1 molar ratio as reported [17,18]. The cobalt(II) complex was prepared by refluxing equimolar proportions (0.001 *M*) of cobalt(II) acetate and the reagent in methanol in a nitrogen atmosphere [20] (Fig. 1).

A Hitachi Model 220 spectrophotometer and a Hitachi Model 655 A liquid chromatograph, connected with a variable-wavelength UV monitor, Rheodyne Model 7125 injector and a Hitachi D 2500 Chromato-integrator were used.

A 3-μm Microsorb ODS column (150 × 4.6 mm I.D.) (Jones Chromatography) and a 5-μm Microsorb C_{18} column (Rainin Instruments, Woburn, MA, USA) were used.

2.1. Solvent extraction of cobalt(II) and iron(II)

Into a well stoppered test-tube were transferred 1–5 ml of solution containing 0–125 μg of cobalt(II) or iron(II), and nitrogen was bubbled through the solution. To the mixture was added sodium acetate–acetic acid buffer (pH 6) (2 ml) for iron(II) or sodium hydrogencarbonate buffer (pH 7.5) (2 ml) for cobalt(II), followed by solid ascorbic acid (4–5 mg) and reagent solution (1% w/v in ethanol) (2 ml). The contents were warmed on a water-bath for 10 min. Chloroform (2 ml) was added and the layers were mixed well. An aliquot of extract (1 ml) was transferred into a sample vial and the solvent was removed on a water-bath. The residue was dissolved in methanol (1 ml) and the solution (5 μl) was injected on to the Microsorb ODS column. The complexes were eluted with methanol–water–acetonitrile (60:39:1, v/v/v) at a flow-rate of 0.9 ml/min. Detection was achieved using a UV monitor fixed at 270 nm.

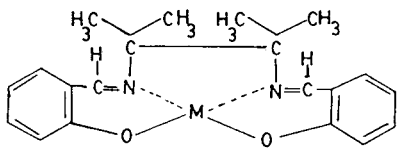

Fig. 1. Structure of metal chelates formed by the reagent.

2.2. Solvent extraction of cobalt(III) and iron(III) for HPLC determination

To an aliquot of solution (1–5 ml) containing 0–150 μg of cobalt or iron was added 37% hydrogen peroxide (0.5 ml) and the solution was evaporated to dryness. The residue was dissolved in ethanol (2 ml) and the procedure in Section 2.1 was followed except that addition of ascorbic acid was omitted.

2.3. Determination of cobalt in vitamin B_{12} syrup

Vitamin B_{12} syrup (Catacon; Glaxo, Karachi, Pakistan) (20 g) was placed in a crucible and potassium hydrogensulphite (0.5 g dissolved in 5 ml of water) was added. The mixture was heated on hot-plate for 15 min, 37% hydrochloric acid (10 ml) and 65% nitric acid (5 ml) were added, the mixture was heated nearly to dryness and the residue was heated on a flame. The white, powdery residue was dissolved in 5 ml of water. A 5-ml sample was taken and the procedure in Section 2.1 was followed.

2.4. Determination of iron in Fefol capsule

To a Fefol capsule (SK&F, Karachi, Pakistan) (0.4197 g) were added 65% nitric acid (5 ml) and 37% hydrochloric acid (10 ml) and the mixture was heated on a hot-plate. When the capsule had completely dissolved, most of the acid was evaporated. Hydrochloric acid (2 ml) was added and the mixture was heated nearly to dryness. The residue was dissolved in water and the volume was adjusted to 25 ml. A 0.1-ml sample was taken and the procedure in Section 2.1 was followed.

3. Results and discussion

Cobalt(II) and iron(II) complexes are easily extractable with chloroform. The effect of pH on the extraction of cobalt(II) and iron(II) with chloroform indicates that cobalt(II) shows maximum extraction in the pH range 7–8, but iron(II) extracts maximally at pH 6. At higher pH (7–8), iron(II) precipitates as iron oxide and does not form a complex. Cobalt(II) can be extracted only above pH 3, but iron(II) shows a colour reaction even at pH 2. For the simultaneous extraction of iron(II) and cobalt(II), pH 6 could be used. The complexes are highly stable and did not show any change in peak shape for up to 24 h.

The cobalt(II) and iron(II) complexes were easily eluted from the Microsorb ODS column when eluted with the methanol–water, giving symmetrical peaks. The excess of reagent added for derivatization eluted after cobalt(II) but before iron(II) and did not interfere. A peak was observed before cobalt(II) when the aqueous solution of cobalt(II) and buffer solution was not deaerated with nitrogen. This signal was considered to be due to cobalt(III) resulting from the air oxidation of cobalt(II) and subsequent complexation and extraction with chloroform. However, when the cobalt(II) solution was deaerated by passing nitrogen, the first peak disappeared. The cobalt(III) complex was therefore prepared by oxidation of cobalt(II) to cobalt(III) with hydrogen peroxide, followed by heating the solution nearly to dryness to remove the hydrogen peroxide. This treatment was considered necessary because in the presence of hydrogen peroxide cobalt(III) formed a coloured complex, which was difficult to extract with chloroform. However, when the cobalt(III) complex extracted with chloroform was injected it gave a single peak and eluted before cobalt(II). The oxidation of cobalt(II) to cobalt(III) was further examined by passing air through freshly prepared cobalt(II) for different times (15–60 min). An enhancement of the peak height of cobalt(III) was observed with a corresponding decrease in the signal for cobalt(II). However, even on passing air for 1 h through cobalt(II) solution, the conversion of cobalt(II) to cobalt(III) was far from quantitative. In contrast, the conversion was complete using nitric acid (1%).

An optimum separation between cobalt(II) and cobalt(III) was obtained when eluted isocratically with methanol–water–acetonitrile (60:39:1, v/v/v) at a flow-rate of 0.9 ml/min (Fig. 2). A similar attempt was made to resolve iron(II) and iron(III), but each time a slightly broader peak was obtained, which could not be resolved into iron(II) and iron(III).

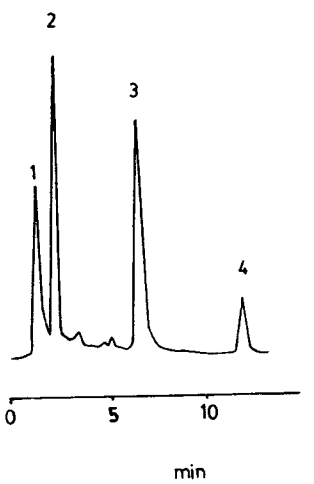

Fig. 2. HPLC separation of (1) cobalt(III), (2) cobalt(II), (3) reagent and (4) iron(II) chelates of H_2SA_2Ten. Column, 3-μm Microsorb ODS II (150 × 4.6 mm I.D.); eluent, methanol–water–acetonitrile (60:39:1, v/v/v), flow-rate, 0.9 ml/min; detection, UV at 270 nm.

Linear calibrations for cobalt(II), cobalt(III), iron(II) and iron(III), measured separately by plotting average peak height ($n = 3$) versus concentration, were found in the range 0–60 μg/ml of extract, using a 5-μl injection. The correlation coefficients for cobalt(II), cobalt(III), iron(II) and iron(III) were 1.0, 0.99, 1.00 and 1.01, respectively. The detection limits measured as three times the background noise were 0.5, 1, 0.5 and 1 μg/ml for cobalt(II), cobalt(III), iron(II) and iron(III), respectively, corresponding to 0.5, 1, 0.5 and 1 ng per injection, respectively.

Cobalt in vitamin B_{12} syrup and iron in a Fefol capsule were determined using the HPLC method. The cobalt concentration found was 0.28 μg/g and that of iron 42.33 mg in 0.42 g with relative standard deviations (R.S.D.) of 3.64% and 2.4%, respectively ($n = 3$). The results obtained were compared with those obtained by atomic absorption spectrometry, which were 0.32 μg/g of cobalt and 43.33 mg in 0.42 g of iron with R.S.D. 1.55% and 0.93%, respectively ($n = 3$).

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Short communication

Microwave-induced rapid synthesis of 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols —a novel derivative for gas chromatography–chemical ionization mass spectrometric study

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Abstract

Structural analyses of fatty alcohols are usually performed as acetate, trifluoroacetate or trimethylsilyl derivatives which produce characteristic molecular ions at $m/z < 400$. We describe a new derivatization technique of fatty alcohol using 4-carbethoxyhexafluorobutyryl chloride. The derivatization reaction requires either 30 min of incubation of the reaction mixture at 60°C, or 4 min of microwave irradiation using 240 W power. The yields of the derivatives were quantitative under both heating condition and microwave irradiation. The 4-carbethoxy hexafluorobutyryl derivatives of fatty alcohols produce characteristic protonated molecular ion peaks in the range of m/z 493 (cetyl alcohol) to m/z 549 (arachidyl alcohol) in the chemical ionization mode using methane as reagent gas. The molecular ion peaks were 54 u more than the conventional heptafluorobutyryl derivatives of fatty alcohols which can also be prepared by microwave irradiation in 3 min (240 W). The new derivatives are less volatile than the conventional heptafluorobutyryl derivatives.

1. Introduction

Fatty alcohols and aldehydes are important natural products found in the lipids of plants, animals and bacteria [1–4]. Surface lipids usually contain wax esters (fatty alcohols esterified to fatty acids), non-esterified fatty acids and sterols. Free and esterified fatty alcohols are also abundant in germinating seeds and in marine organisms, for example, oil of the deep sea fish orange roughy (*Hoplostethus atlanticus*) contains 95% wax esters. The presence of mycobacteria in drinking water represents a significant public health problem. Hydrolysis of those wax esters isolated from bacteria produces characteristic fatty alcohols which are derivatized and analyzed by gas chromatography–mass spectrometry (GC–MS) for identification [5,6]. The Sjogren–Larsson syndrome, an autosomal recessive disorder, is due to a defect in fatty alcohol cycle. Cultured skin fibroblast of these patients accumulate fatty alcohols and analysis of fatty alcohols after derivatization is used as a diagnostic aid for this disease [7,8]. Long-chain fatty acyl coenzyme thioester A have been implicated as substrates for several metabolic reactions. The assay of these compounds can be performed by taking advantage of borohydride reduction of

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these thioesters to fatty alcohols followed by derivatization of fatty alcohols for further analysis [9]. Therefore, analysis of fatty alcohols is widely used in lipid biochemistry.

Fatty alcohols are usually analyzed after conversion to acetate, trifluoroacetate or trimethylsilyl derivatives. These derivatives produce molecular ions at $m/z < 400$. Czarny and Hornbeck [10] recently described derivatization of amphetamine and methamphetamine using 4-carbethoxyhexafluorobutyryl chloride. We studied the derivatization of fatty alcohols using this reagent, hypothesizing the production of molecular ions in a much higher range (492–548).

Recently, microwave irradiation was demonstrated to produce dramatic acceleration of reaction rates of a variety of reactions [11–14]. We previously reported microwave-induced rapid transesterification of lipids and accelerated synthesis of fatty acyl pyrrolidides [15]. We also reported microwave-induced rapid preparation of conventional acetate and trifluoroacetate derivatives of fatty alcohols [16]. Now we would like to report microwave-induced rapid preparation of a novel 4-carbethoxyhexafluorobutyryl derivative of fatty alcohols.

