ISSN 0021-9673

VOL. 477 NO. 2 AUGUST 30, 1989

THIS ISSUE COMPLETES VOL. 477

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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JOURNAL OF CHROMATOGRAPHY

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0021-9673/89/$03.50

Printed in The Netherlands

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Journal of Chromatography, 477 (1989) 187–233
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 587

REVIEW

CHROMATOGRAPHIC METHODS AS TOOLS IN THE FIELD OF MYCOTOXINS

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(First received February 22nd, 1989; revised manuscript received April 18th, 1989)

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0021-9673/89/$03.50

1. INTRODUCTION

Mycotoxins are secondary metabolites of fungi that have been recognized mostly within the last three decades as a potential threat to animal and human health. Various chromatographic techniques have been used for their separation and in only a relatively few instances have mycotoxins been obtained pure using techniques other than chromatography[1] (*e.g.*, secalonic acid, rubratoxin, patulin and citrinin[2–5]).

In the 1980s, various reviews and book chapters on the chromatography of mycotoxins were published (*e.g.*, refs. 1, 6–13). This review describes the most important achievements of chromatography in the field of mycotoxins. The text is divided into the following sections: (2) paper chromatography (PC), (3) thin-layer chromatography (TLC), (4) liquid column chromatography (LCC), and (5) gas chromatography (GC).

The literature on the chromatography of mycotoxins is immense and it would be virtually impossible to give an exhaustive survey. Whereas references to the PC of mycotoxins cover the 1950s and the early 1960s only (in order to show a historical continuity), the literature on TLC, LCC [high-performance liquid chromatography (HPLC) in particular] and GC covers mostly the early and mid-1980s.

In this review, applications of chromatograpic techniques include so-called multi-mycotoxin analysis, followed by data on structurally related families of mycotoxins and individual compounds. Data concerning recent clean-up methods, qualitative analysis, quantitation and preparative methods are also presented.

2. PAPER CHROMATOGRAPHY

In the 1950s and early 1960s, a variety of fungal secondary metabolites, currently classified as mycotoxins, were studied among antibiotics by means of various PC techniques. These techniques have been successfully used to characterize and classify antibiotics, and have also been helpful in establishing isolation procedures for unknown compounds. A few examples are given below.

In the early stages of studies on the metabolites of the toxigenic fungus *Penicillium islandicum,* PC was adopted for the identification of its hydroxyanthraquinones (islandicin, iridoskyrin, catenarin, chrysophanol, erythroskyrin, luteoskyrin, flavoskyrin, rubroskyrin and oxyskyrin) and applied to the detection of anthraquinone-producing strains[14].

Another mycotoxin, cyclochlorotine or chlorine-containing peptide, isolated from *P. islandicum,* has been characterized by PC in several solvent systems[15].

A classification of antibiotics by means of "salting-out paper chromatograms" using nine solvent systems with increasing concentrations of ammonium chloride in water was developed and the analysed antibiotics were divided into six groups (A–F)[16,17]. The classification was extended to fungal metabolites[18], including the following compounds currently classified as mycotoxins: group A, gliotoxin, kojic acid, patulin, penicillic acid and terreic acid; group C, aspergillic acid, neohydroxyaspergillic acid, citrinin and trichothecin; and group D, alternariol, cyanein (brefeldin A), griseofulvin, mycophenolic acid and rugulosin.

Another principle of classification by means of the so-called "pH chromatograms" has been introduced[19,20]. Using this PC technique, the ionic character of

unknown antibiotics and also the general possibilities of their isolation could be determined when only their crude concentrates from Petri dish cultures were available. The principles of "pH chromatography" have been reviewed elsewhere[21,22]. Several fungal metabolites belonging to mycotoxins, such as citrinin, mycophenolic acid, gliotoxin, rugulosin, neohydroxyaspergillic acid and trichothecin, have also been studied by "pH chromatography"[23,24].

PC studies of compounds in several solvent systems for the purpose of their classification and identification have proved useful in systematic analysis. A combination of the sequential and simultaneous analysis of antibiotics was elaborated by the present author. In addition to a series of antibiotics, the following mycotoxins were characterized by the method: kojic acid, penicillic acid, cyanein (brefeldin A), alternariol, rugulosin, citrinin, trichothecin, patulin, gliotoxin, mycophenolic acid, aspergillic acid and griseofulvin[25].

The three above-mentioned PC techniques were found to be helpful in the isolation and identification of citrinin produced by a penicillium strain[26]. When a crude acetone extract of metabolites from an agar culture was chromatographed in four principal solvent systems of systematic PC analysis[25] and detected with *Bacillus subtilis,* the unknown antibiotic was found to belong to subclass Va. Then a "pH chromatogram", using water-saturated ethyl acetate as the mobile phase, showed the unknown antibiotic to be an acid. According to the course of its S-shaped R_F curve it was decided that the antibiotic should be extracted from the culture filtrate with ethyl acetate at pH 2–3 and then transferred from the organic solvent into a phosphate buffer at pH 8.5. The third step was a re-extraction with chloroform at pH 2.5. (It had been found in earlier studies that the S-shaped R_F curves obtained from the "pH chromatograms" represented the dependence of the R_F values on the following factors: pH of the stationary phase, distribution coefficients between the mobile and stationary phases at these pH values and pK values[23,27]). When the purified antibiotic was again characterized chromatographically, its great similarity to citrinin was apparent. The physical, chemical and biological properties of the isolated compound confirmed its identity as citrinin[26].

In our laboratory, several strains of a collection of fungi have been found to produce substances that inhibit mycobacteria. When the active substances, present in crude extracts from agar cultures of four aspergilli, were analysed by systematic PC analysis[25], all of them belonged to subclass IIa and their "summarized chromatograms" were similar to that of kojic acid of the same subclass. The identity to kojic acid was confirmed after the isolation and purification of the active products[28].

PC was used by Sargeant *et al.*[29] in the first separation of aflatoxins from a crude extract of groundnuts. The toxic component produced a single spot that fluoresced under UV light. PC was also used by Holzapfel *et al.*[30] to isolate aflatoxins M_1 and M_2 from a concentrate from the urine of aflatoxin-fed sheep.

However, TLC using silica gel superseded PC and has resulted in the isolation and purification of almost all the aflatoxins and many related fluorescent metabolites.

3. THIN-LAYER CHROMATOGRAPHY

Historically, TLC has been the method of choice for aflatoxin analysis, following the then-recent rediscovery and development of this technique by Stahl. It was

shown as early as in 1962 by Nesbitt *et al.*[31] and, in 1963, by Hartley *et al.*[32] that the single blue-fluorescing spot of toxin-containing extract, observed by PC, could be split into four main components when the extracts were chromatographed on silica gel TLC plates developed in chloroform–methanol. Two of these components, fluorescing blue under UV light, were designated aflatoxins B_1 and B_2 and the other two components, fluorescing turquoise under UV light, were designated aflatoxins G_1 and G_2. Since then various combinations of silica gel and solvent systems have been proposed for separating aflatoxins by TLC.

Pure aflatoxins for structural determination were obtained by preparative TLC (PLC), and quantitative methods have also been developed. In some instances the aflatoxins served as the model compounds for the development of reliable methods to measure trace levels in foods and feeds. The TLC of aflatoxins has received the most attention over the years; consequently, it is the most refined and generally serves as a model for the other mycotoxins[33].

Of the chromatographic techniques applied to mycotoxins, TLC is by far the most widely used in the detection, analysis and characterization of fungal toxins. With advances in techniques, TLC is becoming the method of choice for some mycotoxins.

In the 1980s, numerous reviews and book chapters on the chromatography of mycotoxins in general and on TLC in particular have been published (*e.g.*, refs. 1, 6–13, 33–39). A recent book devoted to methods for the production, isolation, separation and purification of mycotoxins[11] included sections on the TLC of many mycotoxins.

In a chapter on TLC and high-performance TLC (HPTLC) of mycotoxins, Nesheim and Trucksess[33] described the chief procedures for mycotoxins (including multi-dimensional development, screening methods, quantitation and identification) and presented analytical methods for the best known mycotoxins. In a recent review on the TLC of mycotoxins[12], the literature from 1961 to 1984 was covered. Therefore, in this review, only the major achievements of TLC in mycotoxicology published in the 1980s are presented.

3.1. Multi-mycotoxin TLC

Various multi-mycotoxin methods have been published for the simultaneous detection, in natural products, of a number of mycotoxins, which differ in the extraction solvents, clean-up procedures and final detection methods. Among clean-up techniques, disposable Sep-Pak silica cartridges[38] and gel-permeation colums[39] have been applied.

HPTLC was applied by Lee *et al.*[40] to multi-mycotoxin determination (see below). HPTLC and reversed-phase TLC of ten mycotoxins (ochratoxin A, aflatoxins B_1, B_2, G_1 and G_2, zearalenone, sterigmatocystin, T-2 toxin, diacetoxyscirpenol and vomitoxin) with the use of various normal- and reversed-phase solvents and UV detection were reported by Stahr and Domoto[41].

Golinski and Grabarkiewicz-Szczesna[42] published chemical confirmatory tests for ochratoxin A, citrinin, penicillic acid, sterigmatocystin and zearalenone that are performed directly on TLC plates. Later, Grabarkiewicz-Szczesna *et al.*[43] published a multi-detection procedure for the determination of eleven mycotoxins in cereals.

Coman *et al.*[44] reported a TLC analysis of feed samples in which four aflatox-

ins, ochratoxin A, zearalenone, sterigmatocystin and T-2 toxin were detected. A simultaneous TLC detection of aflatoxin B_1 and zearalenone in mixed feed for pigs was described[45]. Another multi-mycotoxin method involving a membrane clean-up step and two-dimensional TLC was published by Patterson *et al.*[46].

Whidden *et al.*[47] developed a method for the simultaneous extraction, separation and determination of eight mycotoxins in corn. Mycotoxins were extracted with acetonitrile, eluted sequentially from a silica gel mini-column and rendered visible by TLC. A flow chart for the extraction and separation of mycotoxins is presented in Fig. 1. Fractions 2 (containing zearalenone and sterigmatocystin), 3 (containing patulin and penicillic acid) and 4 (containing ochratoxin A, aflatoxin B_1 and diacetoxyscirpenol) were analysed on the same TLC plate using external and internal standards. Fraction 5 (containing rubratoxin B) was applied to a separate TLC plate together with external standards.

Zearalenone, T-2 toxin, HT-2 toxin and neosolaniol were detected in grains of oats, wheat and barley[48]. Nowotny *et al.*[49] detected citrinin, ochratoxin A and sterigmatocystin in commercial cheese using TLC and HPTLC. The same group also published a TLC screening method for the determination, in mouldy foods, of 22 mycotoxins[50].

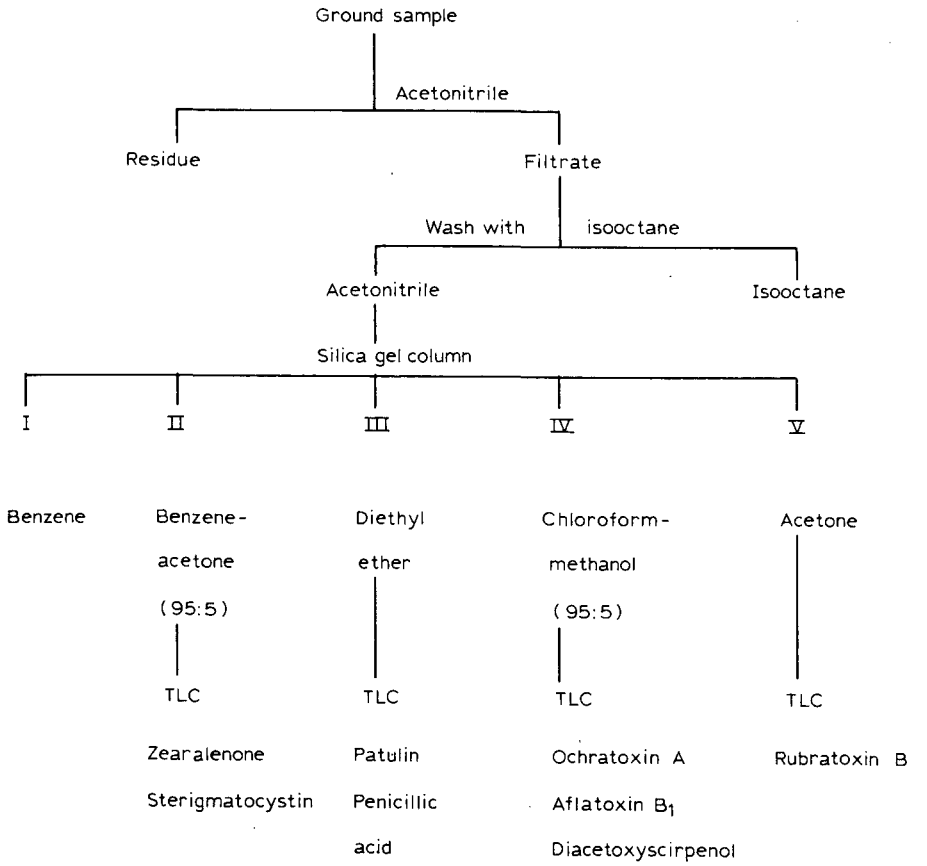

Fig. 1. Flow chart for the extraction and separation of mycotoxins. Adapted from ref. 47.

Gimeno and Martins[51] published a rapid TLC determination of patulin, citrinin and aflatoxin in apples and pears and their juices and jams. The toxins were identified with various developing solvents, spray reagents and chemical reactions and then quantitated by the limit of detection method.

A method for the routine examination of mouldy rice, wheat, bread and other vegetable foodstuffs was described[52]. The mycotoxins were separated by two-dimensional TLC. Aflatoxins were determined fluorimetrically. Ochratoxin A, patulin, penicillic acid and sterigmatocystin were developed on separate plates. Citrinin was chromatographed on an oxalic acid-pretreated plate. Citrinin and ochratoxin A were immediately determined by fluorimetry. The other toxins had to be converted into fluorescent derivatives (penicillic acid using a diphenylboric acid–2-ethanolamine spray, patulin using an N-methylbenzthiazolone-2-hydrazone spray and sterigmatocystin using an aluminium chloride spray reagent) for quantitative determination.

A quantitative TLC method for the analysis of aflatoxins, ochratoxin A, zearalenone, T-2 toxin and sterigmatocystin in foodstuffs was published by Tapia[53]. Detections of *Fusarium moniliforme* toxins[54] and of toxigenic *Fusarium* isolates[55] have been reported. Thrane[55] used griseofulvin as a relative internal standard for *Fusarium* toxins.

Lee *et al.*[40] described a method for the simultaneous determination of thirteen mycotoxins by HPTLC. Using seven continuous multiple developments with two solvent systems of different polarity, a baseline separation of sterigmatocystin, zearalenone, citrinin, ochratoxin A, patulin, penicillic acid, luteoskyrin and aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2) was achieved. About 1 h was required for the separation and quantitation of all thirteen toxins from one spot. By using *in situ* scanning of the HPTLC plate, detection limits in the low nanogram range were obtained by UV–visible absorption and in the low picogram range by fluorescence, with a relative standard deviation of 0.7–2.2% in the nanogram range. Chromatography was performed on 10 × 10 cm HPTLC plates coated with silica gel 60 and impregnated with EDTA. The development stages and spectroscopic properties used for quantitative determination of the individual mycotoxins are presented in Table 1. The mobile phase migration distance was fixed by arranging for a portion of the plate to protrude through the top of the saturated development chamber, at which point the solvent could evaporate freely. The use of multiple development offered the possibility of quantifying the components as they were separated, the use of more than one solvent system and natural refocusing of the sample spot, which occurred when the plate was dried between developments. At each stage of scanning, the migration distance of the spot to be measured was maintained between 1 and 3 cm. Hence the method is capable of providing good resolution of complex mycotoxin mixtures. However, the authors did not show whether comparable results could be obtained with samples extracted from natural products.

Paterson[56] published standardized one- and two-dimensional TLC methods for the identification of fungal secondary metabolites. A simple screening method for moulds producing the intracellular mycotoxins brevianamide A, citreoviridin, cyclopiazonic acid, luteoskyrin, penitrem A, roquefortine C, sterigmatocystin, verruculogen, viomellein and xanthomegnin was developed by Filtenborg *et al.*[57]. After removing an agar plug from the mould culture, the mycelium on the plug is wetted with a drop of methanol–chloroform (1:2). By this treatment the intracellular mycotoxins

TABLE 1

DEVELOPMENT STAGES AND SPECTROSCOPIC METHODS USED FOR THE DETECTION OF MYCOTOXINS BY HPTLC

Adapted from ref. 40.

Development stage	*Time (min)*	*Mycotoxins separated*	*Spectral characteristic used for detection*
Toluene–ethyl acetate–formic acid (30:6:0.5):			
1st development	5.0	Sterigmatocystin	Reflectance, λ = 324 nm
		Zearalenone	Fluorescence, λ_{ex} = 313 nm, λ_{em} = 460 nm
		Citrinin	
2nd development	5.0	No measurement	
3rd development	6.0	Ochratoxin A	Fluorescence, λ_{ex} = 313 nm, λ_{em} = 460 nm
4th development	6.0	Penicillic acid	Reflectance, λ = 240 nm
		Patulin	Reflectance, λ = 280 nm
		Luteoskyrin	Reflectance λ = 440 nm
Toluene–ethyl acetate–formic acid (30:14:4.5):			
5th development	8.0	No measurement	
6th development	8.0	No measurement	
7th development	8.0	Aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2	Fluorescence, λ_{ex} = 365 nm, λ_{em} = 430 nm

are extracted within a few seconds and transferred directly to a TLC plate by immediately placing the plug on the plate while the mycelium is still wet. After removal of the plug, known TLC procedures are carried out. The same procedure was applied to detect aflatoxins, ochratoxin A, citrinin, patulin and penicillic acid in solid substrates.

Multi-mycotoxin TLC has been used succesfully in chemotaxonomic studies of the *Penicillium viridicatum* group[58,59], common asymmetric penicillia[60,61], triverticillate penicillia[62], *Emericella* spp.[63] and other toxigenic moulds[64].

In most TLC screening procedures, extraction and clean-up techniques are applied prior to the TLC analysis. Krivobok *et al.*[65] published rapid and sensitive methods for detecting toxigenic fungi producing aflatoxins, ochratoxin A, sterigmatocystin, patulin, citrinin, penicillic acid and zearalenone. The toxin-producing moulds tested produced detectable amounts of their respective mycotoxins within 2–4 days of incubation at 24°C in a liquid medium. Sterigmatocystin had to be extracted from the mycelium and the rapid production of zearalenone needed to be temperature programmed (24°C for growth and 10°C for toxin production). Detection of the toxins by means of TLC was possible without extraction of the medium or after extraction without purification. The sensitivity of TLC detection and the recovery after extraction were good (see table 2).

Recently, Frisvad and Thrane[66] published a general standardized method for the analysis of mycotoxins and other fungal metabolites, based on HPLC and combined with TLC in two different eluents using R_F values relative to griseofulvin. Data for 182 metabolites were listed in their paper.

3.2. TLC of aflatoxins and related compounds

Various aspects of the TLC of aflatoxins have been discussed in recent years

TABLE 2

TREATMENTS FOR TLC DETECTION AND MINIMUM TOXIN CONCENTRATION DETECTED

Modified from ref. 65.

Mycotoxin	*Treatment*[a]	*Minimum amount detected (ng)*[b]
Aflatoxins B_1, B_2, G_1, G_2	UV	1
Ochratoxin A	UV	10
Citrinin	UV, NH_3, $AlCl_3$	10
Sterigmatocystin	$AlCl_3$	5
Patulin	MBTH	25
Penicillic acid	ANIS	10
Zearalenone	UV, $AlCl_3$	10

[a] Abbreviations: UV, viewed under UV light at 254 or 366 nm; NH_3, ammonia vapour for 15 s; $AlCl_3$, 20% (v/v) aluminium chloride in 96% ethanol; MBTH, 3-methylbenzothiazol-2-one hydrazone hydrochloride solution; ANIS, 0.5% (v/v) *p*-anisaldehyde in ethanol–acetic acid–concentrated sulphuric acid (17:2:1).

[b] ng deposited on TLC plate.

(*e.g.*, refs. 6, 9 and 67–71). A survey of applications was given in a recent review[12] and by Nesheim and Trucksess[33].

Extraction and clean-up procedures, adsorbents and solvent systems have been reviewed[12]. Modifications and improvements of extraction and clean-up techniques have been published[72–78]. Velasco[79] proposed the replacement of benzene as a solvent for aflatoxin standards. Modern methods of aflatoxin extraction use a water–organic solvent mixture, *e.g.*, chloroform–water (10:1)[9,80]. The water wets the substrate and the small amount taken up in the aqueous phase is immediately removed by the solvent, giving a rapid isolation procedure.

The aflatoxins are highly fluorescent and highly oxygenated heterocyclic compounds. Separation of the four main aflatoxins (B_1, B_2, G_1 and G_2) was compared on six commercial silica gel plates, HPTLC giving more compact spots[81]. Procedures for improving aflatoxin spot size and fluorescence intensity have been published[82]. By using strong eluting solvents, diffuse spots could be reduced in size and poorly resolved chromatograms returned to their original state for redevelopment.

Gulyás[74] reported over-pressurized liquid chromatography on Kieselgel 60 HPTLC plates. Two-dimensional TLC of aflatoxin mixtures has been published[83,84]. Eller *et al.*[83] used acetonitrile–acetone–benzene (9:1:1) for the first and diethyl ether–methanol–water (96:3:1) for the second development. Good separation from impurities was achieved[84] and all four aflatoxins were well resolved using chloroform–acetone–water for the first development and toluene–ethyl acetate–formic acid (30:15:5 or 24:20:6) in the second direction. Silufol plates have been applied in the TLC of aflatoxins[83,85].

Aflatoxins have been detected by TLC in groundnuts[86–88], corn[89], black olives[90], raisins[91], ginger[92], groundnut products, corn and nuts[86], vegetable oils[93], food[85,94] and mixed feeds[95,96].

The determination of aflatoxins in vegetable oils is usually based on partition between two immiscible solvents. Aflatoxins are extracted from the oil into a polar solvent and subsequently partitioned into chloroform. Although the recoveries are acceptable, these methods are time consuming, require large volumes of solvents, and frequently involve troublesome emulsions. Miller *et al.*[97] proposed a simple method for the determination of aflatoxins which was succesfully applied to both crude and degummed oils. The oil sample, dissolved in hexane, was applied to a silica gel column and washed with diethyl ether, toluene and chloroform. Aflatoxins were eluted from the column with chloroform–methanol (97:3). Quantitation was then performed by TLC or HPLC.

Leitao *et al.*[98] used TLC for the identification of aflatoxins in extracts from cultures of *Aspergillus* strains isolated from foodstuffs. The aflatoxins were then quantitated by HPLC.

Quantitative determination of aflatoxins in groundnut products using sequential TLC has been reported[86]. The method involves double development with diethyl ether followed by chloroform–acetone–water (88:12:1.5) and triple development with diethyl ether followed by chloroform–acetone–benzene (90:10:10) and chloroform–acetone–hexane (71:12.5:16.5). The aflatoxins were detected spectrometrically (325 nm) at levels of $\geqslant$ 0.05 ng per spot.

In spiked corn samples, greater than 100% recoveries using instrumental HPTLC were observed for aflatoxin analyses[99]. Spots overlying aflatoxins B_1 and B_2 were identified by GC to be C_{16}–C_{18} free fatty acids which enhanced the fluorescence of aflatoxin B_1, resulting in $>100\%$ recoveries. The inclusion of acetic acid in the mobile phase resulted in an increased mobility of the free fatty acids, which eliminated the positive interference on aflatoxin fluorescence.

TLC confirmatory tests were applied in the mini-column chromatography of aflatoxins by Madhyasta and Bhat[100]. Aflatoxins and aflatoxicols in extracts from cereals were detected with and without previous clean-up on a silica gel column. After separation of aflatoxicols from aflatoxins by CC, TLC separated aflatoxicols I and II, aflatoxins M_1 and M_2 being also separated. TLC without previous column separation also separated aflatoxins B, G and M plus aflatoxicols. Fluorodensitometry was used for their quantitation.

Severals papers concerning aflatoxins M have been published. Aflatoxin M_1 has been determined by TLC in milk and various milk products[101–109]. An improved, rapid method for the routine determination of aflatoxin M_1 in milk is based on TLC followed by elution and fluorimetric analysis[110]. TLC data for aflatoxin M_2 have been reported[111]. Koch and Kross[112] described the quantitative determination of harmful aflatoxins in selected cheese samples of food for the military. An assay method for a new hydroxyaflatoxin B_1, aflatoxin M_4, was published by Lafont *et al.*[113].

Reversed-phase HPTLC with fluorimetric detection was used by Blanck *et al.*[114] in their studies of binding of aflatoxin M_1 to milk proteins. A rapid and reproducible method for the extraction and determination of aflatoxin M_1 in milk and dairy products was published[115]. After extraction and clean-up, the aflatoxin was detected by HPLC or TLC. In TLC on silica gel, hexane–acetone (9:1) was used for the first development followed by chloroform–acetone–2-propanol (85:10:5) in the same direction. Fluorescence at 365 nm was observed after spraying the plates with nitric acid and fluorodensitometry was carried out at 440 nm.

Hsieh *et al.*[116] used a sequence of solvent systems for the TLC of aflatoxin B_1 and its metabolites. The plate was first developed in diethyl ether and the separated aflatoxicol was quantitated. The plate was then developed in chloroform–acetone–2-propanol (85:15:15); aflatoxin Q_1 and aflatoxicol H_1 were completely separated after two developments. The final separation of aflatoxins M_1 and B_{2a} was effected by further development in benzene–ethanol (40:4) or chloroform–ethanol (9:1).

Aflatoxins in air samples of refuse-derived fuel were determined by TLC with laser-induced fluorescence spectrometric detection[117]. A rapid determination of aflatoxin together with patulin and citrinin in apples and pears and in products thereof was reported[51]. In a collaborative study, Stubblefield *et al.*[118] carried out the determination and TLC confirmation of the identity of aflatoxins B and M in artificially contaminated beef liver.

TLC was one of the steps leading to the detection of aflatoxin D_1 in ammoniated corn[119]. The most important data on various members of the aflatoxin group have been summarized[67]. They included the hydroxyaflatoxins B_{2a} and G_{2a}, aflatoxins M_1 and M_2, GM_1 and GM_2, M_{2a} and GM_{2a}, parasiticol (aflatoxin B_3) and aflatoxicol (both epimers). Acid dehydration products of aflatoxicol were characterized by TLC[120].

TLC has been applied in studies on aflatoxin biosynthesis[121–123]. Biosynthetically, the aflatoxins are acetate-derived decaketides that are formed via polyhydroxyanthraquinone intermediates. The proposed aflatoxin biosynthetic pathway now consists of the following steps: acetate → norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → *O*-methylsterigmatocystin → aflatoxin B_1[124].

Townsend *et al.*[125] synthesized labelled averufin, which was subsequently incorporated by mycelial suspensions of *Aspergillus parasiticus*. The intact incorporation of averufin into versiconal acetate, versicolorin A and aflatoxin B_1 was demonstrated. Analytical TLC and preparative TLC (PLC) proved to be extremely useful methods in both the synthesis and incorporation of labelled averufin. When combined medial and mycelial extracts were subjected to PLC on silica gel 60 plates, labelled averufin and aflatoxins were separated using hexane–acetone–diethyl ether (7:6:4). The aflatoxin mixture was further separated on a column of silica gel with chloroform–methanol (97:3). In TLC, chloroform–acetone (9:1) provided much cleaner separation of aflatoxin B_1 from the other aflatoxins, with the following R_F values: with hexane–acetone–diethyl ether (7:3:1), averufin 0.40, all aflatoxins 0.18; with chloroform–methanol (97:3), averufin 0.55, aflatoxins B_1 and B_2 0.52, aflatoxins G_1 and G_2 0.44; and with chloroform–acetone (9:1), aflatoxin B_1 0.54, aflatoxin B_2 and averufin 0.50, aflatoxin G_1 0.40, aflatoxin G_2, 0.35.

Aflatoxins, sterigmatocystin and *O*-methylsterigmatocystin were separated and identified by one-dimensional TLC by Bhatnagar *et al.*[126], who identified *O*-methylsterigmatocystin to be an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus*. Identities of the metabolites were established by TLC using six solvent systems as shown in Table 3. The first two of these solvent systems were also used by Cleveland *et al.*[127] in an enzymological study of the conversion of sterigmatocystin to aflatoxin B_1. Analytical TLC and PLC were applied in a study of the biosynthetic origin of aflatoxin G_1[128]. Sterigmatocystin was confirmed to be a precursor of aflatoxins B_1, G_1 and G_2 but no evidence for the conversion of aflatoxin B_1 to aflatoxin G_1 was found.

TABLE 3

TLC SEPARATION OF STERIGMATOCYSTIN (ST), *O*-METHYLSTERIGMATOCYSTIN (OMST), AFLATOXINS B_1 AND G_1 AND THE METABOLITE FROM *ASPERGILLIUS PARASITICUS* (CP461)

Modified from ref. 126.

Solvent system	$R_F \times 100$					*Developing time (min)*[b]
	ST	*OMST*	B_1	G_1	*CP461 product*[a]	
Diethyl ether–methanol–water (96:3:1)	97	44	37	28	44	45
Toluene–ethyl acetate–acetic acid (50:30:4)	75	43	35	24	43	45
Toluene–ethyl acetate–acetone (60:25:15)	86	29	41	30	29	45
Chloroform–acetone (10:0.5)	74	24	22	11	24	40
Chloroform–methanol (10:0.5)	93	69	63	55	69	40
Carbon tetrachloride–methanol (10:2)	81	42	39		41	45

[a] Identical with ST.

[b] The TLC plates were spotted with approximately 50 ng of various compounds and developed for a distance of nearly 14 cm.

Sterigmatocystin and its derivatives have also been included in multi-mycotoxin analyses[40,44,47,52]. TLC determinations of the toxin in cheese have been reported[129,130]. Hu *et al.*[131] reported a two-dimensional TLC determination of sterigmatocystin in cereal grains.

Hence, in the 1980s, TLC has remained a technique of choice in the determination of aflatoxins, their precursors and metabolic products. In addition, PLC has often been used in the isolation and purification of aflatoxins.

3.3. TLC of ochratoxins

Extraction, clean-up procedures and semiquantitative and quantitative methods for ochratoxin A were reviewed by Steyn[132]. Rice starch was recommended as an adsorbent for the TLC of ochratoxin[133]. TLC remains one of the chief methods for the detection, identification and quantitation of ochratoxins.

Stahr *et al.*[134] included TLC among methods of chemical analysis for ochratoxin poisoning. TLC has been applied in the quantitative determination of ochratoxin A in vegetable foods[135].

Problems of streaking of ochratoxin A and B spots in neutral mobile phases accompanied by increasing R_F values with increasing amounts applied and the effects of acidic modifiers on R_F values have been discussed[33].

The fluorescence intensity can change when ochratoxin A is exposed to ammonia–methanol vapour and the magnitude of the change is influenced by the residual mobile phase. This observation was exploited in a method described by Nesheim *et*

al.[136] Samples are spotted on TLC plates in benzene–acetic acid (9:1) and benzene–acetic acid–methanol (90:5:5) is used as the mobile phase. The developed plate is exposed to ammonia–methanol vapour and then is covered with another glass plate to prevent evaporation of the ammonia–methanol. If the ammonia–methanol does escape and the fluorescence intensity drops, it can be restored by re-exposure to fresh ammonia–methanol. The fluorescent spots under these conditions are stable for several days, whereas they occasionally fade in a few minutes on acidic plates. The method is recommended for most commonly contaminated commodities such as corn, barley and pig tissue. The method includes a confirmatory step. Methyl esters are prepared with boron trifluoride as a catalyst. The esters are identified by comparing the R_F values of standard and analyte derivatives.

The heat stability of ochratoxin A in contaminated coffee beans was tested by TLC[137]. Preparative silica gel TLC with benzene–acetic acid (4:1) as the mobile phase was used for the purification of isotopically labelled ochratoxin A[138]. When conversion of ochratoxin C into ochratoxin A in rats was studied, the ochratoxin A-containing fractions from LCC were purified by PLC[139].

Multi-mycotoxin analytical methodology has been applied to ochratoxin A analysis by several workers[44,47,49,52,140]. Lee *et al.*[40] used sequential development on HPTLC plates.

3.4. TLC of patulin and other small lactones

Extraction, clean-up and chromatographic methods for small lactones (patulin, penicillic acid, mycophenolic acid, butenolide and citreoviridin) have been reviewed[12,141].

Several TLC procedures for patulin determination have been published[142–144]. According to one of them[143], quantitation of patulin in fruit and vegetable products is possible. After extraction and clean-up using CC, patulin is chromatographed using toluene–ethyl acetate–85% formic acid (50:40:10) and detected with a fresh 4% solution of *o*-dianisidine in 85% formic acid. Quantitation is based on the yellow fluorescence under longwave UV light (limit 10 ng per spot). Meyer[143] also identified patulin after acetylation. On Kieselgel 60G plates using the above solvent system with 65% formic acid, the R_F values of patulin and of its acetylated product were 0.39 and 0.54, respectively.

Patulin has also been included in multi-mycotoxin TLC[47,51,52].

TLC data for penicillic acid, mycophenolic acid, butenolide and citreoviridin were reviewed[12]. The determination of penicillic acid in extracts from corn, oats, barley and dried beans by TLC was described by Thorpe[145]. TLC of mycophenolic acid in extracts from cheese has been reported[146–148]. Cole *et al.*[149] characterized citreoviridin by TLC on silica gel F_{254} plates using toluene–ethyl acetate–formic acid (5:4:1) as the mobile phase.

3.5. TLC of trichothecenes

About 80 trichothecenes are already known. According to differences in the trichothecene nucleus, the trichothecenes are divided into four types, and of these type A (characterized by a hydrogen atom or a hydroxyl group at the 8-position) and type B (with a ketone group at the 8-position) are the most important in practical analysis. The macrocyclic trichothecenes belong to type C. Group D consist of all the

other trichothecenes which do not fit into the first three categories.

TLC of trichothecenes has been reviewed[12,33]. Romer[150] described the use of small charcoal–alumina clean-up columns in determination of trichothecenes in foods and feeds.

Standard and less frequent detection methods for the TLC of trichothecenes have been described[151–153]. Type A and B trichothecenes have no fluorescence absorption bands under ultraviolet or visible light. Detection requires the TLC plates to be developed with suitable solvents so that the spots can be detected subsequently by colour or fluorescence[154]. Different reagents work best with the different types of trichothecenes. Aluminium chloride is relatively specific for type B trichothecenes whereas type A trichothecenes can be detected with sulphuric acid[33] or chromotrophic acid[152]. Both of these compounds have a poor structural affinity for the 12,13-epoxy group in the trichothecene nucleus[155]. 4-(*p*-Nitrobenzyl)pyridine is reported to interact with the trichothecene nucleus and has been used for the detection of types A, B and D. These reagents react with a wide range of extraneous compounds. Unless the samples are put through several clean-up steps, these reactions can obscure the toxins[155–157]. Some type C compounds fluoresce naturally, but others are detected as fluorescent spots with sulphuric acid and heating. Other detection reagents, such as nicotinamide and 2-acetylpyridine, have been reported[33].

A new, sensitive TLC–HPLC method for detection of trichothecenes was published by Yagen *et al.*[158]. Diphenylindenone sulphonyl (Dis) esters of trichothecenes, when sprayed with sodium methoxide, showed fluorescent spots on silica gel TLC under longwave UV light. The detection limit for trichothecene esters in TLC was 20–25 ng per spot for T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol and iso-HT-2 toxin. A quantitative HPLC analysis of Dis trichothecene esters using UV detection at 278 nm was also developed.

Unlike chemical detection, bioautography is based on the biological effects of the substance to be detected[159]. The study of the toxicity of trichothecene mycotoxins has shown that several genera of yeasts are sensitive[160]. A simple method was described[161] for the detection and quantitative determination of T-2 toxin and its separation from HT-2 toxin on silica gel layers based on growth inhibition of *Kluyveromyces fragilis* and *Saccharomyces cerevisiae*. The detection limit for T-2 toxin is 0.2 n*M* per spot. The area of growth inhibition corresponds logarithmically to the toxin concentration. T-2 toxin could be quantitatively detected from 0.2 to 160 n*M* per spot.

Chemical analysis of the culture filtrates of *Fusarium culmorum* CMI 14764 has demonstrated the presence of seven trichothecene mycotoxins[162]. The crude ethyl acetate extract from the culture filtrates was fractionated on a silica gel column by eluting with diethyl ether–acetone (9:1). Fractions were analysed by TLC and combined as appropriate. Merck silica gel F_{254} plates were developed with diethyl ether–acetone (9:1) and the spots were revealed using 20% sulphuric acid or 4-(*p*-nitrobenzyl)pyridine spray reagents. Unfortunately, R_F values of the seven trichothecenes were not included in the paper.

In the early 1980s, tests for trichothecenes were included in various multi-mycotoxin analyses of feedstuffs and foods (e.g., refs. 44, 47 and 48). Many references to TLC can be found in a monograph on trichothecenes[8].

TLC data on trichothecenes have been reported in studies on mycotoxins in

natural products[163–169]. A revised official method of deoxynivalenol analysis in wheat was published[170]. Analytical and preparative TLC have been used in studies of the bioconversion of T-2 toxin into 3′-hydroxy-T-2 toxin and 3′-hydroxy-HT-2 toxin[171]. A rapid method for the determination of trichothecenes was developed[172]. The trichothecenes occurring in purified extracts of food and feed samples were converted into the corresponding free alcohols by transesterification and then analysed by HPTLC or GC. Harrach *et al.*[173] subjected cleaned-up concentrates of satratoxins G and H to PLC on silica gel. The band with R_F values identical with those of standards of satratoxins G and H was collected, extracted with acetone and used for comparison with satratoxin standards by HPTLC.

TLC and other chromatographic techniques were used extensively in the isolation and characterization of thirteen new macrocyclic trichothecenes from the Brasilian plant *Baccharis megapotamica*[174]. A model 7942 Chromatotron was used for PLC with plates prepared as circular glass disks.

Bata *et al.*[175] described an improved three-step (TLC, GC and HPLC) procedure for the determination of the macrocyclic trichothecenes satratoxin G and H and verrucarin J in cereals.

3.6. TLC of tremorgenic mycotoxins

Maes *et al.*[176] devised simple TLC and HPLC systems for the separation, identification and quantitation of penitrems in culture extracts. As the penitrems are unstable in chloroform when exposed directly to light, all contact of the penitrems with chloroform was avoided throughout their investigation. The only system that gave a complete separation of all the penitrems in TLC was benzene–acetone (85:15) and the best results were obtained by developing the chromatograms twice in this solvent system. The order of decreasing R_F values for the penitrems was F, B, A, E, C and D. Penitrems A–F give blue spots immediately after spraying with cerium(IV) sulphate, which become stable, dark purple after heating. Similar procedures were used by De Jesus *et al.*[177].

PLC has been used in the purification of the janthitrems but CC on Mallinckrodt silica AR CC-7 silica gel was more succesful. The janthitrems are high fluorescent under longwave UV light and can also be detected by spraying the TLC plates with Ehrlich's reagent and exposure to hydrogen chloride vapour, resulting in grey–green spots[178].

TLC was used to check paxilline, another indole-isoprenoid tremorgen, in fractions from CC during purification of the toxin from a submerged fermentation. Spraying with Ehrlich's reagent followed by heating revealed paxilline by its colour, yellow becoming green. Complementary detection involves spraying with 50% ethanolic sulphuric acid and heating at 100°C for 5 min[179].

Territrems A and B were separated by means of TLC in three solvent systems, their detection being based on blue fluorescence[180]. Later, territrem C was discovered and characterized by TLC and other methods. PLC was also used to isolate the methylation product of territrem C and its identity with territrem B was proved[81]. More recently, Peng *et al.*[182] succeeded in isolating another related metabolite. As the R_F values of the compound in TLC were between the R_F values of territrems B and C, the compound was designated territrem B′. TLC fluorodensitometric quantitation of territrems A, B and C was also described[183]. The following mobile phases have been

used to characterize territrems A, B and C[180,181]: (a) benzene–ethyl acetate (1:1); (b) toluene–ethyl acetate–65% formic acid (5:4:1); and (c) benzene–ethyl acetate–acetic acid (55:40:5). The fluorescence intensity of territrem C was quenched when the concentration was higher than 20 μg per spot. The fluorescence intensity also gradually faded after development in system (a), but it was enhanced and turned greenish in acidic solvent systems.