2. Experimental

Cetyl, oleyl, linoleyl, stearyl, arachidonyl and arachidyl alcohols, 11-eicosenol and the internal standard, heptadecanol were purchased from Sigma (St. Louis, MO, USA). The derivatizing agent 4-carbethoxyhexafluorobutyryl chloride was obtained from PCR (Gainsville, FL, USA), while heptafluorobutyric anhydride was procured from Pierce (Rockford, IL, USA). The derivatization reaction was carried out in Reacti-Vials with 1 ml capacity capped with Mini Inert valves, also available from Pierce.

The high-performance thin-layer chromatography (HPTLC) plates coated with silica gel were obtained from EM Separation (Gibbstown, NJ, USA). The developing solvent was ethyl acetate–methanol (90:10, v/v). After developing, bands were visualized by spraying with 4% copper sulfate in 30% phosphoric acid followed by heating. The microwave oven used in this study has a total capacity of 800 W with ten different power settings (Samsung, Model MW 5510 T). The GC–MS analysis was performed by using a Model 5890 gas chromatograph coupled with a 5970 series mass-selective detector for electron impact (EI) MS study and a 5890 series II gas chromatograph coupled to a 5972 series mass-selective detector for chemical ionization (CI) spectra (Hewlett-Packard, Palo Alto, CA, USA). An Ultra-2 capillary column, also obtained from Hewlett-Packard, was used for both instruments. The initial oven temperature of the gas chromatograph was 190°C for analysis of carbethoxyhexafluorobutyryl derivatives of fatty alcohols. After maintaining that temperature for 2 min, the temperature of the gas chromatograph was increased at a rate of 5°C/min to reach a final oven temperature of 290°C. The final temperature was maintained for another 2 min. The solvent delay was 5 min after which the mass spectrometer was turned on. The carrier gas was helium with a column flow-rate of 0.29 ml/min and a linear velocity of 21 cm/s. For the analysis of heptafluorobutyryl derivatives, the initial oven temperature was 160°C. After maintaining that temperature for 2 min, the oven temperature was increased at a rate of 5°C/min to reach a final oven temperature of 290°C. The final oven temperature was maintained for an additional 2 min and the solvent delay was again 5 min. Both mass spectrometers were operated in the scan mode with scanning range of m/z 50–700.

2.1. Preparation of 4-carbethoxyhexafluorobutyryl derivatives

We added 50 μl of 4-carbethoxyhexafluorobutyryl chloride to 0.1–0.2 mg of fatty alcohol and heated the reaction mixture at 60°C for 30 min. Finally the reaction mixture was evaporated to dryness. We took advantage of low volatility of the derivatized fatty alcohol and omitted the addition of anhydrous ethanol to the reaction mixture as described by Czarny and Hornbeck [10]. Instead we evaporated the excess reagent at 37°C and reconstituted the residue in ethyl acetate and injected into GC–MS. Using micro-

wave irradiation (power level 3, 240 W), the reaction was completed only in 4 min.

2.2. Preparation of heptafluorobutyryl derivatives

We also prepared conventional heptafluorobutyryl derivatives of fatty alcohols in 3 min using low power microwave irradiation (power level 3, 240 W). The conventional heating technique requires 20 min incubation of reaction mixture at 60°C. The reaction was quantitative using both microwave irradiation and conventional heating method as evidenced by the complete disappearance of the starting material in HPTLC plates.

3. Results and discussion

3.1. Microwave-induced rapid derivatization

The microwave provides a rapid and convenient method for preparation of 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols for structural analysis. The R_F values of fatty alcohols obtained by the microwave technique were identical to those of the derivatives obtained by conventional heating. The GC retention times as well as MS fragmentation patterns of the derivatives prepared by microwave irradiation were similar to those of the derivatives obtained by conventional heating, indicating that the derivatives obtained by the microwave technique have the same chemical identity as the derivatives prepared by conventional heating (Table 1).

We reported previously the use of microwave irradiation for rapid preparation of acetyl, trifluoroacetyl and *tert.*-butyltrimethylsilyl derivatives of fatty alcohols [16]. We prepared heptafluorobutyryl derivatives of fatty alcohols in 3 min under microwave irradiation (power 3, 240 W) while the conventional technique requires 20 min of heating at 60°C. Again, the GC retention times and MS fragmentation patterns of derivatives formed under microwave irradiation were identical to those obtained by the conventional heating technique.

Table 1
CI-MS characteristics of 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols prepared by microwave irradiation and conventional heating

Compound	MS fragmentation pattern							
	Microwave[a]				Heating[a]			
	M + 1	Base	Other peaks		M + 1	Base	Other peaks	
Cetyl alcohol	493 (5)	225 (100)	169 (8)	155 (13)	493 (4)	225 (100)	169 (8)	155 (13)
Stearyl alcohol	521 (2)	253 (100)	169 (12)	155 (14)	521 (2)	253 (100)	169 (12)	155 (13)
Oleyl alcohol	519 (14)	111 (100)	251 (31)	167 (47)	519 (16)	111 (100)	251 (31)	167 (46)
Linoleyl alcohol[b]	517 (12)	97 (100)	249 (35)	165 (45)	517 (11)	97 (100)	249 (36)	165 (45)
Arachidyl alcohol	549 (2)	281 (100)	183 (11)	169 (13)	549 (3)	281 (100)	183 (9)	169 (11)
Eicosenol	547 (11)	97 (100)	181 (29)	167 (44)	547 (12)	97 (100)	181 (31)	167 (45)
Arachidonyl alcohol	541 (100)	541 (100)	273 (20)	137 (33)	541 (100)	541 (100)	273 (20)	137 (32)
Heptadecanol (I.S.)	507 (1)	239 (100)	183 (8)	169 (11)	507 (1)	239 (100)	183 (7)	169 (10)

I.S. = Internal standard.
[a] m/z, relative abundance in parentheses.
[b] For linoleyl alcohol the M − 1 peak at m/z 515 (relative abundance 27.1%) was stronger than the M + 1 peak.
× I.S. stands for internal standard

3.2. GC retention times and MS characteristics of fatty alcohol derivatives

The advantage of derivatizing fatty alcohols with 4-carbethoxyhexafluorobutyryl chloride is the significantly lower volatility of these derivatives compared to the conventional heptafluorobutyryl derivatives, while retaining excellent chromatographic properties (no significant tailing of peaks). For example, if we set the oven temperature of our gas chromatograph at 160°C, the temperature program we used for the analysis of heptafluorobutyryl derivatives, the retention time of the heptafluorobutyryl derivative of arachidyl alcohol was 16.1 min while the retention time of 4-carbethoxyhexafluorobutyryl derivative of arachidyl alcohol was 24.5 min. The lower volatility of these new derivatives will be helpful in analysis of short- and medium-chain alcohols as well as for fatty alcohols where higher initial oven temperatures should be used. We used heptadecanol as the internal standard.

Another advantage of 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols is the significant increase in the molecular ion peak compared to the conventional derivatives. For example, the molecular ion peaks of acetate and trifluoroacetyl derivatives of arachidonyl alcohol were observed at m/z 332 and 386, respectively [16]. The heptafluorobutyryl derivative of arachidonyl alcohol (a derivative less commonly used) showed a molecular ion peak at m/z 486, while the 4-carbethoxyhexafluorobutyryl derivative showed a molecular ion peak + 1 at m/z 541 (Fig. 1).

The acetyl, trifluoroacetyl or heptafluorobutyryl derivatives of saturated fatty alcohols (cetyl, stearyl and arachidyl) did not show any molecular ion peak in the EI mode [16]. However, 4-carbethoxyhexafluorobutyryl derivatives of saturated fatty alcohols showed weak molecular ion peaks (Fig. 2). The more intense peaks in the EI mode for both heptafluorobutyryl and 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols were observed between m/z 50–100, while the peaks in the higher mass range showed much lower relative abundances. However, in the CI mode using methane as a reagent

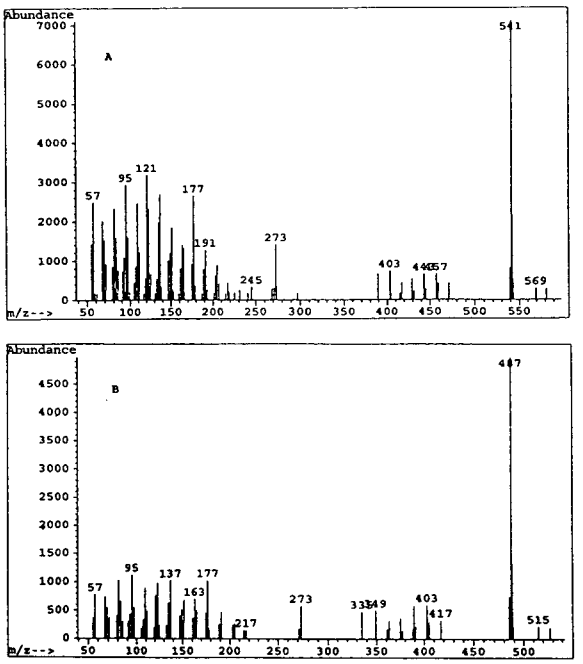

Fig. 1. Chemical ionization mass spectra (methane as a reagent gas) of (A) 4-carbethoxyhexafluorobutyryl derivative and (B) heptafluorobutyryl derivative of arachidonyl alcohol.

gas more intense peaks can be observed in the higher mass range.

Both heptafluorobutyryl and 4-carbethoxyhexafluorobutyryl derivatives of fatty alcohols (saturated and unsaturated) showed distinct protonated and/or M − 1 peak peaks in the CI mode. Another advantage of CI-MS analysis is the distinctively different fragmentation patterns of saturated and unsaturated alcohols. The saturated fatty alcohols after derivatization either with heptafluorobutyryl anhydride or 4-carbethoxyhexafluorobutyryl chloride, showed the same base peaks due to the loss of derivatized part of the molecule including oxygen. We also observed several peaks due to fragmentation of the hydrocarbon part of the molecule after the loss of derivatized part which were similar in both derivatives. As expected, the molecular ion peaks are 54 u higher in 4-carbethoxyhexafluorobutyryl derivatives compared to the heptafluorobutyryl derivatives. The unsaturated al-

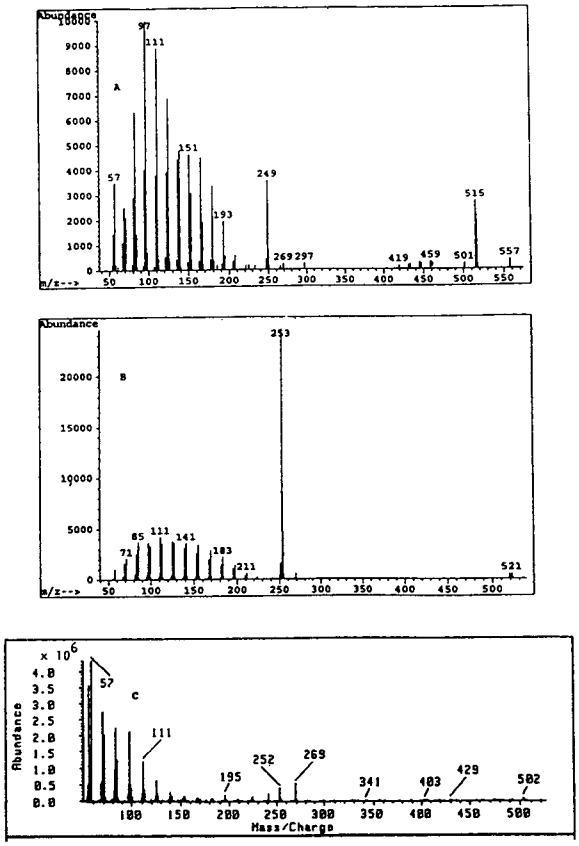

Fig. 2. Chemical ionization mass spectra (methane as a reagent gas) of 4-carbethoxyhexafluorobutyryl derivatives of (A) linoleyl alcohol, (B) stearyl alcohol and (C) stearyl alcohol in the electron impact mode.

cohols, after derivatization, showed much stronger protonated M − 1 peaks while the base peaks were shifted to the lower mass region. However, the peaks in the smaller mass regions of derivatized unsaturated alcohols were more intense than the derivatized saturated alcohols, thus distinguishing MS characteristics of unsaturated alcohols from saturated alcohols. This distinct difference between the MS fragmentation pattern of derivatized saturated and unsaturated alcohol is absent in the EI mode where both saturated and unsaturated compounds showed intense peaks in the m/z 50–100 region. We also observed a distinct peak in the mass spectrum of derivatized unsaturated alcohols due to the loss of the derivatized part of the molecule including oxygen. The straight-chain and branched-chain fatty alcohols after derivatization with 4-carbethoxyhexafluorobutyryl chloride showed different intensities of peaks in the CI mode. The heptafluorobutyryl derivatives of saturated and unsaturated alcohols also showed these distinguishing features in the CI mass spectra using methane as a reagent gas.