TLC of verruculogen has also been reported[184].

3.7. TLC of hydroxyanthraquinones

TLC data for fungal hydroxyanthraquinones have been reviewed[12,15,185]. The hydroxyanthraquinones give yellow, orange or red spots on TLC plates. They are also detected by spraying the plates with a saturated solution of magnesium acetate in methanol or 5% potassium hydroxide in methanol[15]. Detection with methanolic solutions of magnesium acetate and copper acetate was compared[186]. The colour obtained with the latter was more stable, increased for 2 h and then remained stable for 24 h. Spots of hydroxyanthraquinones from *Trichoderma viride* on Silufol plates became intensely orange and violet, respectively, when the plates were exposed to ammonia fumes[187]. Two main anthraquinones from a colour mutant of *T. viride*, 1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxyanthraquinone, were purified by PLC on Silufol plates using benzene–acetone (75:25) with repeated development[188]. Ueno[189] reported the TLC determination of luteoskyrin in rice grains. PLC was used in studies on the conversion of emodin to physcion by a cell-free preparation of *Aspergillus parasiticus*[190].

In studies of anthraquinones produced by *Aspergillus glaucus* group, Anke *et al.*[191] used PLC and oxalic acid-treated silica gel PF_{245} (Machery, Nagel & Co.) to separate and isolate several pigments. Erythroglaucin, physcion and physcion-9-anthrone were well separated in carbon tetrachloride–chloroform (90:10). Catenarin, rubrocristin and viocristin were separated using benzene–ethyl acetate–acetic acid (45:55:1) as the mobile phase.

3.8. TLC of zearalenone

Techniques for the production, isolation, separation and purification of the strogenic mycotoxin zearalenone have been reviewed[192]. Extraction, clean-up, adsorbents, solvent systems and TLC detection of zearalenone have been summarized elsewhere[12]. Some recent techniques and applications are presented here.

A method was developed for the simultaneous extraction, separation and qualitative analysis of zearalenone and seven other mycotoxins in corn[47]. According to Fig. 1, zearalenone was present in the second fraction from the mini-column.

Another extraction and clean-up procedure was proposed[193]. Samples were extracted with acetonitrile–4% potassium chloride (9:1) in 0.1 *M* hydrochloric acid and the extract was defatted with isooctane. The acetonitrile layer was filtered through anhydrous sodium sulphate, which was washed with chloroform and the washings were added to the filtrate. After evaporation, the residue was dissolved in chloroform and used for TLC analysis. Zearalenone was characterized with nine solvent systems and two spray reagents (an ammonium chloride solution and Fast Violet B salt spray). These were also used in quantitation of the toxin. Solvent systems containing formic acid were not satisfactory when the Fast Violet B salt spray was used.

Overpressurized layer chromatography was also used to determine zearalenone in maize[194].

Swanson *et al.*[195] developed a method for the determination of zearalenone and zearalenol in grains and animal feeds. The method involved extraction with 75% methanol, precipitation of pigments with lead acetate and defatting with light petroleum. The mycotoxins were subsequently partitioned into toluene–ethyl acetate, chromatographed on HPTLC plates and detected after spraying with Fast Violet B salt solution. The sensitivity was > 80 ng/g for zearalenone and 200 ng/g for zearalenol.

Two-dimensional TLC with benzene–acetone (60:35) and toluene–ethyl acetate–formic acid (60:30:10) proved to be insufficient in resolving α- and β-zearalenol, which appeared as a single spot[196]. However, the diastereomeric mixture was resolved into two components by HPLC and GC (see also sections 4.4 and 5.2).

In a study of the biosynthesis of zearalenone, the radiochromatographic homogeneity of the isolated [^{14}C]zearalenone was determined by TLC on silica gel G plates using chloroform–methanol (97:3) as the mobile phase. Among fluorescent bands, only that corresponding to zearalenone was radioactive[197].

In studies of the bioconversion of radioactive α-zearalenol and β-zearalenol into zearalenone, the recovery from CC was ascertained by TLC of eluates[198]. Recently, TLC and PLC were used in studies of the microbial conversion of zearalenone. A strain of *Rhizopus* sp. produced zearalenone 4-β-D-glucopyranoside in addition to α- and β-zearalenol[199].

Bennett *et al.*[200] described a method for the determination of zearalenone and deoxynivalenol in cereal grains. After extraction, clean-up and separation by CC, zearalenone was quantitated by TLC and deoxynivalenol by GC of the trimethylsilyl derivative. Multi-mycotoxin TLC studies in which zearalenone was included have been published[40,44,47,48].

3.9. TLC of citrinin

Chromatographic methods, including TLC, using in studies of this mycotoxin have been reviewed[12,201]. The major problems with the TLC of citrinin have been its weak fluorecence, tailing in normal-phase TLC on silica gel and instability.

More intense fluorescence of citrinin and easier detection were accomplished with an aluminium chloride spray followed by heating, which changes the yellow fluorescence to blue[51]. Another improvement has been the incorporation of an acid in the silica gel to reduce tailing. Oxalic acid was used first[201], but more recently glycolic acid was found to be better because of reduced diffusion of the citrinin spots and hence enhanced detectability[202]. This modification was applied to determine citrinin in corn and barley.

TLC was shown to be of importance in the separation and identification of dihydrocitrinone and ochratoxin A as products of conversion of [^{14}C]citrinin by *Penicillium viridicatum*[203]. The major breakdown product, dihydrocitrinone, appeared on the developed TLC plates as a blue spot under longwave UV light.

3.10. TLC of cyclopiazonic acid

The most important data concerning the production, isolation, separation and purification of cyclopiazonic acid and related toxins were summarized by Cole[204].

Because of its neurotoxicity and possible carcinogenicity, cyclopiazonic acid has been intensely investigated in the 1980s. Analytical methodology for its determination in agricultural products has received considerable attention in the past few years. Most of the TLC methodology relies on the reaction of cyclopiazonic acid with Ehrlich's reagent under acidic conditions. Other detection possibilities have been reviewed[12].

TLC systems including densitometry have been reported[205–207]. According to Lansden[207], samples of peanuts or corn are extracted with methanol–chloroform (20:80); the extract is stripped of most interferences by partitioning with aqueous sodium hydrogencarbonate followed by acidification and repartitioning with chloroform. After TLC and derivatization with Ehrlich's reagent, the toxin is quantitated by reflection densitometry at 540 nm. The recovery of the toxin averages 90% for peanuts and 85% for corn. The absolute detection is 25 ng per spot, which represents a detection limit of 125 μg/kg for a 50-g sample.

A simple determination of the toxin in contaminated food and feeds was described[208]. Trucksess *et al.*[209] examined isolates of *Aspergillus* and *Penicillium* species from dried beans, corn meal, macaroni and pecans for their ability to produce cyclopiazonic acid. The toxin in chloroform extracts was semi-quantitatively determined by TLC. Semi-quantitative TLC of the toxin has also been used in studies of its production by *Penicillium* and *Aspergillus* strains[210,211].

TLC has been succesfully applied to discriminate cyclopiazonic acid-producing (CPA^+) from non-producing (CPA^-) strains of *Aspergillus oryzae*[212].

Crude extracts from culture filtrates were characterized by TLC on silica gel $60F_{254}$ that had been previously impregnated with oxalic acid (0.4 *M*) and dried. Development was carried out in isobutyl methyl ketone–chloroform (1:4) and detection was with Ehrlich's reagent. The toxin from CPA^+ strains revealed a blue-violet spot at R_F 0.75, whereas crude extracts from CPA^- strains never did reveal this spot.

TLC has been applied in studies on the production of cyclopiazonic acid by *Penicillium verrucosum* var. *cyclopium*[213] and for screening the toxin in agricultural commodities[214].

Malik *et al.*[213] performed TLC on silica gel G-1500 LS 254 with ethyl acetate–2-propanol–25% ammonia solution (20:15:10). The toxin was measured quantitatively with a spectrodensitometer with a digital counter and integrator at 282 nm. It was detected as a violet spot under ordinary light after spraying with Ehrlich's reagent diluted with 4 volumes of acetone.

Rao and Husain[214] applied PLC to chloroform extracts from culture filtrates. The standard was spotted at one end of the plate. After development (the same system as in ref. 213), the standard was detected with Ehrlich's reagent (the remainder of the plate being covered with a glass plate). When the standard was detected, the covering plate was removed and the TLC plate exposed to iodine vapour. The area with an R_F value corresponding to the standard spot and coloured with iodine vapour was scrapped off, eluted with methanol and used for colorimetric determination of the toxin using a modification of Ehrlich's reagent.

Determination of cyclopiazonic acid in foods by HPTLC was published recently[215].

3.11. TLC of cytochalasans

The family of cytochalasans include compounds with a common skeleton (cy-

tochalasan) such as cytochalasins, zygosporins and chaetoglobosins[124]. TLC data for this large class of fungal metabolites have been reviewed[12].

Chappuis and Tamm[216] used a variety of solvent systems in the analytical TLC and PLC of derivatives and degradation products of cytochalasin D. Analytical TLC was carried out on Fertigplatten 60 F_{254} and Kieselgel 60 PF_{254} was used as the sorbent in PLC. A TLC method for the determination of cytochalasin H production was reported most recently[217].

Chaetoglobosin K was characterized by means of TLC[218]. Decreasing R_F values of five chaetoglobosins on silica gel 60 TLC plates developed in dichloromethane–ethanol (95:5), showing increasing polarity from left to right, were reported as follows: 19-*O*-acetylchaetoglobosin A, chaetoglobosin C, 19-*O*-acetylchaetoglobosin B, 19-*O*-acetylchaetoglobosin D and chaetoglobosin A[219]. TLC has been used in a study on chaetoglobosins A–J[220] and the isolation and identification of two new cytochalasans from *Phomopsis sojae*[221].

3.12. TLC of miscellaneous mycotoxins

In this section, TLC data on the following compounds are included: PR toxin, xanthomegnin, viomellein, vioxanthin, moniliformin, naphtho-γ-pyrones, wortmannin, echinulin, fusaric acid analogues, viridin and several *Alternaria* toxins. Older data were reviewed elsewhere[12].

The production, isolation and chromatographic techniques for PR toxin were reviewed by Scott[222]. Several solvent systems for the TLC of this toxin have been published[223,224]. The toxin was quantitated *in situ* by fluorodensitometry after spraying the plates with 1% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid–acetone (1:10) or in ethanol with subsequent exposure to hydrogen chloride fumes for 10 min, the latter being the preferred method[223].

Methods used for the isolation, separation, purification and detection, including TLC and HPLC, of secalonic acid D were summarized[225]. Ciegler *et al.*[2] quantitated the toxin on pre-coated silica gel F_{254} plates. Secalonic acid D was included in an earlier systematic TLC analysis of 37 mycotoxins[226]. The toxin was detected with *p*-anisaldehyde and iron(III)chloride.

In a screening for toxigenic isolates of *Aspergillus ochraceus* from green coffee beans, Stack *et al.*[227] applied TLC in detecting xanthomegnin, viomellein and vioxanthin in addition to ochratoxins. Standards of xanthomegnin and viomellein were prepared by means of PLC on silica gel plates[228]. Their purity was checked by TLC and HPLC comparisons with reference compounds.

Jansen and Dose[229] described a quantitative TLC determination of moniliformin in vegetable foods and feeds. Crude acetonitrile extracts of *Fusarium moniliforme* cultures were checked for moniliformin[230,231] by spotting, together with a standard, on precoated thin layers of silica gel 60 and developing with chloroform–methanol–formic acid (70:30:0.16). Moniliformin was detected by spraying and heating with 0.5% aqueous 3-methyl-2-benzothiazolinone hydrazone hydrochloride. The limit of detection was approximately 8 μg/g in corn culture.

Monomeric and dimeric naphtho-γ-pyrones, extracted from the mycelium of *Aspergillus niger*, were examined by HPTLC on LHP-KF plates (Whatman) developed with benzene–ethyl acetate–formic acid (10:4:1)[232]. Components were identified by their colour, fluorescence under longwave UV light and colour after spraying with

Gibbs reagent. Data for eight naphtho-γ-pyrones are presented in Table 4.

PLC on silica gel plates developed with chloroform–methanol (97:3) was used to purify a haemorrhagic factor from *Fusarium oxysporum* identical with the antibiotic wortmannin[233].

Echinulin was isolated by means of PLC from acetone extracts of feed refused by swine. The mobile phase was ethyl acetate–hexane (8:2) and the toxin turned blue in the presence of *p*-anisaldehyde reagent at 110°C. The anisaldehyde-reactive material from the PLC was identified with echinulin by its UV and IR spectra[234].

Viridin, a steroid-like antibiotic, is converted by viridin-producing fungi into its dihydro derivative, viridiol, which is ineffective as an antibiotic but is a potent phytotoxin. Both metabolites were isolated from culture extract by means of PLC[235]. TLC was used to characterize two new analogues of fusaric acid from *Fusarium moniliforme*[236].

Alternaria toxins have received much interest in recent years. Production, isolation, clean-up procedures and chromatographic techniques (TLC, GC and HPLC) for the determination of alternariols, altenuene and tenuazonic acid were reviewed[237]. TLC data for alternariol, alternariol monomethyl ether, altertoxin I and II and tenuazonic acid were published[238]. ^{3}H-labelled alternariol and alternariol monomethyl ether were isolated from ethyl acetate extracts of conidia of *A. alternata* by PLC. Two solvent systems were used: (1) toluene–dioxane–acetic acid (95:25:4) and (2) methanol–2*M* HCl (5:1)[239].

Hence, one- and two-dimensional high-performance and preparative TLC have been used extensively in the field of mycotoxins in the 1980s. In addition to comments in this section, achievements and problems with the TLC of mycotoxins are discussed in more detail in the Conclusions.

4. LIQUID COLUMN CHROMATOGRAPHY

Techniques of liquid column chromatography are used in the field of mycotoxins with three main aims: (a) clean-up of mycotoxin-containing extracts for further analysis by other means; (b) large-scale separations and purifications; and (c) qualitative or quantitative analysis of mycotoxins.

TABLE 4

HPTLC DATA FOR NAPHTHOPYRONES

Adapted from ref. 232.

Naphthopyrone	$R_F \times 100^a$	*Gibb's test*	*Fluorescence*
Flavasperone	81	Blue	Violet
Fonsecin monomethyl ether	76	Brown	Violet
Rubrofusarin	72	Blue-green	Orange
Aurasperone A	67	Violet	Yellow
Isoaurasperone A	61	Red-violet	Yellow
Aurasperone B	56	Brown	Yellow
Aurasperone D	53	Violet	Yellow
Aurasperone C	49	Brown	Yellow

[a] With benzene–ethyl acetate–formic acid (100:40:10) on Whatman LHP-KF.

In clean-up procedures, various mini-columns are used. In large-scale separations and purifications, amounts varying between a few miligrams and several grams can be separated on a column. In analytical work, a variety of mini-columns containing different adsorbents are commercially available and may be used as an alternative to TLC. Most analytical work, however, is now performed by means of high-performance liquid chromatography (HPLC).

Almost all large-scale separations and purifications of mycotoxins are performed by column chromatography (CC). The columns used may be normal gravity columns or may run under pressure in the case of the preparative HPLC systems.

In the CC of mycotoxins, four general techniques can be used, *viz.*, adsorption, partition, ion-exchange and gel filtration chromatography. By far the most widely used is adsorption chromatography. Adsorbents such as silica gel (most frequently), alumina, charcoal, cellulose, silicic acid, macroreticular resins, magnesium oxide, magnesium silicate and calcium hydrogenphosphate have been used. Partition chromatography on cellulose impregnated with formamide has given satisfactory results in the purification of some polar toxins such as cyclopiazonic acid. Ion-exchange chromatography has found application in the purification of ochratoxins, cyclopiazonic acid, tentoxin and moniliformin. Gel filtration chromatography has been used to purify verruculotoxin and aflatoxins. The most important techniques of preparative CC of mycotoxins have been described[1] and applications in separations and purifications of the best known mycotoxins have been reviewed[11].

Historically, the gradual introduction of HPLC analysis in the early 1970s for the determination of mycotoxins had to compete with existing widely used TLC-based methods, but the trend has been toward the increased use of HPLC. The rapidly increasing applications of the technique show its inherent advantages over other chromatographic techniques and it seems destined to supplant the other methods in the routine analysis of mycotoxins. Earlier applications of HPLC in the analysis of mycotoxins have been reviewed (*e.g.*, refs. 1, 240 and 241). The most important techniques and applications were summarized by Shepherd[242].

HPLC is applicable to the analysis of nearly all known mycotoxins, in contrast to gas chromatography (GC), which has been used mostly for the trichothecenes, zearalenone, patulin and the anthraquinones[243,244]. In the field of mycotoxins, many of which are highly polar compounds, reversed-phase HPLC has found wide application and most separations or purifications are now performed on reversed-phase columns. The toxins investigated include the aflatoxins, citrinin, the ergot alkaloids, ochratoxin A, patulin, penicillic acid, penitrems A–F, PR toxin, the rubratoxins, sporidesmins, sterigmatocystin and zearalenone.

Among the available detectors, the most frequently used is the variable-wavelength detector, which has a particular application in the mycotoxin field. The main alternative to the UV detector is the spectrofluorimetric detector, which should be inherently more sensitive than UV detectors and has been used in the detection of aflatoxins, ochratoxin A and several other mycotoxins.

Double-beam UV detectors, which can record the UV spectrum of a compound being eluted from the column, have obvious advantages for the characterization of mycotoxins, although in some instances the UV spectrum is not a reliable diagnostic probe. In addition, several mycotoxins exhibit weak absorbance in the UV region and detection at 190 nm is hampered by the background absorption. The introduction of

the recently developed thermospray (TSP) interfacing technique[245] has permitted the measurement of a wider range of organic compounds than previously. This technique was used by Voyksner *et al.*[246] for the HPLC–MS analysis of some *Fusarium* toxins. More recently, Rajakylä *et al.*[247] published a method for the determination of mycotoxins in grain by reversed-phase HPLC and thermospray liquid chromatography–mass spectrometry (TSP–LC–MS), which seems to be a very specific and sensitive method for analysing a wide range of mycotoxins in biological samples.

In the following sub-sections, some applications of HPLC in multi-mycotoxin analysis and in determinations of mycotoxin structural groups or individual toxins are summarized. The data were published in the 1980s.

4.1. HPLC in multi-mycotoxin analysis

Multi-mycotoxin analyses by HPLC have been published[248,249]. Thiel *et al.*[250] studied the natural occurrence of moniliformin together with deoxynivalenol and zearalenone in corn. Grabarkiewicz-Szczesna *et al.*[43] published a simple multi-detection procedure for the determination of eleven mycotoxins in cereals. Griffin *et al.*[251] separated *Alternaria* metabolites. HPLC was used by Scott *et al.*[54] to analyse toxins of *Fusarium moniliforme*.

Hurst *et al.*[252] determined patulin, penicillic acid, zearalenone and sterigmatocystin in artificially contaminated cocoa beans by HPLC. When this method is combined with a method reported earlier for the determination of ochratoxin A[253], it allows the determination of five mycotoxins. Samples are extracted with an acidic acetonitrile solution, partitioned with hexane to remove fat interferences and then partitioned with chloroform to remove the toxins. Interferences are removed by the use of a bonded-phase column followed by the final HPLC determination step. This uses a cyano column with hexane–1-propanol–acetic acid as the mobile phase with dual-channel UV detection at 245 and 280 nm. The method exhibits good linearity, accuracy and precision.

An HPLC method was described[247] for the determination of deoxynivalenol, patulin, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, zearalenone and ochratoxin A using a reversed-phase column and diode-array detector. The combination of the HPLC system together with a modern thermospray (TSP) quadrupole mass spectrometer was shown to be a very specific and sensitive method.

Frisvad[254] developed a general method for most known mycotoxins and other secondary metabolites of fungi based on HPLC, but even using the same type of chromatographic columns the retention times were very variable. The method was also limited by the UV detection wavelength of 254 nm, making the detection of some metabolites with end absorption impossible.

Later, Frisvad and Thrane[66] developed a general standardized method for the analysis of 182 mycotoxins and other fungal metabolites, based on HPLC with an alkylphenone retention index and photodiode-array (PDA) detection combined with TLC in two different solvent systems. Each secondary metabolite was characterized by its bracketed alkylphenone retention time, its UV–VIS absorption maxima and its retardation factor relative to griseofulvin in two TLC systems. The metabolites characterized by this method included aflatoxins B_1, B_2, $G_1$and G_2, ochratoxin A, citrinin, penicillic acid, viomellein, penitrem A, patulin, sterigmatocystin, alternariol, tenuazonic acid, trichothecenes, roquefortines, fusarin C, zearalenone, PR toxin,

citreoviridin, viridicatumtoxin, verruculogen, rugulosin, cyclopiazonic acid and many other alkaloids, polyketides and terpenes. Methods of this type are of great importance also in the chemotaxonomy of fungi.

4.2. HPLC of aflatoxins

Fluorimetry is the detection technique commonly used for the most frequent aflatoxins because of their native fluorescence; λ_{ex} = 360 nm for all four aflatoxins and λ_{em} = 440 nm for B_1 and B_2 and 470 nm for G_1 and G_2[255].

The fluorescence of aflatoxins B_1 and G_1, which is lower than that of B_2 and G_2, can be increased by using strong acids[256] or oxidants such as chloramine T[257], iodine[258–260] or bromine[257,261]. Bromine acts by adding itself to the double bond of the furan ring of aflatoxins B_1 and G_1, thereby increasing their fluorescence by a factor of 20 or more[257]. β-Cyclodextrin also enhances the fluorescence of the two aflatoxins and this effect was utilized in developing a reversed-phase HPLC method for the detection of aflatoxins B_1, B_2, G_1 and G_2 without preparing derivatives of B_1 and G_1[262].

A new approach to the detection of the four aflatoxins involves HPLC with amperometric detection in the differential-pulse mode at a dropping mercury electrode with a 1-s drop time. These aflatoxins can be detected simultaneously with good resolution but with some compromise in sensitivity, The detection limit of underivatized aflatoxin standards is about 5 ng[263].

Although HPLC and flow-injection analysis (FIA) have significant differences (working pressure, presence of interfaces, cost), they involve a number of common components (liquid reservoirs, pumps, injection valves and continuous detectors) and are complementary in nature. Hence attempts to use HPLC and FIA in conjunction are justified[264]. Lázaro *et al.*[257] published a new approach to the joint use of FIA and HPLC in which the flow-injection sub-system allows the total determination of several related compounds. The individual analysis for each analyte is performed by the HPLC sub-system while the flow-injection sub-system acts as a post-column reactor–detector, thereby enhancing the information obtained from the sample. An example of the joint use of these techniques is the analysis of foods for aflatoxins. Bromine was used as the derivatizing agent. The optimum composition of the mobile phase was acetonitrile–methanol–water (23.5:29.5:47), allowing the complete resolution of the four aflatoxins. The concentration and flow-rate of the bromine solution and the flow-rate of the mobile phase were interrelated. The method was used for the determination of aflatoxins in groundnuts and maize.

Other methodological aspects have been studied, such as radial compression separation[265] and post-column derivatization[266–268].

Among many papers published in the 1980s, HPLC has been applied to determine aflatoxins in agricultural products such as heavily contaminated corn[269], corn and groundnuts[270], cotton-seed[271], feedstuffs[266,272,273] and naturally contaminated eggs[274]. The separation of aflatoxin biosynthetic intermediates by HPLC was reported by McCormick *et al.*[275]. A method has been developed for the quantitation of aflatoxins produced by fungal strains in liquid media[98]. HPLC has also been used in toxicological and mutagenic studies of aflatoxins[276–278].

HPLC has been extensively applied in the determination of aflatoxin M_1 in milk and dairy products[105,279–288]. In the 1982 IARC/WHO aflatoxins check sample pro-

gramme[288,289], 21% of the analyses (including 28 out of 115 for aflatoxin M_1 in milk) were completed by means of HPLC. Changes in official methods of analysis of aflatoxins M_1 and M_2 in fluid milk were published[290]. Hsieh *et al.*[291] reported on the production and isolation of aflatoxin M_1 for toxicological studies. An assay method for the contamination of commercial milks by aflatoxin M_4, a new hydroxyaflatoxin B_1, was described by Lafont *et al.*[113].

A recent comparison[292] of a number of published protocols indicated that the best results for aflatoxin M_1 analysis were obtained by direct octadecylsilane solid-phase extraction of liquid milks, followed by reversed-phase HPLC[293]. Recently however, products for the immunological determination of aflatoxins have become available. In the course of evaluating an immunoaffinity column designed for the analysis of aflatoxins B and G in groundnuts and other products, Mortimer *et al.*[294] investigated its suitability for the determination of aflatoxin M_1 in milks. The affinity column clean-up gave excellent results for recovery, sensitivity and sample throughput. The HPLC traces were significantly cleaner than those in alternative methods[292] and the detection limit was as low as 50 ng/l.

Methods for the determination of aflatoxins in human serum[295] and urine[296] have been developed. The determination of aflatoxicol in porcine liver was described by Tyczkowska *et al.*[297] Further HPLC data for aflatoxins have been reviewed[242].

4.3. CC and HPLC of trichothecenes

A variety of preparative and analytical liquid chromatographic methods for trichothecenes have been reported in the 1980s. A review was published[298].

Witt *et al.*[299] purified deoxynivalenol by water-saturated silica gel chromatography. An assessment of extraction procedures for the analysis of naturally contaminated grain products for deoxynivalenol was published[300]. Cohen *et al.*[301] described a preparation of deoxynivalenol using flash chromatography. Isolation and purification of deoxynivalenol and a new trichothecene by HPLC were reported by Bennett *et al.*[302].

The trichothecene fraction produced by a liquid culture of *Fusarium crookwellense* was separated by open-column liquid chromatography on silica gel and by HPLC on a cyano bonded-phase column. The major trichothecene produced was 4,15-diacetoxynivalenol. Other secondary metabolites formed in appreciable amounts were the 7- and 8-hydroxy derivatives of isotrichodermin. Several unknown compounds were isolated and characterized by their mass spectra and ^1H and ^{13}C NMR spectra. Among these compounds were 7,8-dihydroxyisotrichodermin and 4,15-diacetoxy-7-deoxynivalenol[303].

HPLC was used to follow the transmission of [^{14}C]deoxynivalenol to eggs following oral administration to laying hens[304].

Rapid determinations of trichothecenes using small charcoal–alumina clean-up columns have been reported[150,305].

Changes in official methods of analysis of deoxynivalenol in wheat were published[306]. Shepherd and Gilbert[307] described the long-term storage stability of deoxynivalenol standard reference solution. Determination of deoxynivalenol was achieved by HPLC with electrochemical detection[308]. The analysis of several trichothecenes by HPLC was described by Maycock and Utley[309] and the detection of nivalenol, deoxynivalenol, fusarenone X and 3-acetyldeoxynivalenol by HPLC was reported[310].

Analysis of some metabolites of T-2 toxin, diacetoxyscirpenol and deoxynivalenol was achieved by thermospray HPLC–MS[311]. Lauren and Greenhalgh[312] reported on the simultaneous determination of nivalenol and deoxynivalenol in cereals.

The applicability of HPLC with UV detection, based on the absorptivity of the α,β-enone system of type B trichothecenes (such as deoxynivalenol and nivalenol), is often limited by lack of specificity because of the need to use low wavelengths[313]. A pre-column derivatization method using *p*-nitrobenzyl chloride was reported to form a chromophore with an absorption maximum of *ca.* 254 nm[309]. The method has the disadvantages that the reagent gives an interferring peak and its derivatization step is time consuming. HPLC with post-column fluorescence detection should be a suitable method, because it permits efficient separation, selective detection and direct injection of the sample solution after clean-up without further treatment.

Supercritical fluid chromatography with mass spectrometric detection shows interesting possibilities for trichothecenes[314].

A method for the determination of trichothecenes based on the chromogenic or fluorogenic reaction of formaldehyde produced from trichothecenes by an acid decomposition reaction was described by Kato *et al.*[315]. However, application of similar reactions to an HPLC post-column derivatization system is difficult because of the necessity to use viscous concentrated sulphuric acid. Sano *et al.*[313] found that type B trichothecenes give formaldehyde when heated with aqueous alkali. They developed a method based on the HPLC separation of deoxynivalenol, nivalenol and fusarenon-X and a C_{18} column using aqueous acetonitrile, and successive post-column fluorescence derivatization involving an alkaline decomposition to form formaldehyde and a modified Hantzch reaction with methyl acetoacetate and ammonium acetate. With this method, 5–10 ng of the standard trichothecenes could be determined. By employing a clean-up procedure with a Florisil column and a Sep-Pak CN cartridge, 61.4–96.9% recoveries were obtained for deoxynivalenol and nivalenol added to corn, barley and wheat at concentration levels of 0.05–1.0 ppm.

Bata *et al.*[175] published a method for the determination of naturally occurring macrocyclic trichothecene toxins. Certain mycotoxins in this group are either antitumour agents or their precursors. Three trichothecenes produced by the fungus *Myrothecium verrucaria* (roridin J, verrucarin A and roridin K acetate) were separated from a crude extract of the fermentation broth by using a Supelcosil LC-Si column. A step gradient of 20, 30, 40 and 70% ethyl acetate in hexane and 100% ethyl acetate was used as the mobile phase. The ethyl acetate concentration was increased stepwise at 10, 20, 28 and 39 min. Roridin A and 8-β-hydroxyroridin A also have been separated on a Supelcosil LC-Si column[316].

An HPLC analysis of extracts from aerosolized conidia of *Stachybotrys atra* established that conidia contained satratoxins H and G and trichoverrols A and B[317].

The isolation of thirteen new macrocyclic trichothecenes from the Brazilian plant *Baccharis megapotamica* was achieved by Jarvis *et al.*[174]. HPLC and TLC have been used extensively to check the separation and purification procedures. Nine macrocyclic trichothecenes were isolated from *B. coridifolia*. Flash column chromatography was used in their separation and purification[318].

Rood *et al.*[319] developed a rapid screening procedure for the detection of trichothecenes in plasma and urine. HPLC has been applied in studies on the production of deepoxydeoxynivalenol, a metabolite of deoxynivalenol, by *in vitro* rumen

incubation[320] and on the disposition of T-2 toxin in intravascularly dosed swine[321].

Yagen *et al.*[158] described a new, sensitive fluorescence TLC method for the detection of diphenylindenone sulphonyl (Dis) esters of trichothecenes. HPLC of these derivatives using UV detection resulted in a considerable improvement in the detection sensitivity of the trichothecenes diacetoxyscirpenol, T-2 toxin, HT-2 toxin, T-2 triol and T-2 tetraol. The HPLC of Dis trichothecene esters could be used for the quantitation of trichothecenes in plasma and urine.

4.4. HPLC of miscellaneous low-molecular-weight mycotoxins

In addition to the aflatoxins and trichothecenes, the remaining mycotoxins are aranged in this section in an order similar to that used in the TLC sections. Hence, the following toxins are included here: ochratoxin A, citrinin, patulin, citreoviridin, tremorgens, zearalenone, cyclopiazonic acid, secalonic acid D, xanthomegnin, viomellein, moniliformin, anthraquinones, fusaric acid analogues, fusarin C, γ-pyrones, *Alternaria* toxins, PR toxin and rubratoxin B.

HPLC has been applied to determine ochratoxin A in wheat bran[322], feeds, animal tissues and eggs[323]. Abramson[324] measured ochratoxin A in barley extracts by HPLC–MS. Rapid HPLC on a Spherisorb ODS 2 column with fluorescence detection was used for the determination of ochratoxin A in foods[325].

Gareis *et al.*[326] found ochratoxin A in human milk. After extraction and clean-up steps, analysis was performed by reversed-phase HPLC on 5-μm LiChrosorb RP-18 with acetonitrile–water–acetic acid (570:410:20) as the mobile phase and fluorescence detection (λ_{ex} = 330 nm and λ_{em} = 460 nm). Trace amounts of the toxin were found in four out of 36 randomly collected human milk samples.

The occurrence of citrinin in cereal grains was confirmed by HPLC[327]. Citrinin was extracted with chloroform–0.1 *M* phosphoric acid (15:2), passing the chloroform phase through an Extrelut column. The sample was then chromatographed on LiChrospher Si 100, buffered with citrate–phosphate buffer (pH 2.5), with hexane–chloroform (3:2) as the mobile phase and fluorimetric detection (λ_{ex} = 360 nm and λ_{em} = 500 nm).

In the 1980s, several methods have been developed for measuring patulin in food products, including TLC, GC and HPLC. Möller and Josefsson[328] used extraction with ethyl acetate and reversed-phase separation on Spherisorb 50 DS with water as the mobile phase. HPLC was also applied by Ruggieri and Ruggieri[329]. A rapid method was described for the quantitative determination of patulin in ethyl acetate extracts of apple juice[330]. After clean-up with a sodium carbonate solution, patulin was determined by reversed-phase HPLC using a μBondapak C_{18} column and a UV detector at 254 nm.

Citreoviridin levels in *Eupenicillium ochrosalmoneum*-infested maize kernels at harvest were examined by Wicklow *et al.*[331]. The maize was extracted with dichloromethane and the extract was partially purified with silica and amino solid-phase extraction columns. The citreoviridin in the extract was quantitated by using normal-phase HPLC with ethyl acetate–hexane (75:25) as the mobile phase at a flow-rate of 1.5 ml/min. Fluorescence detection with λ_{ex} = 388 nm and λ_{em} = 480 nm gave an optimum response and sufficient densitivity (limit of detection, 2 μg/kg). The main peak, with a retention time of 5.46 min, was citreoviridin. The same method was used to determine citreoviridin in corn and rice[332].

HPLC was applied in combination with TLC to separate and characterize penitrems A–F[176]. The HPLC separation was achieved on an HP 79918A RP-8 reversed-phase column with water–methanol (22:78) as the eluent. The penitrems were detected at 296 nm and it was possible to separate them in one run. For quantitative analysis, penitrem A monoacetate was used as an internal standard.

Lauren and Gallagher[333] developed a method of analysis for the janthitrems using reversed-phase HPLC. The use of both UV and fluorescence detectors allowed the differentiation of the janthitrems from other compounds with similar retention times. A modification of the eluent helped to separate a group of tremorgens as follows: janthitrem B, janthitrem A, verruculogen, penitrem A, fumitremorgin B, janthitrem C and janthitrem D.

Tanaka *et al.*[334] published a method for the rapid and sensitive determination of zearalenone in cereals by HPLC with fluorescence detection. Another method was reported by Cohen and Lapointe[335]. The HPLC determination, with UV and fluorescence detection, of α-zearalenol and zearalenone in corn has been studied collaboratively and the results were published by Bennett *et al.*[336]. Bagneris *et al.*[337] reported the determination of zearalenone and zearalenol in animal feeds and grains, using fluorescence detection. A diastereomeric mixture of α- and β-zearalenol, which could not be resolved by two-dimensional TLC, was resolved into its components by HPLC[196].

Kamimura[199] studied the microbial conversion of zearalenone by various species of fungi and used HPLC, TLC and preparative CC. Joseffson and Möller[338] described an HPLC procedure for the determination of zearalenone and ochratoxin A in cereals.

Another method was reported[339] for the extraction and determination of zearalenone in chicken tissues by HPLC using a reversed-phase radial compression separation system, a UV detector and acetonitrile–water (60:40) as the mobile phase. Recoveries of zearalenone added at levels from 50 to 200 ng/g were in the range 82.6–95.1%.

The HPLC determination of zearalenone and zearalenols in rat urine and liver[340] and in blood plasma and urine[341] has been described. Detection of the fraudulent use of zearalenol and the natural occurrence of zearalenone in cattle urine by HPLC was reported[342].

Zearalenone and α- and β-zearalenol are transmitted into the milk of cows and other animals. They can be extracted with basic acetonitrile and, after acidification, partitioned into dichloromethane and a hydrophilic matrix. After clean-up on an aminopropyl solid-phase extraction column and reversed-phase HPLC, they are detected fluorimetrically. As little as 0.2 ng/ml of zearalenone and α-zearalenol and 2 ng/ml of β-zearalenol can be detected in milk[343].

An HPLC determination of cyclopiazonic acid was reported[344]. A modification of this method based on ligand-exchange HPLC was developed for the determination of cyclopiazonic acid in poultry meat[345]. After extraction and clean-up, the toxin was subjected to ligand-exchange HPLC using a Beckman Ultrasphere ODS column and detection at 284 nm.

According to Goto *et al.*[346], the method reported by Lansden[344] lacks reproducibility, accuracy and sensitivity. To overcome these problems, they developed a sensitive and accurate HPLC method for the analysis of cyclopiazonic acid. The

normal phase with silica gel (Develosil 60-5) gave satisfactory results. The most succesful solvent system was ethyl acetate–2-propanol–25% aqueous ammonia (55:20:5) and the flow-rate was 1.0 ml/min. The detection limit for pure toxin with this system was 0.2 ng, and a linear calibration graph was obtained in the range 0.5 ng–3 μg. This HPLC method was utilized for the analysis of samples contaminated with cyclopiazonic acid. The toxin was extracted from maize, deoiled peanut meal and rice with chloroform–85% phosphoric acid (100:1), purified on Sep-Pak cartridge columns and then analysed by HPLC.

Reddy *et al.*[347] described an HPLC procedure for secalonic acid D and its application to biological fluids. A precolumn before the μBondapack C_{18} column allowed the direct injection of urine and bile without sample clean-up. Rat plasma was acidified and was extracted with ethyl acetate. The extracts were pooled and evaporated under nitrogen. The residue was taken up in the elution solvent system and aliquots were analysed by HPLC. Mixtures of acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5 for system A and 4:3:0.5:0.5 for system B) were used as eluents; system A was used for urine and plasma samples and system B was for bile samples. Detection was effected at 340 nm.

The separation of the 1,4-naphthoquinones xanthomegnin and viomellein by means of HPLC was reported[228]. HPLC has been used for the quantitative determination of xanthomegnin in corn extracts[348], grains and animal feeds[349]. Derivatization of the toxin for fluorimetric determination was reported by Kuan *et al.*[350].

A method was developed for the purification of xanthomegnin produced by *Penicillium viridicatum* on converted rice[351]. The toxin was extracted with dichloromethane and the extract was concentrated to an oil which was partitioned between hexane and methanol–water (9:1). The methanol layer was washed with a second portion of hexane, resulting in deposition of a xanthomegnin-rich precipitate at the interface, which was removed and washed with hexane. Chromatography of the precipitate dissolved in dichloromethane was performed with a Waters Assoc. Prep 500 chromatograph fitted with a Prep Pak 500 silica column and eluted with toluene–methanol–acetic acid (98.5:1:0.5) at a flow-rate of 250 ml/min. The xanthomegnin crystallized in those fractions eluting between 4.5 and 6.5 l. Small amounts of viomellein were eluted between 2.5 and 3.5 l.

A critical analysis of several HPLC systems for separating aflatoxins and their anthraquinone precursors was published recently. An HPLC system with a μBondapak C_{18} column and a solvent system of methanol–tetrahydrofuran acidified with acetic acid was used for the separation of anthraquinone precursors of aflatoxins. The system offers the advantage of a good separation of all the anthraquinones of interest and a running of less than 40 min. It has also been demonstrated that this system can be used as a means of screening fungal strains and mutants for the metabolites which they accumulate, and also for the rapid quantitation of enzymic and non-enzymic interconversions of the metabolites[322].

Monomeric anthraquinones (macrosporin and altersolanol A) and modified anthraquinones (alterporriols A, B and C) from fermentations of *Alternaria porri* were also determined by HPLC[352].

Shepherd and Gilbert[353] developed a method for the determination of moniliformin in maize employing ion-pairing extraction and HPLC. Thiel *et al.*[250] used a paired-ion chromatographic technique in the quantitative determination of monili-

formin together with deoxynivalenol and zearalenone. The same technique and an ion-exchange procedure were used later[354] for the quantitative determinations of moniliformin in corn screenings. Detection was performed at 227 nm. Paired-ion chromatography was carried out on a μBondapak C_{18} column using 0.1 *M* sodium phosphate buffer (pH 7.0)–0.005 *M* tetrabutylammonium hydrogensulphate–8% methanol as the mobile phase. The ion-exchange separations were done on a Partisil 10 SAX column using 0.01 *M* sodium dihydrogenphosphate (pH 5.0) as the mobile phase.