The CI- and EI-MS analysis for derivatized fatty alcohol provide complementary information. A distinct feature in the CI mode is the observation of strong molecular ions peaks for unsaturated alcohols for both heptafluorobutyryl and 4-carbethoxyhexafluorobutyryl derivatives. In addition, both derivatives of saturated fatty alcohols showed molecular ion peaks in the CI mode while molecular ion peaks were not observed for heptafluorobutyryl derivatives in the EI mode, although 4-carbethoxyhexafluorobutyryl derivatives showed weak molecular ion peaks in the EI mode.

We conclude that microwave irradiation can be utilized for rapid synthesis of new 4-carbethoxyhexafluorobutyryl or conventional heptafluorobutyryl derivatives of fatty alcohols for structural analysis.

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Short communication

Determination of secondary amines in various foods by gas chromatography with flame photometric detection

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Abstract

A selective and sensitive gas chromatographic method for the determination of secondary amines in foods has been developed. After extraction of the sample with hydrochloric acid, secondary amines were converted into their N-diethylthiophosphoryl derivatives and then measured by gas chromatography using a DB-1701 capillary column and a flame photometric detector. Primary amines were eliminated through the reaction with *o*-phthaldialdehyde prior to the N-diethylthiophosphorylation. The calibration curves for secondary amines in the range 0.2–50 nmol were linear and sufficiently reproducible for quantitative determination. The detection limits of secondary amines, at a signal-to-noise ratio of 3, were ca. 0.05–0.2 pmol injected. Using this method, secondary amines in food samples could be accurately and precisely determined without any interference from coexisting substances. Analytical results for the determination of secondary amines in various food samples are presented.

1. Introduction

Secondary amines are nitrosated by nitrite in conditions of defined pH and in other conditions similar to those in the mammalian stomach or small intestine [1–4], and some of nitrosamines produced have strong mutagenic and oncogenic activities [5–7]. It is well known that nitrite is widely present in nature and is also produced in human saliva, and the possibility that nitrosation may occur in vivo, particularly in the gastrointestinal tract, upon ingestion of foods containing secondary amines has been reported [8,9]. Therefore, measurement of secondary amines in foodstuff is very important.

The determination of secondary amines has been carried out by high-performance liquid chromatography (HPLC) or gas chromatography (GC), but these methods have some inherent problems related to the difficulty in handling low-molecular-mass amines because of their high water solubility and volatility. HPLC analyses of secondary amines by using ultraviolet [10–13], fluorescence [14–16], electrochemical [17] and chemiluminescence excitation detection [18] require derivatization in order to increase the detection sensitivity. Although some of them are very sensitive, their selectivities for food samples with complicated matrices cannot be guaranteed. GC analyses of underivatized secondary amines result in adsorption and decomposition on the column and readily give tailed peaks [19–21]. In order to solve these problems, many derivatization reagents, such as 2,4-dinitrofluorobenzene [22,23], 2,4-dinitrobenzenesulphonate [24], alkyl chloroformate [25,26], pentafluorobenzoyl chlo-

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ride [27], flophemesyl chloride [28] and benzenesulphonyl chloride [29–32], for GC analyses of secondary amines by flame ionization detection (FID) [24,25], electron-capture detection [27,28], flame photometric detection (FPD) [29,30], thermospecific detection [26], chemiluminescence detection with a thermal energy analyser [32] and GC–mass spectrometry with selected ion monitoring (GC–MS-SIM) [23,31] have been reported. However, GC–FID methods lack sensitivity and selectivity. Other GC methods are highly sensitive, but these methods are not specific for secondary amines, except for the GC–MS-SIM methods which require expensive equipment. On the other hand, GC–FPD methods [29,30] based on the Hinsberg procedure [33] have been reported to be selective for secondary amines, but it was found that these amines overlapped with some primary amines in our experiments [34].

Recently, we have developed a selective and sensitive method for the determination of secondary amines by GC–FPD, in which these compounds are analysed as their N-diethylthiophosphoryl (DETP) derivatives after primary amines were eliminated through the reaction with *o*-phthaldialdehyde (OPA) [35]. By using this method, urinary secondary amines could be accurately and precisely determined without any influence from other constituent substances [35]. This paper reports the extension of this work to the determination of secondary amines in food samples.

2. Experimental

2.1. Reagents

Dimethylamine (DMA) and diethylamine (DEA) as the hydrochlorides, di-*n*-propylamine (DPA), di-*n*-butylamine (DBA), pyrrolidine (PYR), piperidine (PIP), morpholine (MOR), hexamethyleneimine (HMI), N-methylbenzylamine (NMBzA) and N-methylcyclohexylamine (NMCHA) as an internal standard (I.S.) were purchased from Nacalai Tesque (Kyoto, Japan). N-Methylethylamine (MEA) was purchased from Fluka (Buchs, Switzerland). Each amine was dissolved in 0.05 *M* hydrochloric acid containing 50% acetonitrile to make a stock solution at a concentration of 0.2 *M* and used after dilution with 0.05 *M* hydrochloric acid to the required concentration (0.01–0.1 m*M*). OPA (Nacalai Tesque) was used as a 0.2 *M* solution in acetonitrile. Diethylchlorothiophosphate (DECTP) (Tokyo Kasei Kogyo, Tokyo, Japan) was used as a 1% solution in acetonitrile after distillation. All other chemicals were of analytical-reagent grade.

2.2. Preparation of samples

Food samples were purchased at local retail markets and were treated for analyses on the same day without drying. For liquid samples, an aliquot (0.05–0.2 ml) was directly used as the sample for derivatization. For solid samples, an aliquot (0.2–1.6 g) was homogenized in 4 ml of 0.05 *M* hydrochloric acid with a Model LK-21 ultra-disperser (Yamato Kagaku, Tokyo, Japan). After centrifugation at 2000 *g* for 10 min, the precipitate was re-extracted with 2 ml of 0.05 *M* hydrochloric acid. The supernatants were combined and 0.1–0.4 ml of the combined solution was used as the sample for derivatization.

2.3. Derivatization procedure

To the standard solution containing 0.2–50 nmol of secondary amines or the sample prepared by above method were added 0.1 ml of 10 μM I.S., and the total volume was made up to 1.6 ml with distilled water after neutralization with 2 *M* sodium hydroxide. To the mixture was added 0.2 ml of 0.5 *M* phosphate buffer (pH 8) and 0.2 ml of 0.2 *M* OPA, and the mixture allowed to stand for 2 min at room temperature. To the reaction mixture was added 0.2 ml of 10% sodium carbonate and 0.2 ml of 1% DECTP, and then the mixture was incubated at 60°C for 10 min after tightly capping. In order to remove the excess of reagent, the reaction mixture was incubated again at 60°C for 5 min after addition of 0.2 ml of 50 m*M* cysteic acid. The reaction mixture was extracted with 0.2–0.4 ml

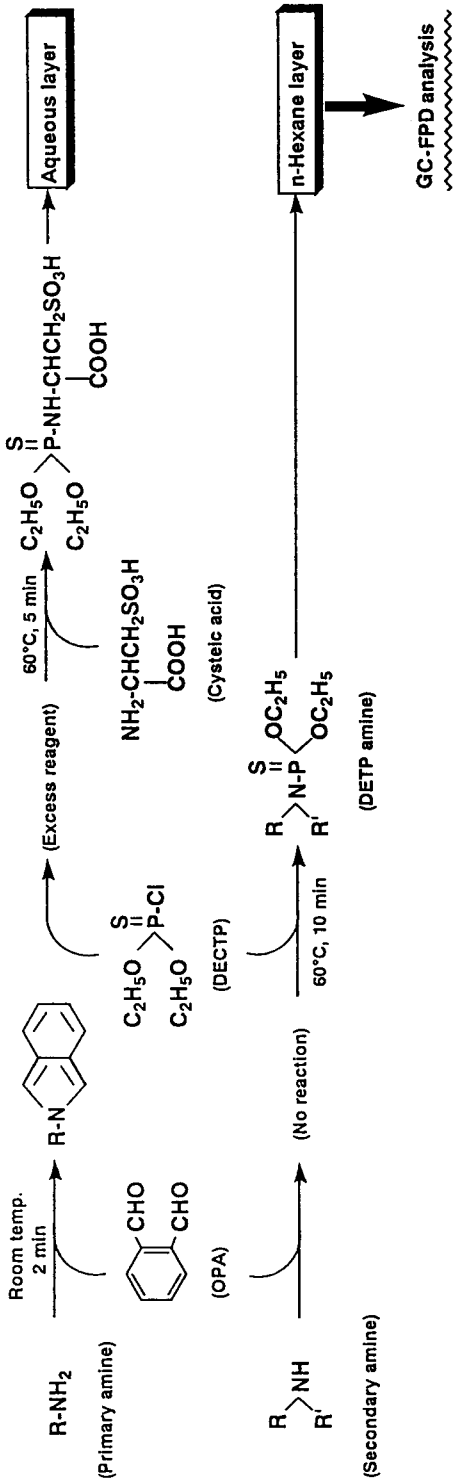

Fig. 1. Outline of selective derivatization of secondary amines. R, R′ = Alkyl or aryl group.

of *n*-hexane and 1 μl of this extract was injected into the GC–FPD system. The derivatization process is summarized in Fig. 1.

2.4. Gas chromatography

GC analysis was carried out with a Shimadzu 14A gas chromatograph equipped with a flame photometric detector (P-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.0 μm film thickness) of cross-linked DB-1701 (J&W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed from 100 to 260°C at 10°C/min; injection and detector temperature, 280°C; nitrogen flow-rate, 10 ml/min. The peak heights of secondary amines and the I.S. were measured and the peak height ratios against the I.S. were calculated.