Preparative separations of two fusaric acid analogues from *Fusarium moniliforme* were reported by Burmeister *et al.*[236].

The determination of fusarin C in corn was reported[355]. After extraction and clean-up, fusarin C was quantified in the column eluate by HPLC on an Ultrasphere column using methanol–chloroform (1:19) as the mobile phase and detection at 360 nm. This method was also applied by Thiel *et al.*[354].

Danieli *et al.*[356] employed reversed-phase HPLC with detection at 250 nm for the determination of PR toxin. The retention time and peak shape were found to be critically dependent on the water–acetonitrile ratio in the mobile phase. HPLC of PR toxin and eremofortin C was described by Chang[357].

It was shown[358] that rubratoxin B is heat labile, at least during clean-up. The toxin was detected in mixed-feed extracts using an acidified mobile phase to maintain it in the non-ionized form.

HPLC of alternariol, its monomethyl ether, altenuene and tenuazonic acid was reviewed by Seitz[237]. An HPLC preparation of alternariol, alternariol methyl ether and altenuene was also reported[359]. A method of ligand-exchange HPLC for the determination of tenuazonic acid has been developed by Scott and Kanhere[360].

4.5. HPLC of peptidic mycotoxins

This sub-section deals with cyclosporin A (cyclosporine), its metabolites, phomopsin A, α-amanitin and phalloidin. Cyclosporin A is a lipophilic neutral and cyclic peptide mycotoxin with exceptional immunosuppressive properties.

Edwards and Lillehoj[361] developed techniques for the quantitative assessment of the toxin in rice. The methods include open-bed CC, TLC and HPLC for the separation and quantitation of cyclosporin A from *Trichoderma polysporum*-inoculated rice. The good baseline resolution at ambient temperature demonstrated no necessity for the higher temperature which have been used in most HPLC analyses of the toxin.

Several HPLC methods have been reported for the determination of cyclosporin A in both human serum and urine[362–370]. A temperature of about 70°C is needed for symmetrical cyclosporin peaks[366,367,370]. To meet this requirement, the mobile phase should be heated before it enters the detector.

For some HPLC methods liquid–liquid extraction (*e.g.*, refs. 362–364) and for the others solid-phase extraction is used (*e.g.*, refs. 370–375). Several procedures use a single column (in either a gradient or isocratic mode; *e.g.*, refs. 362, 371, 376–380). Other, more complex, procedures require column switching[381–386] or multi-step sample preparation[387].

Solid-phase extraction and a highly efficient HPLC analysis were reported to provide accurate measurements of cyclosporin A in whole blood, plasma or serum.

There are two steps in this analysis[370]: extraction of the drug from blood by solid-phase extraction, and analysis on a highly efficient 3-μm silica-based HPLC column. Whole blood samples, rather than serum or plasma, provide the most accurate measurements of cyclosporin A. The detector response for cyclosporins A and D (the internal standard) is optimum at 195–215 nm, a range in which many blood components also strongly absorb. Supelclean LC-CN solid-phase extraction tubes efficiently retain the non-polar cyclosporin molecules, then release them for elution with a solvent of moderate strength. Cyclosporin A was well separated from the internal standard on Supelcosil LC-8, LC-18, LC-DP and LC-CN columns. When methanol was included in the mobile phase, blood or other components remaining in the sample were eluted before the cyclosporins.

Cyclosporin A alone or together with its metabolites can be detected by means of HPLC[388,389].

Brossat *et al.*[390] reported a selective and sensitive HPLC method, which involves solid-phase extraction and ion-pair chromatography. It can be used for the assay of cyclosporin A in serum or urine. Samples were cleaned up on a solid-phase extraction system (cyanopropyl column). The system involved a reversed-phase Ultrasphere C_{18} column maintained at 72°C and a linear gradient of acetonitrile (from 65 to 95%) in 0.14% triethylammonium phosphate. Liquid chromatographic analysis of radioimmunoassay standards showed that some samples contained a contaminant peak. Comparison of cyclosporin A levels obtained by radioimmunoassay and HPLC in clinical investigations showed that the former values were generally, but not always, higher than the latter, and that cyclosporin A is very differently metabolized depending on the patient, disease and treatment.

Other HPLC methods have been published that could be used for the measurement of cyclosporin A and two[391], three[392] or four its metabolites[389].

In trying to reproduce several previously published HPLC methods, Christians *et al.*[389] pointed out one or more of the following disadvantages: a short lifespan of the chromatographic column[362,371], low or variable recovery of cyclosporin A[362,387] and laborious and/or time-consuming extraction and sample preparation[362,371,387]. They developed another HPLC method using cyclosporin D as an internal standard for the routine measurement of cyclosporin A and four of its metabolites. Whole-blood samples were purified on refillable solid-phase glass extraction columns. The chromatographic method included gradient elution using acetonitrile and water (pH 3.0) as eluents and an RP-8 analytical column. More than 1000 samples were analysed without any loss. The inter-assay coefficient of variation (C.V.) was 6.3% and the intra-assay C.V. was 4.9%. A linear correlation was found over a range of 0–3000 ng of cyclosporin A per ml of whole blood. The detection limit was 20 ng and the recovery was 80–90%. Metabolites 1, 17, 18 and 21 could be characterized.

Most recently, Gmur *et al.*[391] reported a column-switching HPLC method for measuring metabolite 17 in whole blood and also separating metabolite 1. New methods for the determination of cyclosporin A alone or cyclosporin A and three of its metabolites (17, 1 and 21) were also published recently[392].

An improved HPLC assay of phomopsin A, the principal hexapeptide mycotoxin responsible for lupinosis, was reported[393]. A reversed-phase Cl column, a methanol–water gradient and UV detection were used.

The diagnosis of suspected poisoning by the mushroom *Amanita phalloides* is a

challenge to clinicians. Several radioimmunoassay (RIA) methods have been developed[394]. An alternative method for the determination of the toxic peptides of poisonous mushrooms (α-amanitin in the amatoxin group and phalloidin in the phallotoxin group of substances) is provided by HPLC. Methods in this direction have been published[395–397]. Most recently, a reversed-phase HPLC assay has been developed for the simultaneous determination of α-amanitin and phalloidin in human plasma[394]. The procedure is based on the enrichment of the toxins on a precolumn, followed by the transfer of both compounds in a foreflush mode to the analytical column. α-Amanitin and phalloidin can be quantified down to a minimum concentration of 10 ng/ml in plasma.

5. GAS CHROMATOGRAPHY

Historically, gas chromatography (GC) was introduced into the field of mycotoxins in the early 1970s. If mycotoxins are sufficiently volatile at the column temperature, or if hydroxylated toxins can be converted into volatile derivatives, GC can be used in their analysis.

In GC, mycotoxins or their derivatives are mostly detected with flame ionization or electron-capture detectors. GC can also be effectively coupled to a mass spectrometer (GC–MS) to obtain qualitative data concerning the identity of the components being analysed.

Some pioneer applications of GC in mycotoxin analysis were reviewed by Gorst-Allman and Steyn[1]. The following mycotoxins were included: alternariol, alternariol monomethyl ether, altenuene, patulin, penicillic acid, sterigmatocystin, trichothecenes and zearalenone. An authoritative review on the GC of mycotoxins was written more recently by Beaver[398]. Vesonder and Rohwedder[243] reviewed the GC–MS analysis of mycotoxins.

As GC is most extensively applied in the field of trichothecenes[399], a selection of papers published in the 1980s are referred to in this section. A few applications for the detection of other mycotoxins have also been selected and are referred to below.

5.1. GC of trichothecenes

5.1.1. Development of techniques. The chromatography of trichothecenes has been reviewed[298,400]. The most sensitive and specific analytical techniques are GC with electron-capture detection (GC–ECD) and GC–MS[401–403]. The most common methods of derivatization are silylation, which converts the trichothecenes into trimethylsilyl ethers (TMSE), trifluoroacetylation and heptafluorobutyrylation, which convert the trichothecenes into trifluoroacetyl and heptafluorobutyryl esters, respectively. Silylation is often preferred because it gives much less complicated gas chromatograms[404,405].

Gilbert *et al.*[406] studied the optimization of the conditions for the trimethylsilylation of trichothecenes. Rizzo *et al.*[407] used trimethylsilylimidazole, with and without trimethylchlorosilane, for the silylation with the aim of improving the detectability of six of the most important trichothecenes by performing selective hydrolysis of the reagent, after silylation, in order to eliminate its interferring effect on the products of derivatization during GC.

Scott and Kanhere[408] compared column phases for the separation of deriv-

atized trichothecenes. Decomposition of trifluoroacetyl derivatives of trichothecenes on fused-silica capillary columns was reported[409]. Visconti *et al.*[410] published mass spectrometric evidence for demethylated homologues occurring at trace levels in trichothecene standards. A fast and sensitive GC–MS method for the simultaneous detection and quantification of several simple trichothecenes with good precision was developed[411]. Two semi-synthetic derivatives, 4-deoxyverrucarol and 16-hydroxyverrucarol, were adequate internal standards for both the detection and quantification of trichothecenes. The detection and quantitation of several polar and thermally labile macrocyclic trichothecenes by GC–negative ion chemical ionization mass spectrometry (GC–NICI-MS) was reported[412]. The method is applicable to the detection and quantitation of these compounds in naturally occurring samples.

5.1.2. Trichothecenes in environmental samples. T-2 toxin has been determined in maize samples by GC–MS[413] and in *Fusarium acuminatum* cultures by GC with ^{63}Ni ECD[414]. The latter procedure was also used to detect T-2 toxin and diacetoxyscripenol in corn samples[355,415]. GC–ECD and GC–MS have been applied to the determination of deoxynivalenol[416–418], nivalenol[419] and nivalenol with deoxynivalenol[420] in cereals.

Applications of GC in multi-trichothecene detection have been published[172,398,421–425]. Black *et al.*[424] described methods for the simultaneous detection of a wide range of trichothecenes, including the most polar ones and some macrocyclics, using either GC–MS with selected ion monitoring or GC–ECD. Trichothecenes have been extracted directly from the various matrices, or from Clin-Elut columns, and cleaned up on Florisil Sep-Pak cartridges. Macrocyclics and neosolaniol have been detected after hydrolysis to verrucarol and T-2 tetraol, respectively. For optimum sensitivity over a wide range, trichothecenes have been detected, both before and after hydrolysis of ester groups, as their heptafluorobutyrate derivatives using a quandrupole mass spectrometer and negative ion chemical ionization. The methods have been used to detect the presence of scirpentriol, nivalenol and 15-monoacetoxyscirpenol in sorghum. Trichothecenes in less complex matrices could be detected, after hydrolysis, using GC–ECD.

More recently, another GC screening method for T-2 toxin, diacetoxyscirpenol, deoxynivalenol and related trichothecenes in feed samples was reported[426]. Feeds were extracted with acetonitrile–water and the toxins were purified on charcoal–alumina–Celite, Florisil, and silica mini-columns. Deoxynivalenol, nivalenol, diacetoxyscirpenol, T-2 toxin and their fungal metabolites were hydrolysed to their parent alcohols by alkaline hydrolysis, derivatized to their pentafluoropropionyl analogues and quantitated by capillary GC with ECD. Identity can be confirmed and the sensitivity increased by using negative chemical ionization MS with no additional sample workup.

5.1.3. Trichothecenes in body fluids. Rood *et al.*[319] published a rapid screening procedure for the detection of trichothecenes in plasma and urine. More recently, they described a diagnostic screening method for the determination of trichothecene exposure in animals[427]. Other workers have used either GC–ECD[428–431] or GC–MS[432,433]. Trichothecenes have been detected in human blood[428,433] and urine[432], dog plasma[430], swine plasma and urine[431] and bovine urine and faeces[429]. Individual or several toxins have been determined as follows: deoxynivalenol and its metabolites[429], diacetoxyscirpenol[431], T-2 and HT-2 toxin[430], T-2 toxin, HT-2 toxin, T-2

triol, diacetoxyscirpenol, deoxynivalenol and verrucarol[433] and eleven trichothecenes of widely varying polarity[428]. Some examples are presented here.

A sensitive and selective method was developed for the simultaneous detection of eleven trichothecenes in human blood. The procedure involved precipitation of blood proteins with acetone followed by a clean-up using reversed-phase Sep-Pak C_{18} cartridges. The extracted trichothecenes were derivatized to their pentafluoropropionyl esters, separated using capillary GC and detected using electron-capture negative ion chemical ionization with methane as the reagent gas and selected-ion monitoring. Optimum sensitivity and selectivity were obtained using low source temperatures (60°C) and high source pressures (1 Torr). Detection limits for 1-ml blood samples were in the range 0.1–5 ppb. The method was readily adaptable to the detection of other trichothecenes and was validated in collaborative studies by the successful analysis of 42 blood samples spiked and submitted blind by two independent laboratories for analysis[428].

Capillary column GC–ammonia chemical ionization MS was found to be an excellent technique for the trace detection and identification of underivatized trichothecenes. Abundant $(M + H)^+$ and/or $(M + NH_4)^+$ pseudo-molecular ions were observed for T-2 toxin, HT-2 toxin, T-2 triol, diacetoxyscirpenol, deoxynivalenol and verrucarol under the conditions adopted. This method was succesfully applied to the analysis of human blood samples spiked with mycotoxins in the range 0–500 ng/g during an interlaboratory exercise. T-2 toxin and diacetoxyscirpenol were detected in these samples in the range 2–180 ng/g. Detection limits of 0.7 and 3.6 ng/g for T-2 toxin and diacetoxyscirpenol, respectively, were possible owing to the specificity of the method[433].

5.1.4. Biotransformations of trichothecenes. GC has been used in studies on biotransformations with the following results. The distribution and metabolism of tritium-labelled T-2 toxin was investigated after oral administration to chickens[434] and a lactating cow[435], and after intravascular administration to swine[436]. In all species, T-2 was rapidly biotransformed to a variety of metabolites. Minor metabolites in the cow and chicken were initially identified as simple hydrolysis products including HT-2 toxin, 4-deacetylneosolaniol and neosolaniol. Other metabolites were characterized later[437,438]. In addition to HT-2 toxin, 4-deacetylneosolaniol, T-2 tetraol and neosolaniol were detected in rat liver homogenates[439]. GC–ECD was used to characterize T-2 toxin metabolites by Knupp *et al.*[440]. Rat liver microsomes transformed T-2 toxin *in vitro* to a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol and 3′-OH-HT-2, in addition to two unidentified compounds.

An *in vitro* rumen system was used by Swanson *et al.*[441] to compare the metabolism of three trichothecenes by rumen microorganisms: T-2 toxin, diacetoxyscirpenol and deoxynivalenol. GC–ECD and GC–MS analyses of extracts indicated that all three toxins were biotransformed to a variety of deepoxy and deacetylated products.

5.2. GC of other mycotoxins

The aflatoxins have been analysed mostly by TLC and less often by HPLC. However, recent reports have described methods for their GC–MS determination. Friedli[442] reported that aflatoxin B_1 could be determined without chemical derivatization by GC using a mass spectrometer as the detector. Trucksess *et al.*[443] were

able to determine aflatoxin B_1 on methylsilicone-coated fused-silica columns. Rosen *et al.*[444] used a fused-silica capillary column coated with a film of bonded 5% phenyl–1% vinylmethylsilicone to chromatograph aflatoxins B_1 and B_2. Dimitrov *et al.*[445] included GC among other methods for the detection of aflatoxins in foods.

Most recently, Goto *et al.*[446] succeeded in determining four major aflatoxins (B_1, B_2, G_1 and G_2) using GC with flame ionization detection (FID) with a capillary column injector and a fused-silica capillary column. A Shimadzu GC-15A gas chromatograph and a Shimadzu GCMS QP1000 mass spectrometer were applied. Two types of stationary phases were tested. The methylsilicone column (DB-1, 10 m) did not separate aflatoxins G_1 and G_2 and barely separated B_1 and B_2. As a result, the shape of the peaks was distorted. In contrast, a 5% phenylmethylsilicone column (DB-5, 10 m) clearly separated aflatoxins B_1 and B_2 and also achieved a 50% separation between aflatoxins G_1 and G_2. A longer column was used to improve the overall separation. Although the four aflatoxins were completely separated on a 25-m DB-5 column, the sensitivity was much lower for aflatoxins G_1 and G_2 than for B_1 and B_2.

In a review chapter on the GC of mycotoxins, Beaver[398] described GC analyses of trichothecenes, zearalenone, patulin, penicillic acid, slaframine, swainsonine, *Alternaria* toxins and aflatoxins. Except for trichothecenes and aflatoxins, most of the methods reviewed were published in the 1970s. Some data published in the 1980s are added below.

Phillips *et al.*[447] described a method for the GC of penicillic acid as its pyrazoline derivative, which was detected by FID. Butenolide was determined by GC–ECD[448]. GC has also been used in the detection of "peptaibols" and other aib-containing peptides of fungal origin, *i.e.*, trichotoxin, alamethicin, suzukacillin, hypelcin and paraselsin[449,450].

Gilbert *et al.*[451] described the derivatization of moniliformin for GC–MS analysis. They found that the reaction of moniliformin with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide containing 1% *tert.*-butyldimethylchlorosilane produced a compound that had a characteristic mass spectrum, was formed quantitatively and in selected ion GC–MS gave a good linear calibration at low levels. The purification of the derivative was achieved by high-performance size-exclusion chromatography.

Bottalico *et al.*[196] extracted zearalenol from *Fusarium*-infected stems of corn. The toxin, which appeared as a single compound in various TLC systems, was resolved into two components by HPLC. A GC–MS examination of a purified fraction confirmed the natural occurrence of zearalenol as a diastereomeric mixture and led to the identification of the α- and β-isomers.

6. CONCLUSIONS

This review was written with the aim of demonstrating the scope of applications of chromatographic techniques in the still developing field of mycotoxins. It is partly arranged with a historical perspective. Although several mycotoxins were recognized before the 1960s (mostly as antibiotic compounds), the origins of the "mycotoxin era" are usually associated with the discovery of aflatoxins in the early 1960s. In that period, the gradual decline of applications of PC was due to the rapid development of TLC. A decade later, the introduction of HPLC and GC had to compete with then-existing methods based on TLC.

Whereas applications of PC in mycotoxicology are now interesting mostly from a historical point of view, it would be impossible to review the mushrooming literature concerning the use of other chromatographic techniques. As earlier contributions of general interest have been repeatedly reviewed, attention is focused here on the achievements of TLC, HPLC and GC published in the selected literature from 1980 onwards.

In most instances, the mycotoxins to be analysed or purified chromatographically are present in contaminated samples. Hence, they must be extracted and cleaned-up prior to TLC, HPLC or GC if reliable results are to be obtained. Extraction procedures include extractions of mycotoxins from feeds and foodstuffs, cultivation media and/or mycelia of toxigenic fungi and body fluids or tissues.

Clean-up procedures include CC, gel-permeation chromatography, liquid–liquid partition and precipitation. In these procedures, contaminating substances have to be removed from the mycotoxin samples. Several recent clean-up methods were included into the sections covering TLC, HPLC and GC.

TLC is by far the most widely used chromatographic technique applied to mycotoxins owing to its relatively simple, fast and inexpensive character. One- and two-dimensional TLC and HPTLC have been used. Preparative TLC is also of great importance in purification procedures. With HPTLC and in quantitations, TLC becomes more expensive owing to the need for densitometers and spectrophotometers.

In addition to its use in clean-up procedures, CC is applied either in large-scale separations and purifications or in some analytical methods using various mini-columns.

HPLC is applied mostly for analytical purposes but preparative columns are also used in mycotoxicology.

It appears that countercurrent chromatography (CCC) has not yet been applied in the field of mycotoxins, although applications of procedures such as droplet CCC, centrifugal droplet CCC, rotation locular CCC and planet coil centrifugal CCC have proved advantageous for the isolation and purification of a diverse array of natural products[452]. For example, CCC greatly facilitated the isolation of a variety of structural types of antibiotics and was more convenient than other chromatographic techniques[453]. Avoidance of contamination with traces from solid chromatographic supports by CCC facilitated instrumental analyses of daunomycin reduction products[454]. The resolution of the actinomycin C complex into its components by CCC has been very successful[455]. Actinomycin C2 contains just one methylene group more than actinomycin C1 and one methylene group less than actinomycin C3. Nonetheless, these components were conveniently resolved utilizing only 600 ml of mobile phase. Hence, one would expect that CCC will find its place among other chromatographic techniques hitherto used in the field of mycotoxins.