3. Results and discussion

It is well known that OPA reacts only with primary amino groups. Therefore, it is considered that secondary amines might be selectively detected as suitable derivatives if a sample was previously treated with OPA. On the other hand, FPD with 526-nm interference filter inserted in the optical path is sensitive and selective for

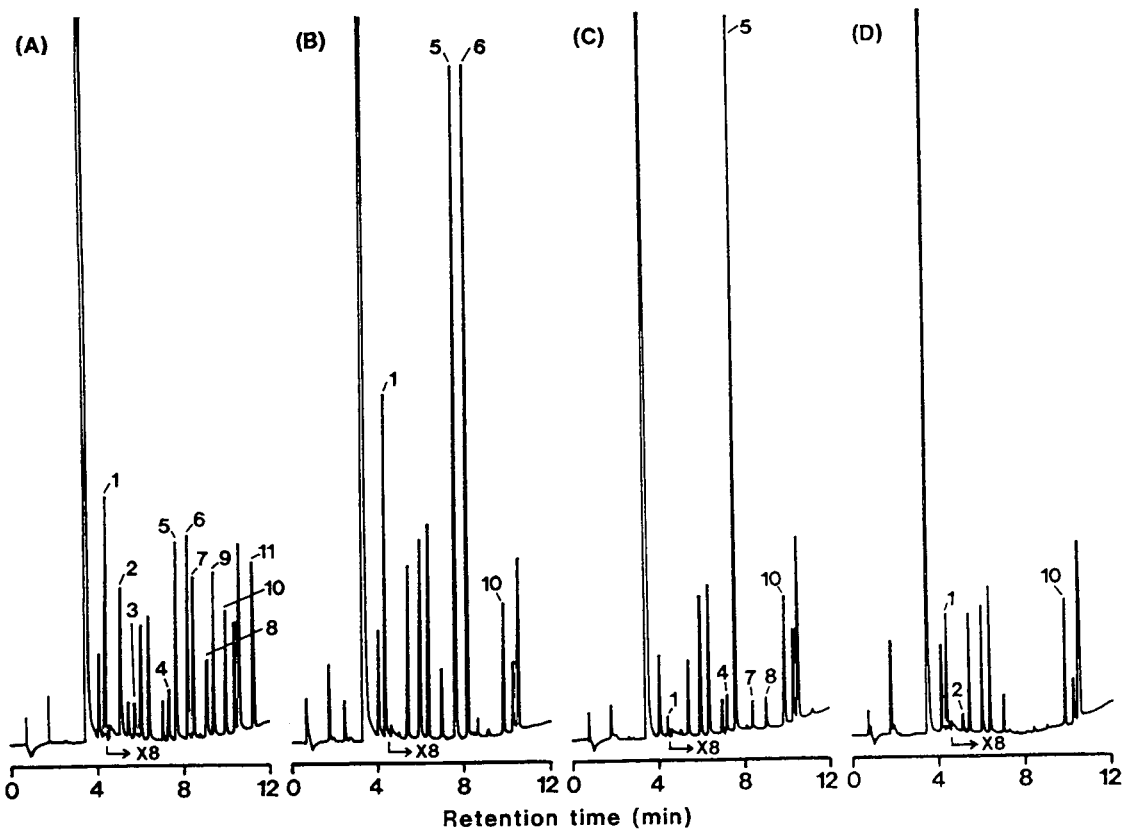

Fig. 2. Typical gas chromatograms obtained from (A) standard (containing 10 nmol of dimethylamine and 1 nmol of other secondary amines), (B) milk (0.1 ml), (C) red pepper (6.4 mg) and (D) oyster (52 mg). GC conditions are given under Experimental. The arrows show that the recorder response was raised up to 8-fold after ca. 4.5 min from sample injection. Peaks: 1 = dimethylamine; 2 = N-methylethylamine; 3 = diethylamine; 4 = di-*n*-propylamine; 5 = pyrrolidine; 6 = piperidine; 7 = morphorine; 8 = di-*n*-butylamine; 9 = hexamethyleneimine; 10 = N-methylcyclohexylamine (I.S.); 11 = N-methylbenzylamine.

phosphorous compounds. Therefore, secondary amines might be sensitively and selectively detected by GC–FPD if these amines were converted into volatile phosphorous-containing derivatives. On the basis of these ideas, we investigated a selective and sensitive method for the determination of secondary amines. The derivatization process of secondary amines is shown in Fig. 1. Primary amines in food samples were eliminated by the reaction with OPA as previously described [35]. Secondary amines did not react with OPA and therefore these compounds were selectively derivatized by the subsequent reaction with DECTP. Reaction conditions for diethylthiophosphorylation of secondary amines were established in the previous investigation [35]. This reaction was completed within 5 min at 60°C and excess DECTP was removed by reaction with cysteic acid. The DETP derivatives of cysteic acid and other amino acids in the samples were not extracted into *n*-hexane in alkaline media. On the other hand, the DETP derivatives of secondary amines were quantitatively extracted into *n*-hexane. These derivatives were volatile and stable, and eluted as separate symmetrical peaks although some peaks originated from reagents were observed (Fig. 2A). The derivatives provided an excellent FPD response and minimum detectable amounts of DMA, DEA, PYR, PIP, MOR and NMBzA to give a signal three times the noise under our instrumental conditions were ca. 0.08, 0.2, 0.06, 0.05, 0.07 and 0.08 pmol injected, respectively. NMCHA was chosen as an internal standard because it was well separated from the amines investigated. The calibration curves for DMA and the other amines were linear in the range 2–50 and 0.2–5 nmol, respectively, and the correlation coefficients were above 0.991.

For secondary amine analysis of foodstuff, hydrochloric acid was used to precipitate proteins. Secondary amines were quantitatively extracted from food samples by extraction twice with 0.05 *M* hydrochloric acid. Fig. 2B–D show the chromatograms obtained from several food samples. The recorder response was raised up to 8-fold at ca. 4.5 min from sample injection because of the large difference in concentration between DMA and the other amines. Secondary amines in these samples could be detected with-

Table 1
Recoveries of secondary amines added to several food samples

Sample	Amine[a]	Added	Amount found[b]		Recovery (%)
			Non-addition	Addition	
Wine	DMA	100 nmol/ml	26.4 ± 0.2 nmol/ml	125.0 ± 5.0 nmol/ml	99
	PYR	10	7.5 ± 0.6	17.4 ± 0.3	99
	PIP	10	7.9 ± 0.5	17.3 ± 0.3	94
	MOR	10	8.9 ± 0.04	18.5 ± 0.5	96
	DBA	10	ND[c]	10.1 ± 0.4	101
	NMBzA	10	29.8 ± 0.9	40.3 ± 0.8	105
Salted pollack roe	DMA	663 nmol/g	2086 ± 73 nmol/g	2792 ± 203 nmol/g	106
	PYR	66.3	15.0 ± 1.2	82.7 ± 1.2	102
	PIP	66.3	6.4 ± 0.3	77.4 ± 2.3	107
	MOR	66.3	4.9 ± 0.4	66.6 ± 1.2	93
	DBA	66.3	ND	60.6 ± 2.3	91
	NMBzA	66.3	6.7 ± 0.5	81.6 ± 1.3	113

[a] DMA = Dimethylamine; PYR = pyrrolidine; PIP = piperidine; MOR = morphorine; DBA = di-*n*-butylamine; NMBzA = N-methylbenzylamine.
[b] Mean ± S.D. ($n = 4$).
[c] Not detectable.

Table 2
Secondary amine contents in various foods obtained from commercial sources

Sample	Content (nmol/g or nmol/ml)[a]									
	DMA	MEA	DEA	DPA	PYR	PIP	MOR	DBA	HMI	NMBzA
Rice	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bread	10.3 ± 0.4	ND	ND	ND	3.4 ± 0.3	3.5 ± 0.3	ND	4.2 ± 0.1	ND	ND
Red beans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soy beans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Almond	38.1 ± 1.0	ND	ND	ND	ND	ND	ND	1.1 ± 0.05	ND	ND
Black pepper	165 ± 12	ND	ND	ND	453 ± 38	6351 ± 368	ND	9.0 ± 0.5	ND	ND
Red pepper	42.9 ± 2.5	ND	ND	120 ± 3	661 ± 36	ND	20.4 ± 0.1	21.6 ± 1.3	ND	ND
Wasabi	ND	ND	ND	ND	3.0 ± 0.03	ND	ND	4.2 ± 0.3	ND	2.0 ± 0.1
Garlic	96.1 ± 2.9	19.4 ± 0.01	ND	110 ± 0.4	277 ± 0.3	ND	ND	ND	ND	ND
Onion	10.2 ± 0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cabbage	14.3 ± 0.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Banana	4.8 ± 0.2	ND	ND	ND	1.6 ± 0.1	2.1 ± 0.05	ND	ND	ND	ND
Dried fungi	212 ± 10	ND	ND	ND	45.9 ± 5.3	ND	ND	ND	ND	ND
Salted algae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salted pollack roe	2086 ± 73	ND	ND	2.5 ± 0.2	15.0 ± 1.2	6.4 ± 0.3	4.9 ± 0.4	ND	ND	6.7 ± 0.5
Cod (dried)	1043 ± 38	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mackerel	2129 ± 390	ND	ND	ND	16.4 ± 1.9	15.9 ± 2.3	ND	ND	ND	ND
Oyster	39.3 ± 2.0	1.6 ± 0.1	ND	ND	ND	ND	ND	ND	ND	ND
Beef	26.4 ± 0.4	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pork	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chicken	18.9 ± 0.6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Egg white	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Egg yolk	109 ± 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yogurt	483 ± 50	ND	ND	ND	19.2 ± 1.9	18.0 ± 1.5	ND	ND	ND	ND
Cheese	29.8 ± 2.9	ND	ND	ND	17.4 ± 1.2	29.3 ± 3.1	ND	ND	ND	ND
Cow's milk	147 ± 7	ND	ND	ND	50.1 ± 0.4	62.4 ± 3.1	ND	ND	ND	ND
Wine	26.4 ± 0.2	ND	352 ± 25	52.9 ± 6.1	7.5 ± 0.6	7.9 ± 0.5	8.9 ± 0.04	ND	ND	29.8 ± 0.9
Beer	64.5 ± 5.7	2.5 ± 0.1	ND	ND	7.9 ± 1.1	22.2 ± 1.2	ND	31.2 ± 2.1	ND	ND
Sake	ND	ND	ND	ND	10.3 ± 0.1	ND	ND	14.5 ± 1.3	ND	ND
Bean jum	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Mean ± S.D. ($n = 4$).
[b] Not detectable.

out any interference from coexisting substances. As shown in Table 1, the overall recoveries of secondary amines added to several food samples were 91–113% and the relative standard deviations were 0.4–8.2% ($n = 4$). The secondary amine contents in various food samples determined by this method are summarized in Table 2. It can readily be seen from our data that fish and fish products contained high concentrations of DMA (39–2129 nmol/g; literature value, 33–3556 nmol/g) and spices contained high concentrations of PYR (3–661 nmol/g; literature value, ND–1296 nmol/g) and PIP (ND-6351 nmol/g; literature value, ND–7553 nmol/g). On the other hand, secondary amine contents in grain, vegetable and meat were very low or not detectable. DMA was distributed in most of the foods investigated, but MEA, DEA, DPA, MOR, HMI and NMBzA were seldom detected in our study. These tendencies are similar to the results reported by Pfundstein et al. [9].

4. Conclusions

A convenient and reliable method for the determination of secondary amines in food samples has been established. This method is selective and sensitive, and food samples can be directly analysed without pre-treatment except for deproteinization and without any interference from other coexisting substances. We believe that this method provides a useful tool for routine analysis of foodstuffs.