The advantages and disadvantages of the use of TLC and HPLC in mycotoxicology were excellently compared as follows[242]:

"The most significant advantage of TLC is that it can be a very inexpensive technique, although in its more sophisticated forms it requires a considerable capital investment in items such as spotters and densitometers. In addition, if one-dimensional TLC gives adequate resolution, a considerable number of samples may be analysed on one plate. Should two-dimensional TLC be found necessary, several plates can be developed simultaneously. Hence results can be obtained rapidly by TLC, even when

TABLE 5

COMPARISON OF VARIOUS FORMS OF AUTOMATED CHROMATOGRAPHIC SYSTEMS

Modified from ref. 456.

Comparisons	*TLC (proposed)*	*LC*	*GC*
Mode	Stepwise, batch	Continuous, flow	Continuous, flow
Limit of detection	pg	pg	pg, fg
Analysis time (per throughput)	Minutes	Minutes	Minutes
Sample per throughput	As many as 72[a]	One	One
Effective analytical time (per sample)	Seconds	Minutes	Minutes[b]
Potential sample capacity (per day)	As many as 10 000[c]	72	72

[a] Per 20-cm HPTLC plate[457].

[b] A high-speed chromatographic gas analyser has been described[458].

[c] Assuming a constant time of 10 min.

taking into account the time required for spotting the plates. The two principal disadvantages of TLC analysis are its lack of potential for automation and the subjective nature of the quantitation step. Use of a densitometer overcomes the latter objection but at a cost equivalent to that of a single HPLC system. Autosamplers permit unattended running of HPLC equipment and allow the sample throughput in this sequential method of analysis to be as great as that for TLC, while the recent development of short, very high-efficiency columns has demonstrated the capability of HPLC to provide extremely rapid results. Because of the general growth in the use of HPLC, many laboratories possess the necessary instrumentation and could therefore perform mycotoxin analysis should it appear to offer definite advantages over the more conventional TLC methods. Comparing HPLC and TLC techniques, a similar high degree of competence is necessary when establishing procedures and validating methods, but it is sometimes not appreciated that although TLC may be carried out using very simple equipment, it then demands greater operator skills and attention to detail in use then does HPLC.

Separation by HPLC may be preferred for other reasons. One factor to consider is safety; liquid chromatography offers greater protection, particularly for preparative work, because toxins are maintained in solution and contaminated silica dust does not arise. Equally, moisture- or oxygen-sensitive samples, such as xanthomegnin, are more readily chromatographed on a column. One potentially important advantage of HPLC lies in its suitability for on-line clean-up of crude extracts, and it is possible that this will ultimately be seen as one of the more compelling reasons for employing HPLC rather than TLC as the analytical technique".

According to Shepherd's comparison of TLC and HPLC, one of the two principal disadvantages of TLC analysis is "its lack of potential for automation"[242]. However, in his "Considerations for automating TLC", Rogers[456] was less pessimistic. He proposed the following operational parameters of automated TLC: (i) plate insertion/retrieval; (ii) sample application; (iii) development, first dimension; (iv) development, second dimension; (v) derivatization, reagent spray; (vi) derivatization, energy imput;

(vii) microdensitometric scanning; and (viii) documentation. His comparison of various forms of automated chromatographic systems are presented in Table 5.

Except for trichothecenes, GC has hitherto been much less used than TLC or HPLC in mycotoxicology. It has been applied in determining some small lactones (patulin, penicillic acid, butenolide), sterigmatocystin, aflatoxins, zearalenone, moniliformin and some *Alternaria* toxins. The derivatives of trichothecenes prepared for GC analysis have usually been trimethylsilyl, heptafluorobutyryl and trifluoroacetyl. However, the ability to detect some mycotoxins in an underivatized state using fused-silica capillary columns has recently been demonstrated (*e.g.*, ref. 433).

In GC, mycotoxins or their derivatives may be detected by FID or ECD. GC can also be effectively coupled with mass spectrometry to obtain qualitative data concerning the identity of compounds being analysed.

7. SUMMARY

Achievements in the applications of chromatographic techniques in mycotoxicology are reviewed. Historically, column chromatography (CC) and paper chromatography (PC) were applied first, followed by thin-layer chromatograhy (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Although PC techniques are no longer used in the analysis of mycotoxins, selected applications of PC are included to underline historical continuity. The most important achievements published from 1980 onwards are described. They include clean-up methods, TLC, CC, HPLC and GC of mycotoxins in environmental samples, foods, feeds, body fluids and in studies on biosynthesis and biotransformations of mycotoxins. Advantages and disadvantages of chromatographic techniques used in mycotoxicology are also evaluated.

NOTE ADDED IN PROOF

Further possibilities of the use of HPLC techniques in the field of mycotoxins can be documented with the following examples.

A simple LC–MS procedure has been developed recently for the analysis of some of the most toxic (roridins) and few known benevolent (baccharinoids) and isomeric macrocyclic trichothecenes[459]. Roridins and baccharinoids were separated on a reversed-phase HPLC column and effectively ionised under thermospray ionisation conditions. A semisynthetic macrocyclic trichothecene, 8-ketoverrucarin A, was used as the internal standard. Minimum detectable limits were to be 2–5 ng.

In chromatography of cyclosporin A, only reversed-phase HPLC techniques have been developed and the use of normal-phase techniques has not been reported so far (see Section 4.5.). Most recently, Oka *et al.*[460] have developed a normal-phase HPLC method to be conducted in conjunction with rapid flow fractionation for sample pretreatment. The method was used to determine cyclosporin A concentrations in the serum of kidney transplant patients. HPLC rather than the conventional radioimmunoassay (RIA) provided more precise and reliable values for the concentration of cyclosporin A.

The advent of computer-controlled photodiode-array UV detectors in HPLC[461] could even increase the use of HPLC in the field of mycotoxins. For exam-

ple, profiling of mycotoxins in fungal-contaminated foodstuffs by HPLC coupled to detectors that are unable to give retention data corroborated by peak purity and peak identity may result in unreliability owing to the complexity of the matrix. Computer-controlled photodiode-array UV detectors provide considerable help solving such a complex analytical problem by greatly improving peak identification, peak purity assessment and quantitation. The high spectral acquisition rate during elution provides a matrix of absorbance wavelength time data that can be treated by computer-aided techniques for their reduction, manipulation and presentation. The potential of HPLC with diode-array detection for the profiling of mycotoxins in food samples has been demonstrated very recently[462]. A gradient elution reversed-phase chromatographic method was devised that simultaneously separated and detected major *Alternaria* mycotoxins in foodstuffs. According to the authors of this paper, "the multi-signal plotting capability may be an aid for a first identification (through the choice of selective wavelength) and at the same time can optimize the detection sensitivity for compounds (or classes) having different absorption maxima. In addition, three-dimensional spectrochromatograms give UV spectra and the possibility of identification of those toxins having characteristic spectra... Although UV spectra alone can rarely give an absolutely certain identification of a compound, a reasonable degree of confidence may be reached in most instances. Coupling of a diode-array UV detector to a functional group-specific detector, *e.g.*, electrochemical, should provide additional evidence (and sensitivity)".

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Journal of Chromatography, 477 (1989) 235–248
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 582

MICROCOMPUTER-AIDED CHARACTERIZATION OF MOBILE PHASES FOR NORMAL-PHASE LIQUID–SOLID CHROMATOGRAPHY BASED ON SNYDER'S THEORY AND DATA

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(First received March 3rd, 1989; revised manuscript received April 19th, 1989)

SUMMARY

A microcomputer program of practical importance is described that permits the characterization of any mobile phase for normal-phase separations on silica or alumina by means of Snyder's parameters solvent strength, ε, localization, m, and polarity, P'. The calculations follow the guidelines proposed by Snyder. In addition, an algorithm is developed that permits the choice of mobile phases of equal strength, the so-called equi-eluotropic mobile phases.

The microcomputer program was tested against approximately 250 mobile phases which were characterized by values of the above three parameters. Good agreement was found with the calculated or experimental values of either ε and m reported earlier by Snyder and co-workers for some of the mobile phases. Some of the computer-calculated ε values are presented in an easy-to-use nomograph form.

INTRODUCTION

The application of Snyder's theory[1–3] for the treatment of the thin-layer chromatographic (TLC) behaviour of numerous diastereoisomers[4–6] convinced us that this theory is very useful for the characterization of mobile phases. Solvent strength and selectivity effects of the latter in normal-phase separations can be described in terms of certain parameters introduced by Snyder. These parameters are derived from the displacement model for normal-phase retention. The parameter ε is a direct measure of solvent strength. Overall changes in sample retention as a function of mobile phase composition can be predicted if values of ε for various mobile phases are known. Likewise, a second parameter, localization, m, is useful for characterizing the selectivity effects of various mobile phases.

The polarity P' is a third parameter of interest, derived by Snyder[7,8] on the basis of a given solubility parameter[9]. Solvent strength in normal-phase separations seems to be better understood by both ε and P' as discussed at the end of this paper. The P' value of any mobile phase is obtained in a simple manner[7,8].

0021-9673/89/$03.50

Procedures for obtaining values of ε and m for different mobile phases and either alumina and silica as adsorbent have been described by Snyder and co-workers[1–3,5,10–13]. However, the associated calculations are tedious[14], which accounts for the fact that the useful Snyder theory is rarely used in every-day chromatographic practice. We undertook this study for several reasons. First, because of the relative complexity of the calculations, it seemed worthwhile to verify independently the originally reported values of ε and m in another laboratory. Second, a considerable number of experimental ε values have been reported in the literature[3,5,6,10–13], and it seemed appropriate to compare these data with values calculated by the present program. Finally, many workers would like to see the results of such calculations expressed as an expanded eluotropic series for silica or alumina. One such example has appeared[14], but it is based on older calculation procedures that are less accurate, and does not include many solvents that are now more popular for either high-performance liquid chromatographic (HPLC) or TLC separations.

THEORY

The calculations of solvent strength, ε, localization, m, and polarity, P', follow the guidelines proposed by Snyder and co-workers[1–3,5,10–13], but are described here in sufficient detail to allow other workers to write similar programs.

Calculation of mobile phase strength, ε

Solvent strength values for pure solvents commonly used in liquid–solid chromatography (LSC) and other characteristics are given in Table I in ref. 3. According to the developed displacement model[3,5,10–13], the calculation of ε values for mobile phases containing 2–z solvents includes the steps described below.

(1) Calculation of molar fraction, N_i, for each solvent of the mobile phase on the basis of its volume percentage P_i using the following equation:

$$N_i = (d_i P_i/\mathrm{MW}_i)/\sum_{i=1}^{z}(d_i P_i/\mathrm{MW}_i) \tag{1}$$

where d_i is the density of solvent i and MW_i is its molecular weight. Let us arbitrarily assume that i corresponds to the solvent number when the solvents of the mobile phase are arranged in increasing order of their individual ε values with the first and last solvents having $i = 1$ and z, respectively.

(2) Calculation of the "mean" area $\bar{n}$ which the solvents of the mobile phase occupy on the adsorbent surface is based on the equation

$$\bar{n} = \sum_{i=2}^{z}(N_i n_i)/(1 - N_1) \tag{2}$$

where n_i is the molecular area of the solvent i, denoted by n^b in Table I in ref. 3. The value obtained is further specified in (8) because two equations are proposed for calculation of $\bar{n}$ (see ref. 3, p. 217).

(3) Calculation of R_i, *i.e.*, the ratio of the molar fractions of solvent i and the preceding solvent $i-1$:

$$R_i = N_i/N_{i-1} \tag{3}$$

(4) Values of the parameters ε_i' and ε_i'' are necessary for the following calculations. The two parameters correspond to strength ε_i of the solvent i when its molar fraction N_i in the mobile phase approaches 0 or 1, respectively. ε_i'' values are included in Table I in ref. 3 together with ε_i' values which are valid if the first solvent has $\varepsilon_1'' = 0$. In contrast, ε_i' values are calculated by means of the following equation:

$$\varepsilon_i' = \varepsilon_{i(\text{Table})}' - [f_1(C)/n_i]\varepsilon_1'' \tag{4}$$

where $\varepsilon_{i(\text{Table})}'$ is the ε' of solvent i given in Table I in ref. 3 and $f_1(C)/n_i$ is a parameter whose values are included in the same table. The calculation of ε' should ensure a value that is not lower than the corresponding ε_i'' value of the same solvent.

(5) The strength ε_i of solvent i in the mobile phase is calculated according to the equation

$$\varepsilon_i = \varepsilon_i'' + \%_{1c(i)}(\varepsilon_i' - \varepsilon_i'') \tag{5}$$

The parameter $\%_{1c}$ is a measure of the localization of solvent i on the adsorbent surface and is arbitrarily assumed to be 50% at the beginning of an iterative procedure.

(6) The equilibrium constant K for adsorption of the solvent i with desorption of the preceding solvent $i-1$ is calculated using the following equation:

$$K_i = 10^{\alpha'\bar{n}(\varepsilon_i - \varepsilon_{i-1})} \tag{6}$$

where α' is an adsorbent activity function which is usually equal to 0.57 for silica and 0.65 for alumina.

(7) The molar fraction in the adsorbed phase of the first solvent θ_1 and the following solvents up to the last one, θ_z, are obtained on the basis of the following equations:

$$\theta_1 = 1/\{1 + R_2K_2[1 + \ldots R_{z-1}K_{z-1}(1 + R_zK_z)]\} \tag{7}$$

$$\theta_z = \theta_{z-1}R_zK_z \tag{8}$$

(8) Specification of parameter $\bar{n}$ is done on the basis of the following equation:

$$\bar{n} = \sum_{i=2}^{z} (\theta_i n_i)/(1 - \theta_i) \tag{9}$$

(9) The parameter $\%_{1c(i)}$ is then calculated:

$$\%_{1c(i)} = (1 - \theta_i)[1/(1 - 0.94\theta_i) - 14.5\theta_i^9] \tag{10}$$

If the value of this parameter is not equal to 50% as assumed at the beginning, then the arithmetic mean of the two values is taken and the calculations return to entry 5. This

iteration continues until the input value of $\%_{1c}$ in entry 5 becomes equal to its output value in entry 9. The current value of θ_i is then registered.

The final value of strength ε of the mobile phase, denoted originally by ε^0, is calculated by means of the following equation:

$$\varepsilon = \varepsilon_1 + \log(N_1/\theta_1)/\alpha'\bar{n} \tag{11}$$

Calculation of localization, m

The calculations are performed according to the equation

$$m = \sum_{i=2}^{z} \left\{ m_i \left[\mathrm{f} \sum_{j=1}^{z} (\theta_j) - \mathrm{f} \sum_{k=i+1}^{z} (\theta_k) \right] \right\} \tag{12}$$

where the localization parameters of any solvent i and the last solvent z are denoted by m_i and m_z, respectively. The values of the localization function $\mathrm{f}(\theta)$ are obtained on the basis of the corresponding θ values using the plot in Fig. 5 in ref. 13.

Calculation of polarity, P'

The polarity parameter, P', of a given mobile phase is calculated by means of volume percentage P_i of each solvent and its own polarity P'_i value:

$$P' = \sum_{i=1}^{z} P_i P'_i \tag{13}$$

The values do not depend on the adsorbent used.

EXPERIMENTAL

A Pravetz-16 microcomputer compatible with an IBM-PC and having a memory of 256 kbytes is used. The microcomputer program is written in Turbo Pascal and registered elsewhere[15].

RESULTS AND DISCUSSION

Description of the microcomputer program

The microcomputer program has three main modes:

(1) organization of a file containing the basic literature data necessary for the calculations;

(2) characterization of mobile phases by values of ε, m and P'; and

(3) choice of equi-eluotropic mobile phases of any desired ε value.

Each mode is described below.

The first mode permits the organization of a file containing the characteristics of pure solvents, namely density d, molecular weight MW, polarity P', molecular area n, solvent strength ε' and ε'', the parameter $f_i(C)/n$ and localization m^0. Such a file was made for 28 pure solvents as the data are taken from Table 6-1 in ref. 2 and Table I in ref. 3, replacing some of them by more recent values in ref. 6. The values of the latter four parameters are different for silica and alumina whose α' values are 0.57 and 0.65,

TABLE I

VALUES OF THE SOLVENT LOCALIZATION FUNCTION $f(\theta_i)$ *VS.* THE FRACTIONAL COVERAGE θ_i OF THE ADSORBENT SURFACE BY THE LOCALIZING SOLVENT (FROM FIG. 5 IN REF. 13)

θ_i	$f(\theta_i)$	θ_i	$f(\theta_i)$	θ_i	$f(\theta_i)$	θ_i	$f(\theta_i)$	θ_i	$f(\theta_i)$
0.00	0.000								
0.01	0.005	0.21	0.118	0.41	0.338	0.61	0.700	0.81	0.930
0.02	0.008	0.22	0.128	0.42	0.350	0.62	0.710	0.82	0.940
0.03	0.011	0.23	0.132	0.43	0.362	0.63	0.730	0.83	0.945
0.04	0.016	0.24	0.142	0.44	0.380	0.64	0.748	0.84	0.948
0.05	0.020	0.25	0.150	0.45	0.395	0.65	0.760	0.85	0.952
0.06	0.025	0.26	0.162	0.46	0.410	0.66	0.780	0.86	0.960
0.07	0.030	0.27	0.170	0.47	0.420	0.67	0.793	0.87	0.965
0.08	0.035	0.28	0.180	0.48	0.440	0.68	0.805	0.88	0.968
0.09	0.038	0.29	0.190	0.49	0.470	0.69	0.820	0.89	0.970
0.10	0.040	0.30	0.200	0.50	0.478	0.70	0.830	0.90	0.975
0.11	0.048	0.31	0.210	0.51	0.495	0.71	0.840	0.91	0.980
0.12	0.052	0.32	0.220	0.52	0.510	0.72	0.850	0.92	0.982
0.13	0.060	0.33	0.235	0.53	0.530	0.73	0.860	0.93	0.985
0.14	0.068	0.34	0.248	0.54	0.550	0.74	0.870	0.94	0.988
0.15	0.072	0.35	0.260	0.55	0.560	0.75	0.880	0.95	0.990
0.16	0.080	0.36	0.270	0.56	0.580	0.76	0.890	0.96	0.992
0.17	0.088	0.37	0.280	0.57	0.600	0.77	0.900	0.97	0.994
0.18	0.095	0.38	0.295	0.58	0.620	0.78	0.910	0.98	0.996
0.19	0.102	0.39	0.308	0.59	0.650	0.79	0.920	0.99	0.998
0.20	0.110	0.40	0.320	0.60	0.680	0.80	0.925	1.00	1.000

respectively. The file also contains the data necessary for calculating the function $f(\theta_i)$ on the basis of the molar fraction in the adsorbed phase θ_i. The data are read from the plot in Fig. 5 in ref. 13 and are presented in Table I. The file is automatically used by the computer during the two calculation modes.

Choosing the second mode, the user inputs the adsorbent and the mobile phase of interest, specifying its solvents and their volume percentages. The computer calculates and prints out the values of the solvent strength, ε, localization, m, and polarity, P', using the algorithm described under Theory.

When the third mode of the program is activated, the user inputs the adsorbent, the solvents of the mobile phases and the desired ε value. The computer calculates and prints out a set of mobile phases composed of the input solvents and the corresponding percentages in which they should be mixed in order to ensure the input ε value. The algorithm used to this end is described below.

(1) To start the calculations, given values of the volume percentage P_i of each solvent are automatically input by computer. A ratio of 50:50 is arbitrarily used for mobile phases composed of two solvents. For the case of more than two solvents, the input value for the first solvent P_1, the last solvent P_z and any other solvent P_i is calculated on the basis of the following equations:

$$P_1 = P_z = (100 - A)/2 \tag{14}$$

$$P_i = A/(Z - 2) \tag{15}$$

where A is a variable parameter equal to 10 at the beginning and Z is the total number of the solvents of the mobile phase. Alternatively, the possibility is given for the user to change the automatically input values of P_i according to his or her own requirements.

(2) Current values of the mobile phase strength, $\varepsilon_{\text{current}}$, and percentages of the solvents, $P_{i(\text{current})}$, are obtained on the basis of the algorithm described under Theory.

(3) The difference between the input value and the current value of the solvent strength ε is found:

$$D = \varepsilon_{\text{input}} - \varepsilon_{\text{current}} \tag{16}$$

(4) If the value of D differs from zero, then the percentages of the first and the last solvents only are changed. As the last solvent is the strongest of all solvents of the mobile phase, it has the greatest effect on mobile phase strength, ε. It is assumed that ε is proportional to the percentage of the last solvent raised to the power of 1/5 because the plot of such a relationship has a steep part at the beginning followed by a part with small changes. To find the new percentage of the last solvent P_z which is necessary to continue the calculations, the following equations are written:

$$\varepsilon_{\text{current}} = kP_{z(\text{current})}^{1/5} \tag{17}$$

$$\varepsilon_{\text{input}} = kP_z^{1/5} \tag{18}$$

where k is a constant.

The ratio between these two equations leads to the following equation:

$$P_z = P_{z(\text{current})}(\varepsilon_{\text{input}}/\varepsilon_{\text{current}})^5 \tag{19}$$

Then the percentage of the first solvent P_1 is found from the equation

$$P_1 = P_{1(\text{current})} + [P_{z(\text{current})} - P_z] \tag{20}$$

(5) Using the values of P_1 and P_z found without any change in the percentages of the remaining solvents, the calculations return to entry 2. The iterative procedure from entry 2 to entry 4 continues until the difference D becomes equal to zero, which means that the input ε value is obtained. Then the current percentage of each solvent in which we are interested is printed out, together with the values of localization, m, and polarity, P', calculated by means of eqns. 12 and 13, respectively.

The calculations in the third mode of the microcomputer program continue further with repeated increases of 10 in the value of the variable parameter A, which leads to a set of mobile phases composed of the solvents initially specified by the user but with different proportions. However, all mobile phases of this set have the input strength $\varepsilon_{\text{input}}$.

Application of the microcomputer program

The microcomputer program was applied to calculate the values of ε, m and P' of approximately 250 mobile phases, 92 of which had already been characterized by Snyder and co-workers[3,11–13] by their calculated and experimental ε or m values. The

TABLE II

COMPUTER ε, m AND P' VALUES OF MOBILE PHASES COMPOSED OF TWO TO THREE SOLVENTS: COMPARISON WITH LITERATURE $\varepsilon_{calc.}$ AND $\varepsilon_{exptl.}$ VALUES

No.	*Mobile phase*	*Proportions (%, v/v)*	*Computer values*			*Literature values*		
			m	*P'*	ε	$\varepsilon_{calc.}$	$\varepsilon_{exptl.}$	*Ref.*
Silica as adsorbent								
1	Hexane–diethyl ether	95.95:4.05	0.56	0.21	0.207	0.207	0.222	12, Table II
2		91.82:8.18	0.61	0.32	0.243	0.243	0.248	
3		83.3:16.7	0.63	0.55	0.277	0.277	0.279	
4		65.16:34.84	0.65	1.04	0.316	0.316	0.308	
5		34.82:65.18	0.66	1.86	0.378	0.379	0.358	
6	Benzene–diethyl ether	97.65:2.35	0.03	2.70	0.264	0.267	0.216	
7		77.26:22.74	0.37	2.72	0.348	0.358	0.351	
8		26.69:73.31	0.64	2.77	0.406	0.404	0.409	
9	Hexane–ethyl acetate	98.48:1.52	0.53	0.17	0.204	0.204	0.185	
10		84.12:15.88	0.59	0.78	0.302	0.302	0.318	
11		36.21:63.79	0.60	2.84	0.436	0.433	0.444	
12	Benzene–ethyl acetate	97.79:2.21	0.04	2.74	0.267	0.267	0.259	
13		78.31:21.69	0.44	3.07	0.362	0.361	0.356	
14	Hexane–acetone	98.86:1.14	0.79	0.16	0.215	0.242	0.238	
15		87.6:12.4	0.92	0.72	0.337	0.346	0.332	
16		43.08:56.92	0.95	2.95	0.475	0.475	0.477	
17	Benzene–acetone	98.33:1.67	0.08	2.74	0.281	0.292	0.300	
18		82.8:17.2	0.77	3.11	0.416	0.421	0.415	
19		64.35:35.65	0.88	3.56	0.458	0.476	0.463	
20	Hexane–dioxane	85.87:14.13	–	0.76	0.323	0.324	0.340	
21		39.44:60.56	–	2.95	0.471	0.471	0.477	
22	Benzene–dioxane	80.55:19.45	–	3.11	0.429	0.428	0.445	
23		30.74:69.26	–	4.16	0.474	0.473	0.498	
24	Benzene–acetonitrile	87.12:12.88	0.50	3.10	0.367	0.388	0.373	
25		42.01:57.99	1.02	4.50	0.475	0.477	0.488	
26	Hexane–isopropanol	72.3:27.7	–	1.15	0.459	0.477	0.458	
27	Hexane–methylene chloride	59.82:40.18	0.10	1.31	0.223	0.223	0.223	11, Table III
28	Hexane–methyl *tert.*-butyl ether	96.14:3.86	0.72	–	0.249	0.223	0.226	
29	Hexane–methylene chloride–methyl *tert.*-butyl ether	82.9:16.03:1.07	0.41	–	0.260	0.223	0.229	
Alumina as adsorbent								
30	Pentane–dioxane	90:10	–	0.48	0.388	0.383	0.372	3, Table V
31		50.05:49.95	–	2.40	0.549	0.547	0.549	
32	Pentane–pyridine	89.98:10.02	1.22	0.53	0.476	0.465	0.435	
33		75.01:24.99	1.22	1.32	0.572	0.566	0.565	
34	Pentane–benzene–methylene chloride	60:30:10	0.01	1.12	0.266	0.266	0.220	11, Table I
35		30:30:40	0.24	2.05	0.345	0.344	0.320	
36	Pentane–benzene–dioxane	60:38:2	–	1.12	0.366	0.365	0.350	
37		60:35:5	–	1.19	0.402	0.404	0.400	
38		60:30:10	–	1.29	0.429	0.431	0.440	
39	Pentane–methylene chloride–dioxane	60:38:2	–	1.27	0.382	0.382	0.390	
40		60:35:5	–	1.33	0.442	0.439	0.430	
41		60:30:10	–	1.41	0.472	0.472	0.470	
42		60:20:20	–	1.58	0.490	0.493	0.520	

TABLE III

COMPUTER ε, m AND P' VALUES OF MOBILE PHASES COMPOSED OF TWO TO FOUR SOLVENTS: COMPARISON WITH LITERATURE $m_{calc.}$ AND $m_{exptl.}$ VALUES

No.	*Mobile phase*	*Proportions (%, v/v)*	*Computer values*			*Literature values*	
			ε	P'	m	$m_{calc.}$	$m_{exptl.}$
Silica as adsorbent[a]							
43	Hexane–methylene chloride	59.82:40.18	0.223	1.31	0.10	0.10	0.09
44	Hexane–chloroform	35.05:64.95	0.225	2.70	0.10	0.10	0.05
45	Hexane–methyl *tert.*-butyl ether	95.95:4.05	0.252	–	0.72	0.74	0.81
46	Hexane–methylene chloride–methyl *tert.*-butyl ether	83.19:15.37:1.44	0.278	–	0.49	0.36	0.44
47	Hexane–chloroform–methylene chloride	46.8:34.48:18.72	0.225	2.04	0.10	0.10	0.16
48	Hexane–chloroform–methylene chloride–methyl *tert.*-butyl ether	89.08:4.61:2.53:3.78	0.293	–	0.71	0.71	0.60
49	Hexane–methyl *tert.*-butyl ether	97.98:2.02	0.216	–	0.67	0.68	0.71
50		91.97:8.03	0.284	–	0.76	0.77	0.66
51	Hexane–methylene chloride–acetonitrile	92.48:5.02:2.5	0.302	0.39	0.84	0.93	0.85
52	Hexane–methylene chloride–acetonitrile	90.95:5.02:4.03	0.336	0.48	0.90	1.02	0.83
53	Hexane–methyl *tert.*-butyl ether	96.14:3.86	0.249	–	0.72	0.73	0.68
54	Hexane–methylene chloride–acetonitrile	71.8:28.04:0.16	0.222	0.95	0.17	0.13	0.16
Alumina as adsorbent[b]							
55	Pentane–acetonitrile	99.9:0.1	0.126	0.01	0.50	0.45	0.39
56		99.86:0.14	0.152	0.01	0.65	0.57	0.46
57		99.7:0.3	0.207	0.02	0.93	0.85	0.87
58		99.6:0.4	0.225	0.02	1.00	0.91	0.95
59		99.4:0.6	0.248	0.04	1.07	0.94	0.98
60		99.3:0.7	0.257	0.04	1.09	1.04	1.09
61	Pentane–pyridine	98:2	0.304	0.11	1.20	1.14	1.16
62		95:5	0.399	0.27	1.21	1.17	1.14
63	Pentane–acetone	99.8:0.2	0.133	0.01	0.61	0.58	0.42
64		99.6:0.4	0.171	0.02	0.80	0.74	0.79
65		99.4:0.6	0.192	0.03	0.85	0.80	0.77
66		99.2:0.8	0.205	0.04	0.88	0.83	0.91
67	Pentane–tetrahydrofurane	98:2	0.194	0.08	0.74	0.72	0.73
68		95:5	0.240	0.20	0.78	0.77	0.77
69	Pentane–triethylamine	95:5	0.188	0.10	0.78	0.77	0.77
70	Pentane–ethyl acetate	99:1	0.171	0.04	0.67	0.65	0.65
71		96:4	0.255	0.18	0.74	0.72	0.72
72	Pentane–diethyl ether	98:2	0.070	0.06	0.19	0.26	0.32
73		95:5	0.128	0.14	0.42	0.47	0.55
74		91:9	0.168	0.25	0.52	0.53	0.47
75		77:23	0.231	0.64	0.59	0.58	0.43
76	Pentane–1,2-dichloroethane	85:15	0.244	0.53	0.34	0.33	0.33
77	Pentane–chloroform	85:15	0.179	0.62	0.31	0.30	0.23
78		70:30	0.246	1.23	0.33	0.33	0.41
79	Pentane–methylene chloride	87:13	0.193	0.40	0.26	0.25	0.25
80		77:23	0.253	0.71	0.28	0.27	0.26
81		65:35	0.298	1.09	0.29	0.28	0.33

TABLE III (*continued*)

No.	*Mobile phase*	*Proportions (%, v/v)*	*Computer values*			*Literature values*	
			ε	P'	m	$m_{calc.}$	$m_{exptl.}$
82		40:60	0.354	1.86	0.29	0.29	0.22
83	Pentane–ethyl sulphide	92:8	0.141	–	0.23	0.20	0.18
84		85:15	0.191	–	0.27	0.25	0.27
85	Pentane–chlorobenzene	70:30	0.207	0.81	0.12	0.12	0.12
86	Pentane–bromoethane	60:40	0.253	0.80	0.08	0.08	0.08
87	Pentane–tetrachloromethane	50:50	0.117	0.80	−0.08	−0.08	−0.08
88	Pentane–benzene	85:15	0.158	0.41	−0.14	−0.13	−0.04
89		72:28	0.210	0.76	−0.15	−0.14	−0.02
90		50:50	0.261	1.35	−0.15	−0.15	−0.25
91		20:80	0.302	2.16	−0.15	−0.15	−0.29
92	Pentane–toluene	70:30	0.197	0.72	−0.16	−0.15	−0.15

[a] Literature values from ref. 13 (Table V).
[b] Literature values from ref. 13 (Table II).

TABLE IV

AGREEMENTS WITHIN THE COMPUTER-CALCULATED VALUES OF ε OR m, AND CORRESPONDING CALCULATED AND EXPERIMENTAL VALUES REPORTED BY SNYDER AND CO-WORKERS FOR THE MOBILE PHASES PRESENTED IN TABLES II AND III

Compared values[a]	*S.D.*	*Compared values*[a]	*S.D.*
$\varepsilon_{comp.}$ *vs.* $\varepsilon_{calc.}$	0.010	$m_{comp.}$ *vs.* $m_{calc.}$	0.05
$\varepsilon_{comp.}$ *vs.* $\varepsilon_{exptl.}$	0.018	$m_{comp.}$ *vs.* $m_{exptl.}$	0.08
$\varepsilon_{calc.}$ *vs.* $\varepsilon_{exptl.}$	0.017	$m_{calc.}$ *vs.* $m_{exptl.}$	0.07

[a] Abbreviations of subscripts: comp. = computer-calculated values; calc. = literature calculated values; exptl. = literature experimental values.

TABLE V

COMPUTER ε, m AND P' VALUES OF SOME SETS OF MOBILE PHASES COMPOSED OF 2–5 SOLVENTS

In each column of three values, ε is the upper value, m is the middle value and P' is the lower value.

Set No.	*Volume percentage x of the most polar solvent of the indicated set of mobile phases*										
	1	*5*	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*
Silica as adsorbent											
93	Pentane–ethyl acetate [(100−x):x]										
	0.183	0.246	0.274	0.310	0.340	0.370	0.398	0.423	0.442	0.458	0.470
	0.51	0.56	0.58	0.59	0.59	0.60	0.60	0.60	0.60	0.60	0.60
	0.04	0.22	0.44	0.88	1.32	1.76	2.20	2.64	3.08	3.52	3.96
94	Pentane–methyl *tert.*-butyl ether [(100−x):x]										
	0.168	0.256	0.289	0.321	0.340	0.356	0.374	0.397	0.423	0.447	0.467
	0.56	0.73	0.76	0.79	0.80	0.80	0.81	0.81	0.82	0.82	0.82
	—										

(Continued on p. 244)

TABLE V (*continued*)

Set No.	*Volume percentage x of the most polar solvent of the indicated set of mobile phases*										
	1	*5*	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*
95	Pentane–methylene chloride–methyl *tert.*-butyl ether [(90 − *x*):10:*x*]										
	0.224	0.322	0.357	0.390	0.409	0.422	0.433	0.442	0.452	0.461	
	0.43	0.70	0.75	0.78	0.78	0.79	0.80	0.80	0.81	0.81	
	–										
96	Chloroform–diethyl ether [(100 − *x*):*x*]										
	0.265	0.282	0.302	0.335	0.362	0.380	0.393	0.401	0.405	0.409	0.417
	0.01	0.05	0.12	0.27	0.43	0.52	0.57	0.61	0.63	0.64	0.65
	4.09	4.04	3.97	3.84	3.71	3.58	3.45	3.32	3.19	3.06	2.93
97	Chloroform–acetone [(100 − *x*):*x*]										
	0.275	0.325	0.369	0.420	0.446	0.462	0.472	0.481	0.490	0.503	0.518
	0.04	0.24	0.47	0.75	0.84	0.88	0.90	0.92	0.93	0.94	0.95
	4.11	4.15	4.20	4.30	4.40	4.50	4.60	4.70	4.80	4.90	5.00
98	Methylene chloride–ethyl acetate [(100 − *x*):*x*]										
	0.302	0.311	0.322	0.343	0.362	0.380	0.398	0.415	0.432	0.448	0.464
	0.01	0.02	0.07	0.16	0.28	0.41	0.49	0.54	0.57	0.59	0.59
	3.11	3.17	3.23	3.36	3.49	3.62	3.75	3.88	4.01	4.14	4.27
99	Methylene chloride–methyl *tert.*-butyl ether–ethyl acetate–isopropanol [(80 − *x*):10:10:*x*]										
	0.384	0.402	0.423	0.459	0.489	0.516	0.540	0.561	0.581		
	–										
	–										
100	Diethyl ether–acetonitrile [(100 − *x*):*x*]										
	0.432	0.440	0.449	0.464	0.476	0.485	0.493	0.500	0.506	0.511	0.516
	0.01	0.06	0.15	0.37	0.59	0.82	0.91	0.98	1.01	1.03	1.04
	2.83	2.95	3.10	3.40	3.70	4.00	4.30	4.60	4.90	5.20	5.50
101	Tetrahydrofuran–acetonitrile [(100 − *x*):*x*]										
	0.481	0.483	0.486	0.492	0.497	0.501	0.505	0.509	0.512	0.515	0.518
	0.01	0.04	0.09	0.22	0.40	0.59	0.80	0.90	0.98	1.02	1.04
	4.02	4.09	4.18	4.36	4.54	4.72	4.90	5.08	5.26	5.44	5.62
Alumina as adsorbent											
102	Pentane–toluene–pyridine [(90 − *x*):10:*x*]										
	0.306	0.408	0.477	0.551	0.592	0.620	0.641	0.658	0.671	0.682	
	1.15	1.20	1.21	1.22	1.22	1.22	1.22	1.22	1.22	1.22	
	0.29	0.51	0.77	1.30	1.83	2.36	2.89	3.42	3.95	4.48	
103	Pentane–diethyl ether–methylene chloride–acetone [(80 − *x*):10:10:*x*]										
	0.388	0.467	0.496	0.523	0.539	0.550	0.559	0.566	0.573		
	0.75	0.94	0.97	0.99	1.00	1.00	1.00	1.01	1.01		
	0.64	0.85	1.10	1.61	2.12	2.63	3.14	3.65	4.16		
104	Pentane–diisopropyl ether [(100 − *x*):*x*]										
	0.010	0.044	0.076	0.122	0.153	0.177	0.197	0.215	0.233	0.250	0.266
	–										
	0.02	0.12	0.24	0.48	0.72	0.96	1.20	1.44	1.68	1.92	2.16
105	Pentane–diisopropyl ether–chloroform [(90 − *x*):10:*x*]										
	0.091	0.135	0.174	0.226	0.261	0.286	0.306	0.323	0.336	0.348	
	–										
	0.28	0.45	0.65	1.06	1.47	1.88	2.29	2.70	3.11	3.52	
106	Pentane–diisopropyl ether–chloroform–tetrahydrofuran [(80 − *x*):10:10:*x*]										
	0.265	0.346	0.378	0.410	0.430	0.446	0.461	0.473	0.485		
	–										
	0.69	0.85	1.05	1.45	1.85	2.25	2.65	3.05	3.45		
107	Pentane–diisopropyl ether–chloroform–tetrahydrofuran–acetonitrile [(70 − *x*):10:10:10:*x*]										
	0.597	0.655	0.675	0.687	0.689	0.688	0.685	0.682			
	–										
	1.11	1.34	1.63	2.21	2.79	3.37	3.95	4.53			

data obtained by means of the second mode of the microcomputer program together with the corresponding literature data are presented in Tables II and III and were used for some comparisons, summarized in Table IV. Table V shows the data obtained for the remaining mobile phases that had not been studied previously and Fig. 1 presents their ε values in nomograph form. We shall discuss the previously made[16,17] choice of mobile phases for given TLC separations by means of the third mode of the microcomputer program at the end of the paper.

It is noteworthy that the calculation of "mean" mobile phase area $\bar{n}$ based on eqns. 2 and 9 leads to the values of ε and m as presented in Tables II, III and V which were very close to those obtained when eqn. 9 was excluded from the calculation procedure. The agreement between these two types of values was ± 0.012 units in ε and ± 0.03 units in m. This shows the accuracy of eqn. 2.

The mobile phases included in Tables II and III were selected from about 400 mobile phases studied so far[1–3,5,6,10–13,16,17] by means of Snyder's theory.

The 42 mobile phases presented in Table II are composed of two or three of the solvents pentane, hexane, benzene, methylene chloride, diethyl ether, methyl *tert.*-butyl ether, ethyl acetate, acetone, dioxane, acetonitrile, isopropanol and pyridine. Mobile phases 1–29 and 30–42 have been used for separations on silica and alumina, respectively, and characterized by calculated and experimental literature values of ε. The former values were obtained by means of the algorithm described under Theory but with some differences in the input values of the solvent molecular area n, solvent strength ε' and ε'' and localization parameter $f_1(C)/n$.

Table III includes 50 mobile phases composed of two to four of the solvents pentane, hexane, chloroform, methylene chloride, 1,2-dichloroethane, ethyl sulphide, bromoethane, tetrachloromethane, chlorobenzene, benzene, toluene, diethyl ether, methyl *tert.*-butyl ether, acetone, ethyl acetate, tetrahydrofuran, acetonitrile, triethylamine and pyridine. Mobile phases 43–54 and 55–92 have been used for separations on silica and alumina, respectively, and characterized by literature calculated and experimental values of m. The former values were obtained using eqn. 12 and m^0 values which differ in some instances from those recently reported and used in this study.