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Short communication

Extraction of pesticides using supercritical trifluoromethane and carbon dioxide

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Abstract

The off-line supercritical fluid extraction of pesticides using trifluoromethane (CHF_3) and carbon dioxide (CO_2) is described. Pesticides containing nitrogen or phosphorus heteroatoms were used as analytes to determine the extraction ability of CHF_3 in comparison with CO_2 at various pressures and densities. A 15% increase in extraction efficiency was obtained when CHF_3 was used as the extraction solvent rather than CO_2. No selectivity was observed for different groups of pesticides, although CHF_3 has a dipole moment whereas CO_2 does not. Because in environmental matrices the hydrocarbons are mostly present in high excess compared with the pesticides, extractions were carried out without and with addition of alkanes and polycyclic aromatic hydrocarbons (PAHs). The extraction efficiency of the pesticides with CHF_3 performed without addition of alkanes and PAHs was significantly decreased.

1. Introduction

An attractive alternative to conventional liquid solvent extraction for the recovery of organic analytes from adsorbents and solids is analytical-scale supercritical fluid extraction (SFE). Owing to the physical properties of supercritical fluids (SCFs), i.e., higher diffusion coefficients than liquids and high solubility, the extraction is fast and high recoveries are obtained. Many researchers have reported the use of supercritical carbon dioxide to extract various pollutants, e.g., polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and pesticides, from different matrices such as soil, sediments and biological material [1–13]. The solvation power of pure carbon dioxide, which is the most often used SCF, is limited to apolar or slightly polar compounds even at very high densities. Therefore, SFE of polar analytes requires the addition of organic polarity modifiers to CO_2, mostly methanol, to enhance the solvation power and the mobile phase selectivity [14–18].

Another method for extracting polar analytes from solid matrices is the reaction of derivatizing reagents with the analyte under supercritical conditions [19,20]. Hills et al. [21] investigated simultaneous supercritical fluid derivatization and extraction (SFDE) using silylation reagents for the extraction of roasted coffee beans, tea and marine sediment. They found that a silylation reagent not only served as a derivatizing reagent but also acted as a coextracting reagent,

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which increased the extraction yield of medium-polarity compounds [22]. In situ chemical derivatizations of 2,4-D from soil, of phenols from C_{18} sorbent discs and of fatty acids from whole cells using trimethylphenylammonium hydroxide and boron trifluoride in methanol have been reported by Hawthorne et al. [23]. An on-line supercritical fluid derivatization and extraction procedure coupled with capillary gas chromatography was performed by Hillmann and Bächmann [24] to extract phenoxycarboxylic acids from a C_{18} sorbent and to eliminate excess of derivatizing reagent by fractionated SFE. On-line SFDE was used by King et al. [25] to determine the fatty acid composition of oilseeds.

Many investigators have used alternative fluids to overcome the limited solvation power of non-polar CO_2 and the problems presented by mixed mobile phase systems [26–28]. Hawthorne et al. [29] compared the SFE recoveries for native pollutants including PCBs from a standard reference material and PAHs from a petroleum waste sludge and from a railroad bed using supercritical $CHClF_2$, N_2O and CO_2. Sulfur hexafluoride (SF_6), nitrous oxide and SF_6-modified carbon dioxide were used in on-line SFE–GC to characterize complex environmental and petroleum matrices [30].

The use of trifluoromethane (CHF_3) as a supercritical fluid for extracting polar analytes, has several advantages over $CHClF_2$, which has a comparable polarity. CHF_3 has a lower critical temperature and a less harmful environmental effect than $CHClF_2$. Howard et al. [31] evaluated the use of CHF_3 to extract sulfonylurea herbicides, which are polar and thermally labile, from Celite and PAHs from filter-paper and clay.

This paper describes the comparison of the SFE rates obtained for the off-line extraction of polar pesticides at the low ppm level from silanized glass beads using CHF_3 and CO_2. Different classes of pesticides, i.e., triazines, organophosphorus compounds, carbamates and anilides, were used as analytes. The effect of pressure and density of the fluids on extractability and the addition of alkanes and PAHs to the inert, non-adsorptive matrix is described.

2. Experimental

2.1. Reagents

The pesticides used, atrazine, desethylatrazine, diazinon, fenpropimorph, malathion, metazachlor, metribuzine, propiconazol and triallate (CAS numbers 1912-24-9, –, 333-41-5, 67564-91-4, 121-75-5, 67129-08-2, 21087-64-9, 60207-90-1, 006-039-00-X) were supplied by Riedel-de Haën (Seelze, Germany). Chrysene, pyrene, benzo[*ghi*]perylene, dodecane, tetradecane and hexadecane were purchased from Supelco (Deisenhofen, Germany). Solutions of the pesticides were prepared at 200 μg/ml each in acetone. The solutions of the PAHs and the alkanes had concentrations of 200 and 250 μg/ml, respectively, in acetone.

2.2. Off-line SFE

Extractions with CHF_3 (Linde, Unterschleissheim, Germany; purity of 99.999%) were performed using a Milton Roy (Riviera Beach, FL, USA) Model CP3000 cryogenically cooled (6°C) dual-head reciprocating pump. Extractions with CO_2 (Messer Griesheim, Frankfurt, Germany; supplied in a cylinder with a dip tube, purity of 99.9995%) were performed using a computer-controlled, high-pressure syringe pump (Series 600; Lee Scientific, Salt Lake City, UT, USA). The SFE pumps were connected to the extraction cell with 1/16 in. O.D. stainless-steel tubing and finger-tight Dynaseal connectors (Knauer, Berlin, Germany). Extraction cells were placed inside a Sichromat II-GC oven (Siemens, Karlsruhe, Germany) to maintain the extraction temperature. Extraction cells with internal volumes of 120 μl were filled with silanized glass beads and spiked with 5 μl of the pesticide solution. The solvent was allowed to evaporate for 10 min. Fused-silica tubing (SGE, Weiterstadt, Germany) of 25 μm I.D. was used to control SFE flow-rates at 100–200 ml gaseous carbon dioxide and trifluoromethane, respectively, depending on the operating pressure. The extracted analytes and the supercritical fluid

were collected by inserting the outlet end of the restrictor into empty vials, which were designed for the concentration of liquid samples (6-ml volume). The vials were cryogenically cooled with liquid nitrogen during extraction. The end of the restrictor was heated with a resistance heater to avoid clogging of the restrictor. At the end of the extraction, the resistance heater was shut off and the vial was removed from the liquid nitrogen. After venting the carbon dioxide or the trifluoromethane at room temperature, the analytes were dissolved in 200 μl of acetone and analysed without further steps.

2.3. GC instrumentation

The extracted pesticides were analysed using a Model 5890 Series II gas chromatograph (Hewlett-Packard, Bad Homburg, Germany) with nitrogen–phosphorus detection (NPD). The split–splitless injector and the detector were operated at 250 and 280°C, respectively. The pesticides were separated on a 50 m × 0.25 mm I.D. SE-54 column (Macherey–Nagel, Düren, Germany) with a film thickness of 0.25 μm. The carrier gas was helium at an initial linear velocity of 40 cm/s. The column oven temperature was held at 100°C for 1 min, then programmed at 10°C/min to 150°C and at 5°C/min to 290°C.

3. Results and discussion

The aim of this investigation was to compare the extraction abilities of two different fluids, CHF_3, and CO_2. Trifluoromethane was selected because it has nearly the same critical temperature as CO_2 and because of its analogous structure to chloroform, which is a good organic solvent and is capable of hydrogen bonding. The comparison of the extraction abilities, which are an indication of the solubility of the pesticides, was carried out at constant temperature in order to remove the effect of vapour pressure.

The analytes used were nitrogen- and phosphorus-containing pesticides, some of which show good water solubility and therefore harmful environmental effects in ground water. They are widely used in agriculture and the European Community Drinking Water Directive sets a limit of 0.1 μg/l for individual pesticides and 0.5 μg/l for the sum of all pesticides.

The trapping efficiency of the collection unit was investigated in an earlier study with C_8–C_{16} *n*-alkanes [9] and found to be quantitative. During extraction the collection vials were cryogenically cooled with liquid nitrogen. Because of the very low vapour pressures of the selected pesticides (between 10^{-4} and 10^{-7} hPa at 20°C) compared with *n*-octane (15 hPa at 20°C) and their polarity, the trapping efficiency in this investigation was assumed to be quantitative. The reproducibility of all extractions for the standard solutions was between 2% and 9% (n = 3). Table 1 summarizes the pressure dependence of the extractability of the selected pesticides from silanized glass beads with supercritical CHF_3 at a constant temperature. Only the extraction profiles for atrazine, fenpropimorph, and diazinon under various extraction conditions are shown in Fig. 1 because those for the other

Table 1
Effect of pressure on the recovery (%) of pesticides from glass beads by SFE using CHF_3

Analyte	Pressure (MPa)[a]			
	13.8	20.7	28.0	34.5
Desethylatrazine	72.9	96.5	68.5	84.0
Atrazine	78.4	95.4	63.6	70.8
Diazinon	69.4	86.5	62.5	61.7
Triallate	72.8	84.5	61.2	66.7
Metribuzine	77.4	94.6	66.2	64.2
Malathion	73.5	92.2	66.1	64.7
Fenpropimorph	66.7	84.3	71.3	75.6
Metazachlor	72.5	86.3	60.8	72.6
Propiconazol	64.6	87.7	73.6	76.5

The R.S.D.s (n = 3) for all percentage recoveries were between 2% and 9%.

[a] Conditions: extraction temperature = 26°C; extraction time = 30 min; matrix = silanized glass beads; trap temperature = liquid nitrogen temperature; extraction vessel size = 120 μl.

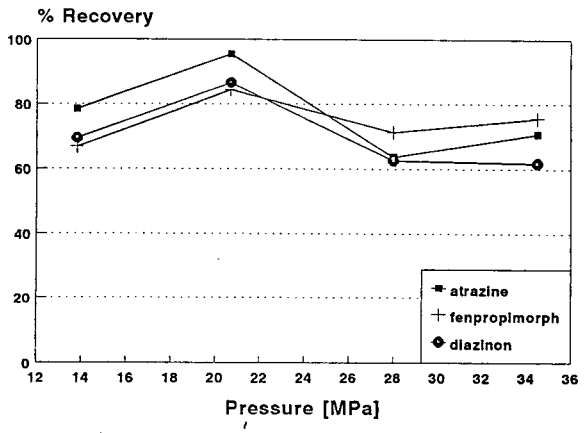

Fig. 1. Effect of pressure on the extraction of (□) atrazine, (+) fenpropimorph and (○) diazinon from silanized glass beads at 26°C and with an extraction time of 30 min. Percentage recoveries are based on those reported for CHF_3 extractions in Table 1.

pesticides differed in the total recovery, but not in the profile. At the lowest pressure the extractibility for most pesticides is greater than 72%, which is acceptable for multi-component analysis [32]. It can be seen that at pressures >20 MPa (i.e., reduced pressure >4) the recovery slopes down again for all pesticides. This phenomenon can be described as a consequence of repulsive forces "squeezing" the solute out of solution [33]. This investigation showed no selectivity for the different groups of pesticides if they were extracted with CHF_3 at 26°C and various pressures.

Tables 2 and 3 give the results obtained following the extraction at various densities with trifluoromethane and carbon dioxide. The temperature and the extraction time were kept constant at 50°C and 15 min, respectively, for both supercritical fluids during extraction. The highest recoveries for all the pesticides except for triallate and fenpropimorph were found at a density of 0.8 g/ml for both supercritical fluids. The reduced density and therefore the pressure needed to obtain this density was lower for CHF_3 than CO_2 because of the higher density and lower pressure of CHF_3 at its critical point. Fig. 2 shows as an example the extraction profiles for atrazine at various reduced densities

Table 2
Effects of density on the recovery (%) of the pesticides from glass beads by SFE using CHF_3

Analyte	Density (g/ml)[a]			
	0.6	0.7	0.8	0.9
Desethylatrazine	72.8	72.0	77.9	66.7
Atrazine	80.8	75.0	91.8	60.8
Diazinon	68.0	67.4	73.3	48.1
Triallate	73.3	71.0	70.7	55.8
Metribuzine	77.2	72.0	86.8	59.0
Malathion	75.1	71.4	82.1	55.0
Fenpropimorph	67.8	77.3	61.1	57.2
Metazachlor	69.9	72.7	78.4	57.6
Propiconazol	48.1	72.0	76.6	57.8

The R.S.D.s ($n = 3$) for all percentage recoveries were between 2% and 9%.
[a] Conditions: extraction temperature = 50°C; extraction time = 15 min; matrix = silanized glass beads; trap temperature = liquid nitrogen temperature; extraction vessel size = 120 μl.

because the extraction profiles for the other pesticides differed only in total recovery. No selectivity could be observed for different groups of pesticides, e.g., the polar triazines having aromatic amino groups and the less polar or-

Table 3
Effects of density on the recovery (%) of the pesticides from glass beads by SFE using CO_2

Analyte	Density (g/ml)[a]			
	0.6	0.7	0.8	0.9
Desethylatrazine	67.1	60.1	66.7	30.2
Atrazine	49.4	52.5	72.3	39.0
Diazinon	40.6	41.5	64.1	36.5
Triallate	41.5	44.8	65.7	34.6
Metribuzine	47.0	55.0	71.6	35.6
Malathion	47.4	45.9	70.2	40.1
Fenpropimorph	52.5	55.9	72.0	44.2
Metazachlor	50.0	58.1	66.3	38.8
Propiconazol	56.1	67.4	83.5	50.4

The R.S.D.s ($n = 3$) for all percentage recoveries were between 2% and 9%.
[a] Conditions: extraction temperature = 50°C; extraction time = 15 min; matrix = silanized glass beads; trap temperature = liquid nitrogen temperature; extraction vessel size = 120 μl.

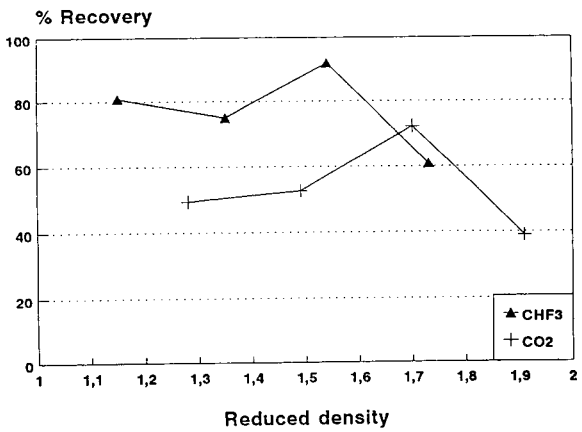

Fig. 2. Effect of the reduced density on the extraction of atrazine from silanized glass beads by SFE with (+) CO_2 versus (▲) CHF_3. Extractions were performed at 50°C for 15 min at densities of 0.6, 0.7, 0.8 and 0.9 g/ml for each supercritical fluid. Percentage recoveries are based on those reported for CHF_3 and CO_2 extractions in Tables 2 and 3, respectively.

ganophosphorus insecticides, although CHF_3 is capable of hydrogen bonding and has a large dipole moment of 1.6 D whereas CO_2 is without a dipole moment.

Because in environmental matrices the hydrocarbons are mostly present in large excess compared with the pesticides, all previous extractions were performed with the addition of alkanes and PAHs. Table 4 lists the results observed following the extraction of the pesticides without an addition of alkanes and PAHs. They were compared with the results shown in Table 2. The extractions were performed using only CHF_3 at different densities because the use of this supercritical fluid yields higher recoveries in the previous investigations compared with CO_2. Fig. 3 shows as an example the extraction profiles for atrazine. It can be seen that the extraction ability of CHF_3 for the selected pesticides without addition of alkanes and PAHs was significantly decreased. It is assumed that the hydrocarbons act as modifiers whereby the polarizability of the supercritical fluid was increased because CHF_3 is a small, hard molecule with a large dipole moment. Dobbs et al. [34] noted that a non-polar modifier such as octane has the same effect on polar and non-polar solutes to increase their

Table 4
Recovery of pesticides from glass beads without addition of PAHs and alkanes by SFE using CHF_3

Analyte	Density (g/ml)[a]			
	0.6	0.7	0.8	0.9
Desethylatrazine	n.d.	60.1	66.7	30.2
Atrazine	19.2	52.5	72.3	39.0
Diazinon	16.4	41.5	64.1	36.5
Triallate	19.0	44.8	65.7	34.6
Metribuzine	18.9	55.0	71.6	35.6
Malathion	19.1	45.9	70.2	40.1
Fenpropimorph	17.2	55.9	72.0	44.2
Metazachlor	17.7	58.1	66.3	38.8
Propiconazol	15.0	67.4	83.5	50.4

The R.S.D.s ($n = 3$) for all percentage recoveries were between 2% and 9%.

[a] Conditions: extraction temperature = 50°C; extraction time = 15 min; matrix = silanized glass beads; trap temperature = liquid nitrogen temperature; extraction vessel size = 120 μl.

solubility in a supercritical fluid if the molecular masses or polarizabilities are similar. In contrast, a polar modifier may increase markedly the solubility of a polar solute, but may not affect that of a non-polar solute. However, Schmitt and Reid [35] showed that fluoroform is a poor

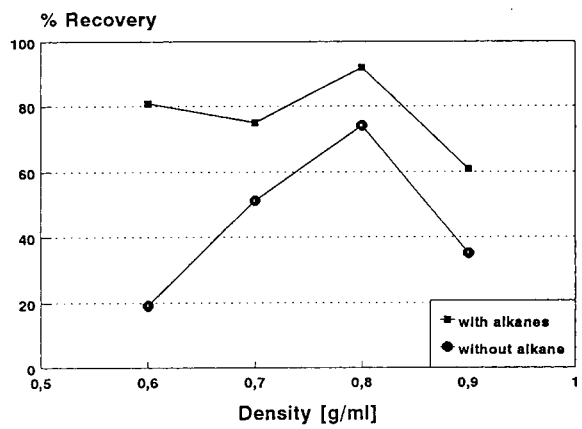

Fig. 3. Extractability of atrazine from silanized glass beads (□) with and (○) without PAHs and alkanes by SFE using CHF_3. SFE was performed at 50°C for 15 min. After additionally spiking the glass beads with 5 μl of the alkane/PAH solution, the solvent was allowed to evaporate for 10 min.

solvent for hydrocarbons, but found it to be a good solvent for molecules containing functional groups capable of hydrogen-bond association with the acid proton on fluoroform, such as carbonyl and amine.

4. Conclusions

The extraction of pesticides, i.e., triazines, organophosphorus insecticides, carbamates and anilides, using CHF_3 yields higher recoveries than CO_2 but the recoveries are not quantitative. No selectivity for pesticides of different polarity can be observed, although CHF_3 has a dipole moment whereas CO_2 does not. Extractions were carried out without and with the addition of alkanes and PAHs. The extraction efficiency of the pesticides with CHF_3 performed without the addition of alkanes and PAHs was significantly decreased.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Adsorption chromatography on cellulose XII. General effects of aqueous solutions of α-cyclodextrin as eluent

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Abstract

Adsorption chromatography on cellulose was examined for compounds that form complexes with cyclodextrins using aqueous solutions of α-cyclodextrin as eluent. Interesting effects were observed with methyl orange and similar diazo dyes and with aromatic nitro compounds.

1. Introduction

In a previous paper in this series we described the improvement of chiral separations of substituted tryptophans when aqueous solutions of α-cyclodextrin solutions were used as eluents [1]. R_F differences of 0.21 could be obtained in some instances whereas without α-cyclodextrin the R_F differences were 0.06–0.10. It was also noted that some substituted tryptophans increased their R_F values considerably in the presence of α-cyclodextrin, e.g., L-6-methyltryptophan from 0.24 to 0.58, but others much less, e.g., L-4-fluorotryptophan from 0.30 to 0.44, although in both instances the improvement in the chiral separation was almost identical. We felt that a general survey of the effect of α-cyclodextrin in an aqueous eluent would be interesting and report our findings here.

A preliminary survey of the compound groups that separate well on cellulose with aqueous solvents was disappointing. A random selection of alkaloids (berberine), acridine dyes (acriflavine), inks of felt pens, mercurochrome and some pH indicators such as methylene blue, α-naphthyl red, methyl red, bromocresol green and eosine, all of which yield good chromatograms on cellulose, do not notably change their R_F values when α- or β-cyclodextrin is added as the eluent. When we considered compounds known to form complexes with cyclodextrins, we were more successful in finding interesting chromatographic behaviour. In this paper we describe results obtained with methyl orange and similar diazo compounds and with nitrophenols, all of which have been extensively studied [2].

2. Experimental

Standard paper and thin-layer chromatographic techniques were used as reported previously

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[3]. The cyclodextrins were obtained from Fluka (Buchs, Switzerland).

3. Results

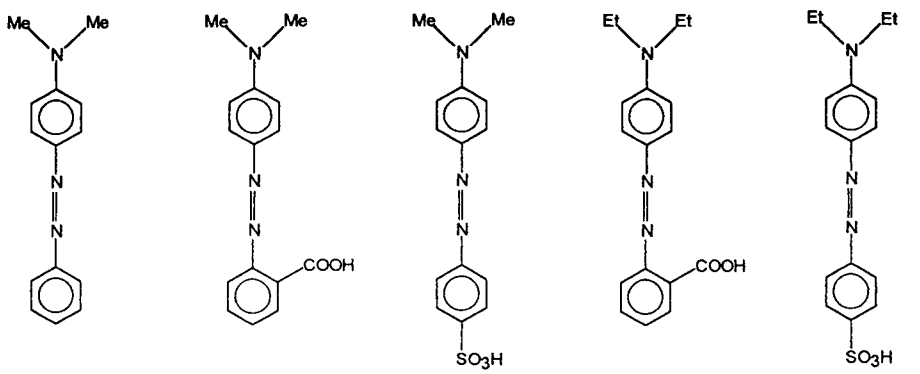
3.1. Methyl orange and similar diazo compounds

The α-cyclodextrin complex of methyl orange has been studied extensively and its crystal structure has been investigated [4].

Our first chromatograms showed a large increase in R_F value when α-cyclodextrin was added to the aqueous eluent. We therefore chromatographed a number of similar dyes (see formulae) together (the pH ranges given were those for which these compounds are used as indicators).

In chromatographing these compounds, not only the complexation with α-cyclodextin but also the degree of ionization must be taken into account. As shown in Table 1, pH values from 1 to 10 were examined. There remained the question of whether the anion of the buffer solution could play a role in so far as ion pairs could form between the cationic form of the compounds and the anions of the eluents.

We found there were only minor differences between 1 *M* $HClO_4$, 1 *M* HNO_3, 1 *M* HCl and 1*M* H_2SO_4, so that ion pairing could be considered of little importance. The R_F values in 2 *M* acetic acid were different, but it has a higher pH and exhibits considerable organic solvent prop-



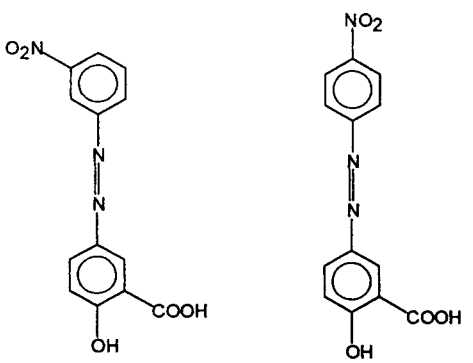


Table 1
R_F values of methyl orange and similar diazo compounds on microcrystalline cellulose thin layers (Merck No. 5577) with aqueous eluents in presence and in absence of α-cyclodextrin

Compound	0.5 *M* HCl (pH ≈ 0)	0.5 *M* HCl + 1% α-CD	1 *M* NaOAc + 1 *M* HOAc (pH ≈ 4.5)	1 *M* NaOAc + 1 *M* HOAc + 1% α-CD	1 *M* Na_2CO_3 (pH ≈ 10)	1 *M* Na_2CO_3 + 1% α-CD
Methyl yellow	0.31	*0.73*	0.11	*0.22*	0	*0.40*
Methyl red	0.19	0.17	0	0.09	0.11	0.14
Methyl orange	0.38	*0.77*	0.10	*0.43*	0.01	*0.72*
Ethyl orange	0.78	*0.97*	0.35	*0.69*	0.04	*0.73*
Ethyl red	0.37	0.35	0.26	0.28	0.17	0.23
Alizarin yellow 2G	0	0	0.08	*0.19*	0.01	*0.51*
Alizarin yellow R	0	*0.22*	0.03	*0.10*	0.01	*0.43*

erties. Therefore it cannot be compared with the other acids.

In Table 1, important R_F increases (≥0.10) on addition of α-cyclodextrin to the solvent are indicated in italics. Methyl red and ethyl red with a carboxylic group in the *ortho* position do not seem to interact with α-cyclodextrin. Ethyl orange, although much less adsorbed than methyl orange, shows very strong desorption in the presence of α-cyclodextrin. Alizarin yellow 2G and alizarin yellow R show the greatest effects in alkaline medium. The sulphonic group on methyl orange and ethyl orange is not of importance for complex formation, as shown by the fact that methyl yellow is desorbed fairly well in presence of α-cyclodextrin.

With this group of compounds we have the largest effects due to α-cyclodextrin so far encountered, and although most of them have both an acidic and an amino group, the effect is present in both acidic and alkaline media.

The effect of the α-cyclodextrin concentration was examined for 1 *M* NaCl as eluent, as shown in Table 2, and with 0.5 *M* HCl, as shown in Fig. 1. Fig. 1 shows that the plot of R_F versus concentration of α-cyclodextrin is an S-shaped curve. However, methyl orange and methyl yellow give parallel curves. Without data on the activities of the various constituents, it is difficult to draw conclusions. A methyl orange–α-cyclodextrin complex of ratio 1 : 2 has been characterized [4]. The S-shaped curve suggests the transition from a 1 : 1 to a 1 : 2 complex in solution. Methyl yellow exhibits behaviour very similar to that of methyl orange. In both compounds the remarkably high R_F increase due to α-cyclodextrin may be due to the formation of a very stable 1 : 2 complex.

Table 2
R_F values of methyl orange and similar diazo compounds on microcrystalline cellulose thin layers (Merck No. 5577) with 1 *M* NaCl as eluent and various concentrations of α-cyclodextrins

Compound	0% α-CD	1% α-CD	2% α-CD	4% α-CD	1β-CD
Methyl yellow	0	0.66	0.66	0.73	0
Methyl red	0.08	0.08	0.09	0.08	0.12
Methyl orange	0.10	0.90	0.75	At solvent front	0.60
Ethyl orange	0.44	0.86	0.87	At solvent front	0.72
Ethyl red	0.12	0.11	0.11	0.23	0.38
Alizarin yellow 2G	0.03	0.52	0.57	0.77	0.24
Alizarin yellow R	0	0.38	0.48	0.65	0.11

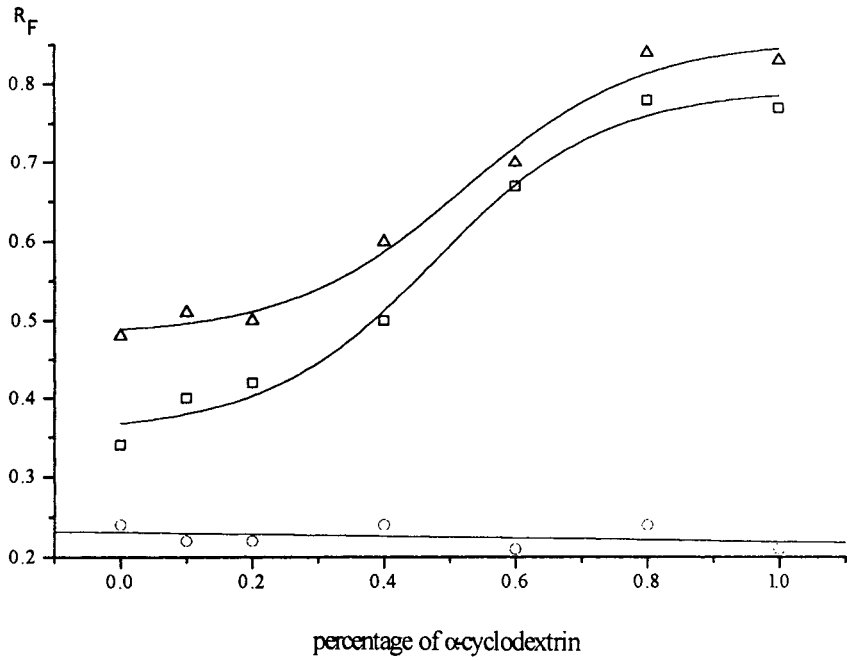


Fig. 1. Graph of R_F values of (□) methyl yellow, (○) methyl red and (△) methyl orange plotted against concentration of α-cyclodextrin in the eluent on Merck No. 5577 microcrystalline cellulose thin layers with 0.5 *M* HCl as eluent.

In considering the results in Table 2 and Fig. 1, the movement of the azo dyes depends on their adsorption on cellulose and their complexation with cyclodextrin. The forces involved in both interactions are believed to be mainly hydrophobic interactions and hydrogen bonds.

In the case of α-cyclodextrin, methyl yellow, methyl orange, ethyl orange, alizarin yellow 2G and alizarin yellow R all seem to favour complexation over adsorption on cellulose whereas methyl red and ethyl red favour adsorption on cellulose. When eluted with β-cyclodextrin the situation is not the same: methyl yellow is not eluted, ethyl red is more eluted than with α-cyclodextrin and alizarin yellow 2G and alizarin yellow R are not as strongly eluted. The structure of the cyclodextrin seems to play an important role.

3.2. Nitrophenols

Complexes between *p*-nitrophenol in its non-ionized and ionized forms and α-cyclodextrin have been characterized and the dissociation constants measured [2]. Here 2-, 3- and 4-nitrophenol, 2,4-dinitrophenol and picric acid were examined. These compounds give intermediate R_F values in various eluents (acidic, neutral and alkaline) on cellulose and their R_F values increase when α-cyclodextrin is added as the eluent. Care has to be taken with 2-nitrophenol: as it is fairly volatile it can be lost from the chromatogram.

There is an increase in the R_F values of all the compounds examined when α-cyclodextrin is added (Table 3). Comparison with the results obtained with methyl orange and other diazo compounds seems to suggest that all of them react with α-cyclodextrin, irrespective of the pH of the solution. Several methyl-*p*-nitrophenols

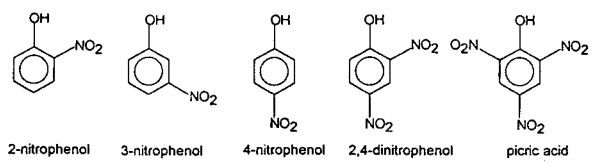


Table 3
R_F values of nitrophenols on microcrystalline cellulose thin layers (Merck No. 5577) with acidic, neutral and alkaline eluents containing α-cyclodextrin

Nitrophenol	1 *M* Na_2CO_3	1 *M* Na_2CO_3 +4% α-CD	40% sat. $(NH_4)_2SO_4$	40% sat. $(NH_4)_2SO_4$ +5% α-CD	1 *M* H_2SO_4	1 *M* H_2SO_4 + 4% α-CD
2-Nitrophenol	0.60	0.69	0.38	0.75	0.54	0.80
3-Nitrophenol	0.53	0.68	0.38	0.59	0.51	0.64
4-Nitrophenol	0.43	At solvent front	0.38	0.73	0.51	0.70
2,4-Dinitrophenol	0.33	0.78	0.25	0.71	0.51	0.59
Picric acid	0.33	0.53	0.24	0.53	0.44	0.55

have been reported to form complexes [2], so it is not surprising that the compounds listed in Table 3 also show R_F increases when α-cyclodextrin is added to the eluent.

4. Conclusions

Many compounds do not change their behaviour when α- or β-cyclodextrin is added to the eluent. Substantial effects could be obtained with compounds known to form inclusion complexes such as methyl orange and similar diazo compounds and nitrophenols. Increases in R_F values (in some instances from 0.1 to 1.0) could be obtained and were usually observed with neutral, acidic and alkaline solvents. This effect could provide an interesting diagnostic method for detecting cyclodextrin inclusion compounds. However, its limitation is that it could only be used for compounds that adsorb on cellulose.

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Adsorption chromatography on cellulose XIII. Chromatography with aqueous solutions of carbohydrates as eluents

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Abstract

The effect of adding carbohydrates to the aqueous eluent in adsorption chromatography on cellulose was investigated. No R_F shifts were obtained when sucrose or linear dextrans were added to the eluent. R_F changes occurred with soluble starch, but these are smaller than those with α-cyclodextrin and discriminate less between enantiomers (tryptophans) or between positional isomers (azo dyes). They could be obtained only in neutral or alkaline solutions.

1. Introduction

There is an extensive literature on the application of cyclodextrins in chromatography [1–3]. Cyclodextrins have been used as part of the stationary phase or as a constituent of the mobile phase. The forces involved were considered and Menges and Armstrong [4] concluded that hydrophobic interactions and hydrogen bonding in addition to specific steric effects due to the ring structure could account for the phenomena observed. Adsorption on cellulose was found to be essentially due to the same effects and all three interactions, hydrophobic, hydrogen bonding and chiral effects, were found to differ between native and microcrystalline celluloses [5].

When cellulose thin layers or papers are developed with aqueous solutions of cyclodextrins, good separations of enantiomers [6] and diazo dyes [7] were obtained. In these systems we thus have a stationary and a mobile carbohydrate phase and solute molecules interact with both phases in a similar manner.

To our knowledge, so far there have been no studies of adsorption on cellulose with carbohydrates other than cyclodextrins in the eluent. Adducts between saccharides and other compounds have been reported. Weichert [8] obtained a sucrose–tryptophan adduct that exhibited chiral specificity. We therefore felt that it might be rewarding to study a number of carbohydrates dissolved in an aqueous mobile phase.

In this work, we concentrated on the behaviour of substituted tryptophans and on diazo dyes similar to methyl orange (for structures of the azo dyes and the pH ranges for which they are used, see Ref. [7]), as both give substantial effects with α-cyclodextrin [6,7]. It is possible

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that these two compound groups are not representative, but at least we could use them for a comparison with α-cyclodextrin, with which they exhibit very marked effects.

2. Experimental

Standard ascending paper and thin-layer chromatographic techniques were employed.

The starches were obtained from Fluka (Buchs, Switzerland), except for Amidon soluble pour analyses, lot 86022, which was supplied by Prolabo (Paris, France). Dextran samples were obtained from Fluka.

Tryptophans were detected either with 1% ninhydrin in acetone or with iodine vapour. No reagents were needed for the azo dyes.

3. Results

3.1. Sucrose in the eluent

Weichert [8] reported adducts with sucrose obtained by evaporation, i.e., at very high sucrose concentrations. In this work, no effect was observed when 8% sucrose in 1 *M* NaCl was used as the eluent for diazo dyes, as shown in Fig. 1. No changes in enantiomeric separations were noted when sucrose was added to the eluent in the separation of substituted tryptophans. Perhaps one reason for the lack of effects is the high molecular mass of sucrose (342.30). An 8% solution is thus only 0.23 *M*. Higher concentrations tend to give very viscous solutions, which are impractical as eluents.

3.2. Starch in the eluent

We examined mainly two soluble starches, namely Amidon soluble pour analyses, Prolabo lot 86022, and Starch from potatoes, Fluka lot 85642. Both yield clear solutions when dissolved in 1 *M* NaCl.

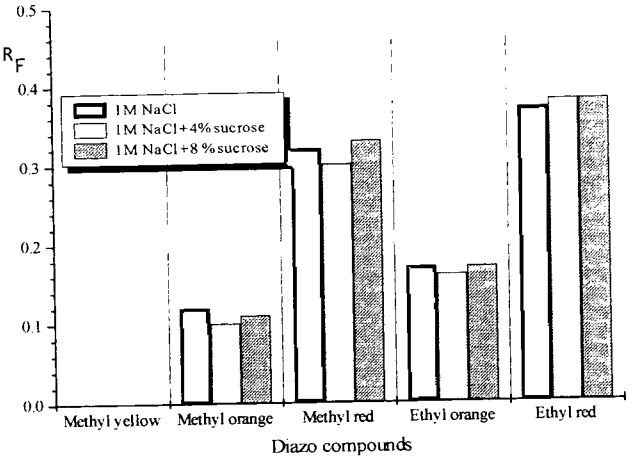

Fig. 1. Effects of different concentrations of sucrose in the eluent on the R_F values of azo dyes on Merck No. 5577 microcrystalline cellulose thin layers.

Substituted enantiomeric tryptophans give a measurable increase in R_F differences with 8% Amidon, and this seemed greatest with tryptophans substituted in the 4-position (4-methyl and 4-fluoro). The differences were about 0.1, but the effect is usually less than that with the same concentrations of α-cyclodextrin [7].

On microcrystalline cellulose plates, there is a considerable effect when methyl orange and related diazo compounds are chromatographed in the presence of starch (Fig. 2). Similar results were obtained on Whatman 3MM paper. This behaviour is different from that obtained earlier with α-cyclodextrin [7]: α-cyclodextrin interacts also at acidic pH (0.5 *M* HCl), whereas starch does not (Fig. 2a); α-cyclodextrin does not interact with methyl red and ethyl red [7], whereas starch does (fig. 2b and c); and the R_F shifts are less with starch than with α-cyclodextrin but they give linear R_M versus log[starch] plots (Fig. 3), which is not the case with α-cyclodextrin.

Complexes between azo dyes and amylose have already been studied by spectral methods by Sensse and Cramer [9] and it was suggested that the addition could be explained by the helical structure of amylose. In soluble starch, however, there are also branched structures of

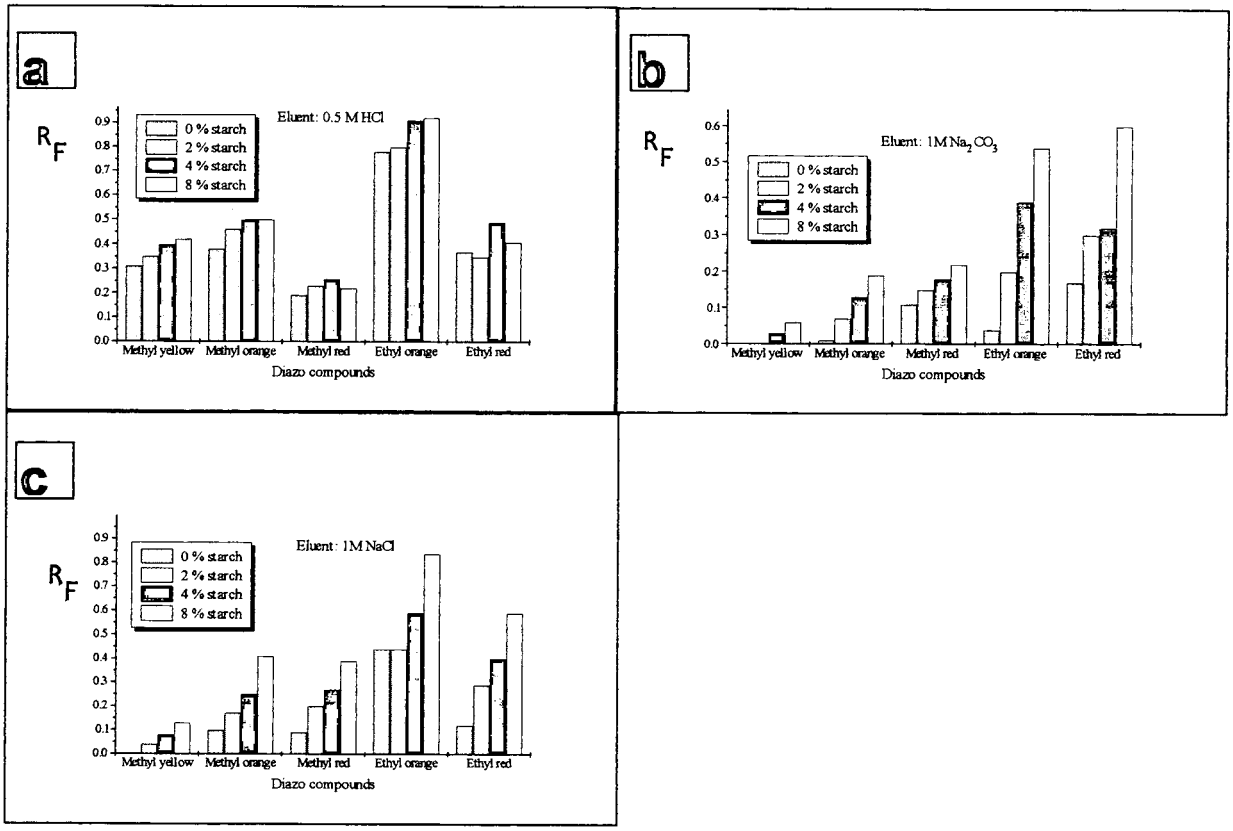

Fig. 2. Effects of starch in the eluent on the R_F values of some azo dyes on Merck No. 5577 microcrystalline cellulose thin layers with eluents (a) 0.5 *M* HCl, (b) 1 *M* Na_2CO_3 and (c) 1 *M* NaCl.

the amylopectin, which may also have a similar action. Table 1 shows a comparison of potato starch with wheat and maize starch. The latter two are only poorly soluble (less than 1%) and saturated solutions were prepared. It can be seen that no great difference exists between them once concentrations are taken into account.

3.3. Dextrans in the eluent

Dextrans are linear α-1–6-linked D-glucose polymers of bacterial origin and are available in a range of molecular mass fractions. Unlike starch, there are no R_F changes worth discussing either in enantiomeric separations of substituted tryptophans or in the separation of diazo dyes.

3.4. Differences between microcrystalline cellulose thin layers and Whatman 3MM paper

In this work we noted for azo dyes that when 1 *M* NaCl is used as the eluent different sequences are obtained with microcrystalline cellulose and Whatman 3MM paper, whereas in all other systems that we have investigated [5–7] there is little difference between these two supports.

The problem is illustrated in Table 2 (the values in italics indicate R_F differences >0.10). Here we compare also R_F values in alkaline and acidic media, which show the expected agreement. Whatman 3MM paper must be assumed to be rich in calcium and even to contain $CaCO_3$ on the surface owing to its manufacture with very hard water in the UK. For this reason, Whatman

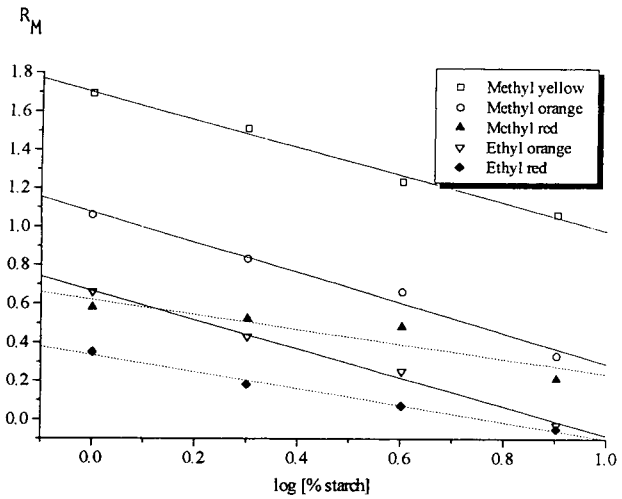

Fig. 3. Relationship between R_M values and percentage of added potato starch for some azo dyes chromatographed on Whatman 3MM paper using 1 *M* NaCl as eluent.

papers must be washed with acid for separations involving phosphate (see the last column in Table 2). Chromatography on acid-washed papers was tried and the same sequence as on the thin layer was obtained (Table 2). In presence of 8% of starch essentially the same chromatogram was obtained as on unwashed paper.

4. Discussion

After observing substantial R_F shifts when α-cyclodextrin was added to aqueous solvents in adsorption chromatography on cellulose [6,7], we wanted to investigate whether other polysaccharides also exhibited similar behaviour. Sucrose and linear dextrans did not produce any R_F shifts with substituted tryptophan enantiomers or with azo dyes whereas soluble starch showed measurable effects. However, the interactions seem to be different from those observed with cyclodextrins, as they do not occur in acidic medium and they seem to be less discriminatory with respect to molecular shapes. We did not observe essential differences between starches of different origins.

Table 1
R_F values of azo dyes on Whatman 3MM paper developed with 1 *M* NaCl containing different starches

Compound	Eluent: 1 *M* NaCl		
	+1% potato starch	Saturated with wheat starch	Saturated with maize starch
Methyl yellow	0.02	0.02	0.02
Methyl orange	0.17	0.09	0.09
Methyl red	0.36	0.31	0.32
Ethyl orange	0.30	0.16	0.18
Ethyl red	0.49	0.38	0.40

Table 2
Comparison of R_F values on Merck No. 5577 microcrystalline cellulose thin layers (TL) and Whatman 3MM paper with different eluents

Compound	0.5 *M* HCl		1 *M* NaCl		1 *M* Na_2CO_3		1 *M* NaCl
	Whatman 3MM	Merck TL	Whatman 3MM	Merck TL	Whatman 3MM	Merck TL	Acid-washed Whatman 3MM
Methyl orange	0.59	0.50	0.12	0.10	0.03	0.01	0.20
Methyl red	0.30	0.25	*0.32*	*0.08*	0.17	0.11	0.07
Ethyl orange	0.78	0.78	*0.17*	*0.43*	0.05	0.04	0.67
Ethyl red	0.44	0.37	*0.37*	*0.12*	0.24	0.17	0.12

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