Let us discuss the comparisons within the various values of ε or m presented in the corresponding three columns in Tables II and III. According to Table IV, the agreement between the computer values and literature calculated values is very good, especially for the ε values, where S.D. $= \pm 0.01$. This means that the calculation procedures presented under Theory are properly incorporated in the microcomputer program. In addition, the process of specifying the values of the parameters $n, f_1(C)/n$ and m^0 for pure solvents is successful but still requires further attention.

Table IV also shows the important fact that both the computer and literature calculated values agree equally well with the literature experimental values. The agreement is again better for the ε values, with S.D. $\approx \pm 0.02$ units.

To generalize, the data discussed here and the other data reported so far show agreement between the calculated and experimental values within ± 0.02 units for ε and ± 0.08 units for m. This conclusion is based on hundreds of HPLC and TLC separations performed on different types of silica or alumina using different types of solutes and approximately 400 mobile phases composed of two to six solvents. The experimental data were mainly obtained by Snyder and co-workers, but also by Hara

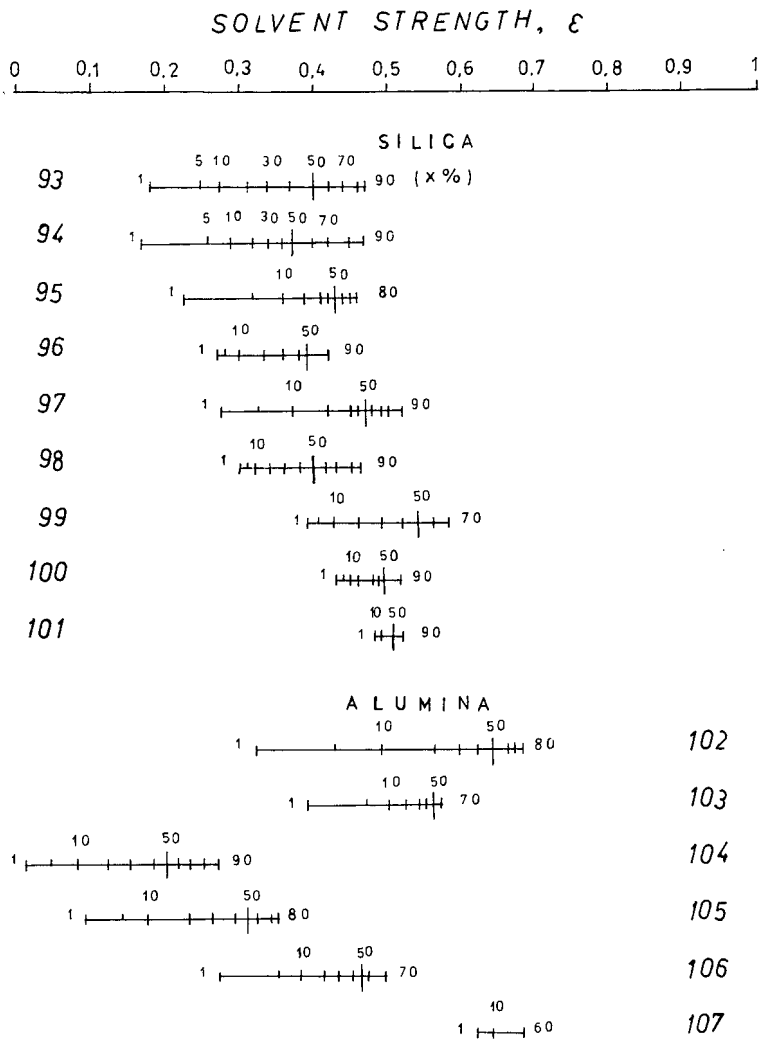

Fig. 1. Nomogram form expressing the solvent strength, ε, of sets of mobile phases 93–107 as a function of the volume percentage x of the most polar solvent. The composition of these sets is as follows: 93 = pentane–ethyl acetate [(100−x):x]; 94 = pentane–methyl *tert.*-butyl ether [(100−x):x]; 95 = pentane–methylene chloride–methyl *tert.*-butyl ether [(90−x):10:x]; 96 = chloroform–diethyl ether [(100−x):x]; 97 = chloroform–acetone [(100−x):x]; 98 = methylene chloride–ethyl acetate [(100−x):x]; 99 = methylene chloride–methyl *tert.*-butyl ether–ethyl acetate–isopropanol [(80−x):10:10:x]; 100 = diethyl ether–acetonitrile [(100−x):x]; 101 = tetrahydrofuran–acetonitrile [(100−x):x]; 102 = pentane–toluene–pyridine [(90−x):10:x]; 103 = pentane–diethyl ether–methylene chloride–acetone [(80−x):10:10:x]; 104 = pentane–diisopropyl ether [(100−x):x]; 105 = pentane–diisopropyl ether–chloroform [(90−x):10:x]; 106 = pentane–diisopropyl ether–chloroform–tetrahydrofuran [(80−x):10:10:x]; 107 = pentane–diisopropyl ether–chloroform–tetrahydrofuran–acetonitrile [(70−x):10:10:10:x]. Characterization of given mobile phases from the sets by values of ε, m and P' is presented in Table V.

and co-workers (see ref. 12) and ourselves[4,6,16,17]. It should be mentioned that m is better expressed by m^0 of the most polar solvent instead of by the corresponding calculated values in a given study[6], but this seems to be a separate case[16]. Consequently, the data available show unequivocally the ability of Snyder's theory to predict the solvent strength and selectivity of mobile phases in normal-phase liquid–solid chromatography (LSC).

As seen under Theory, ε of a given mobile phase is a function of the molar fractions of individual solvents. Hence, a considerable part of the data are reported together with corresponding molar fractions (*e.g.*, see ref. 12) instead of with the convenient to use volume percentages. The 153 mobile phases in Table V characterized for the first time by calculated values of ε, m and P' belong to fifteen sets as the ratio among the solvents is expressed in percentages as in Tables II and III. The data of sets 93–101 refer to silica and those of sets 102–107 are valid for alumina. Each set is composed of two to five solvents and the percentage of the last solvent increases with the corresponding decrease in that of the first solvent. Table V shows that the values of ε and m increase within any set because the last solvent has the greatest individual values of these two parameters. A slight deviation in ε values for the last three mobile phases of set 107 is observed.

All mobile phases studied show a great difference in their TLC properties as the computer-calculated values of ε, m and P' vary within the ranges 0.010–0.682, -0.16 to 1.22 and 0.01–5.50, respectively (see Tables II, III and V). The mobile phases characterized can easily be used because they are composed of practically important solvents and cover a considerable part of the experimentally accessible ranges of the three parameters. The plot in Fig. 1 is a nomograph presentation of the ε values in Table V which enables mobile phases of intermediate ε values which are not included in the table to be found.

Although the microcomputer program is universal for normal-phase LSC, we applied it first[16,17] to choose mobile phases of constant ε value for optimizing TLC separations of different diastereoisomers on silica. (Note: eqn. 9 was not yet incorporated in the program.) Thus, 41 mobile phases having three different ε values and composed of two to six solvents were found by means of the third mode of the microcomputer program. The corresponding experimental R_F values of the three groups of compounds studied vary within the ranges 0.12–0.82, 0.11–0.89 and 0.01–0.75, independently of the number and nature of solvents included in their composition and independently of the presence of complicating factors such as the formation of a second front in some instances. The agreement between the computer and experimental ε values is within ± 0.016 units, which shows for the first time the applicability of Snyder's theory to characterizing mobile phases composed of more than four solvents.

The above-mentioned scatter of the experimental R_F values, when there is no second front formation, is probably due to the fact that the mobile phases used for a given group of compounds have different P' values in spite of their constant ε value. The tendency found[16,17] for an increase in R_F values with an increase in P' values is reasonable, bearing in mind that a higher P' value means a better solution of a solute in the corresponding mobile phase. Concerning HPLC, another example for the importance of P' can be given. Snyder and Glajch have established a surprisingly poorer agreement of ± 0.043 units between calculated and experimental values of ε in the case

of some acetonitrile-containing mobile phases (see Table IV and related discussion of ref. 12). The data show a tendency for higher $\varepsilon_{exptl.}$ values which can be explained by the fact that acetonitrile has the highest P' value (5.8) among all solvents investigated. Thus, solvent strength is mainly determined by ε but influenced by P' too. Consequently, polarity P' appears to be a co-parameter in determining solvent strength in normal-phase LSC (*cf.* ref. 2, p. 258).

CONCLUSION

The parameters solvent strength, ε, localization, m, and polarity, P', proposed by Snyder characterize well the solvent strength and selectivity effects of mobile phases for normal-phase LSC techniques such as HPLC and TLC using silica or alumina as adsorbents. The microcomputer program described calculates in a few seconds the values of the three parameters for mobile phases composed of two to several solvents and finds equi-eluotropic mobile phases of any desired ε value. This permits the easy application of Snyder's useful theory in every-day chromatographic practice.

ACKNOWLEDGEMENT

We express our great thanks to Dr. L. R. Snyder for his generous support of this study with various examples of calculations, very helpful comments on the manuscript and editorial notes.

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Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF SMALL MOLECULES: CHARACTERIZATION OF THE RETENTION OF ACYL COENZYME A HOMOLOGUES

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SUMMARY

The hydrophobic interaction chromatography (HIC) of C_2–C_{10} acyl coenzyme A (CoA) homologues was investigated in the isocratic mode. Retention of the homologues and of CoA fragments is well described by the empirical salting-out equation, $\log k' = \log k'_0 + mM_s$. Both slope and intercept in this equation increase with carbon number of the homologous series. Simple additivity with carbon number was not found for either slope or intercept. Each additional methylene group had a greater effect on increasing retention than the preceding methylene group. Comparison of the retention of structural fragments of CoA to that of acyl CoA homologues shows that the alkyl moiety of the homologues dominates their retention. The average free energy of transfer of a methylene group from water to a propyl HIC stationary phase was estimated as -280 cal/mol. The dependence of this free energy of transfer on salt concentration is found to be -50 cal/mol per 1 M increase in ammonium sulfate concentration.

INTRODUCTION

Small-molecule investigations in hydrophobic interaction chromatography (HIC) are uncommon since HIC is usually associated with protein separations. Although HIC has proven to be useful in separating proteins under stabilizing conditions, the retention mechanism(s) are not well understood. A study of the retention parameters of smaller molecules should provide a simpler interpretable system.

Although homologous series have been used extensively in reversed-phase chromatographic studies, there have been no reports of analogous studies in HIC. A homologous series of acyl coenzyme A (CoA) derivatives provides a useful means for probing the effect(s) of analyte structure on retention in HIC.

A linear increase has been reported in the log of the capacity factor (k') with increasing carbon number for several reversed-phase systems[1–4]. These observations have given rise to empirical models relating analyte structure to retention[2,5–9]. The idea that retention parameters of structural components of an analyte are additive in

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the retention of the entire analyte molecule is not new. The Martin rule of additivities of molecular increments[10,11] has been extensively applied to predict retention of an analyte based on its structural characteristics[12–14]. For example, quantitative structure–retention relationships (QSRR) based on this premise are widely applied in drug development as a means of characterizing molecular structure of analytes[15,16].

The present studies were carried out with a typical HIC stationary phase, using solvent compositions and operating conditions that are conventionally applied in HIC. Systematic capacity factor determinations are reported for several acyl CoA homologues as a function of ammonium sulfate concentration in the mobile phase. Additivity of the log of the capacity factor with increasing carbon number is not found for this series. The influence of ammonium sulfate concentration in the mobile phase on the free energy of transfer of a structural unit, the methylene group, to the stationary phase is discussed.

EXPERIMENTAL

Materials

Acetyl, *n*-propionyl, *n*-butyryl and *n*-hexanoyl CoA were obtained as their lithium salts from Sigma (St. Louis, MO, U.S.A.). *n*-Octanoyl and *n*-decanoyl CoA were obtained in the free acid form from Sigma and PL Biochemical (Milwaukee, WI, U.S.A.), respectively. CoA was obtained as its lithium salt from Pharmacia (Piscataway, NJ, U.S.A.). Adenosine and adenosine 5′-monophosphoric acid were obtained from Sigma. Ammonium sulfate (ultrapure grade) was obtained from Schwarz-Mann Biotech (Cleveland, OH, U.S.A.). All other reagents were of A.C.S. certified analytical-reagent grade.

Methods

The chromatographic system consisted of two Waters Model M6000A pumps, a Rheodyne Model 7125 injection valve, a 6.5-μm, 150 × 4.6 mm I.D. SynChropak propyl hydrophobic interaction column (SynChrom, Lafayette, IN, U.S.A.), and a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A reporting integrator. The column dead time was determined by water injection.

Mobile phases were prepared with high-purity high-performance liquid chromatography (HPLC)-grade water obtained in-house with a Millipore (Bedford, MA, U.S.A.) Milli-Q water purification system as follows: mobile phase A: 2.1 *M* ammonium sulfate, 0.02 *M* potassium dihydrogen-phosphate, adjusted to pH 7.0 with a sodium hydroxide solution; mobile phase B: 0.02 *M* potassium dihydrogen phosphate, adjusted to pH 7.0 with a sodium hydroxide solution.

The composition of the mobile phase was controlled by a Waters Model 660 solvent programmer. Stock solutions of each CoA homologue were prepared by using high-purity HPLC-grade water (unbuffered) at a concentration *ca.* 0.2 mg/ml. Mobile phases and stock solutions were filtered through a Millipore HA (0.45 μm) filter and stored at 4°C when not in use. A 20-μl injection loop was used for all injections (approximately 4 μg per injection). The acyl CoA homologues were detected at 260 nm using a Waters Lambda-Max Model 480 spectrophotometer. The flow-rate was 1.0 ml/min throughout the study. The chromatographic column was maintained at 30 $\pm$ 0.2°C with a circulating-water jacket.

RESULTS AND DISCUSSION

It has been proposed that in HIC the log of the capacity factor, k', is linearly related to the surface tension (σ) of the mobile phase[17]

$$\log k' = A + B\sigma \tag{1}$$

and that surface tension is a linear function of salt concentration, M_s[18]

$$\sigma = \sigma_o + tM_s \tag{2}$$

Therefore, the relationship of log k' to ammonium sulfate concentration is given by eqn. 3

$$\log k' = \log k'_0 + mM_s \tag{3}$$

This equation is of the same form as the Setschenow equation for the salting out of non-polar compounds from aqueous solution[19], and presumably reflects a similar mechanism. The coefficient m is presumed to reflect the contact area between the analyte and the stationary phase.

For each of the six CoA derivatives, isocratic HIC data were acquired with mobile phases ranging in concentration from 2.1 to 0.0 M ammonium sulfate. A plot of eqn. 3 for each of the CoA derivatives is shown in Fig. 1.

Initially, the effect of added salt at low salt concentrations on ionized species is to reduce electrostatic interaction between the analytes and the stationary phase[20]. Retention of highly negatively charged molecules like acyl CoA homologues will probably involve substantial electrostatic interaction at very low salt concentrations.

Intercept (log k'_0) and slope (m) values for each acyl CoA homologue obtained from the lines of best fit of log k' *versus* ammonium sulfate concentration (eqn. 3) are plotted against carbon number of the homologue (Figs. 2 and 3). The values of log k' at

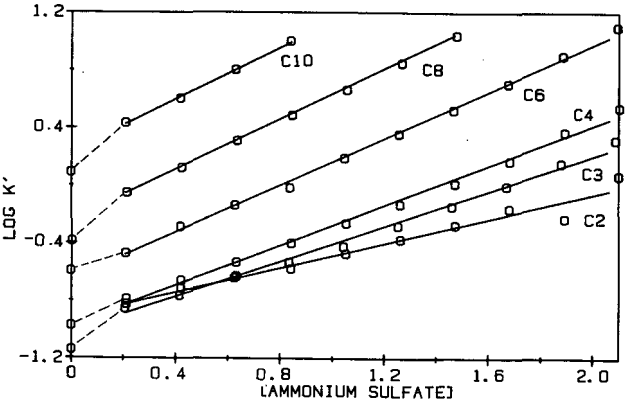

Fig. 1. Retention of acyl CoA derivatives on a SynChropak propyl column as a function of ammonium sulfate concentration. The carbon number of the acyl group identifies each curve. Each point represents the average of 3 or 4 t_R measurements.

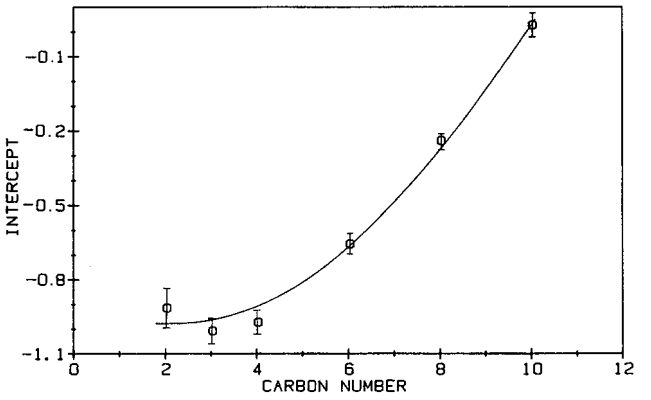

Fig. 2. Intercepts from log k' *versus* $[(NH_4)_2SO_4]$ plot, Fig. 1, as a function of carbon number in the acyl group. Error brackets indicate the 95% confidence interval, computed on the conservative premise that each point in Fig. 1 represents a single observation.

zero ammonium sulfate concentration were omitted from regression analysis because of the electrostatic considerations mentioned above. In recent versions of Sinanoglu's solvophobic theory[21], the slope (B) of eqn. 1 is taken to measure the change in solvent-exposed non-polar area on binding; the slope m eqn. 3 may be likewise identified. Fig. 2 and 3 show a kind of complementarity: slopes change most rapidly with carbon number in the low end of the range where intercept is statistically invariant, and intercepts change rapidly at the high end of the range where slope appears to approach a limiting value. Without providing any details, these results suggest some difference in the kind of interaction for the low carbon number and the high carbon number homologues with the stationary phase. The trends shown in Figs. 2 and 3 show that simple additivity is not found in slope or intercept.

Isocratic log k' values for each of the CoA derivatives were plotted *versus* carbon number (Fig. 4). Above carbon number 3 these plots yield approximately linear

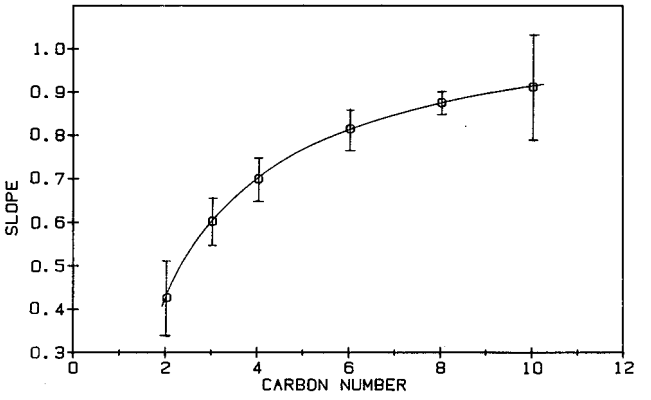

Fig. 3. Slopes from log k' *versus* $[(NH_4)_2SO_4]$ plot, Fig. 1, as a function of carbon number in the acyl group. Error brackets indicate the 95% confidence interval as in Fig. 2.

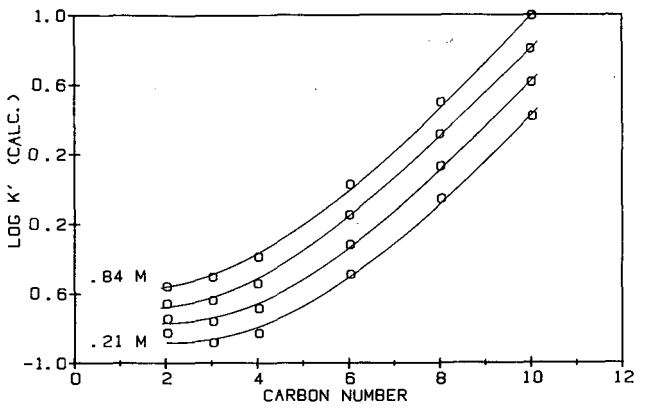

Fig. 4. Variation of k' (data from Fig. 1 smoothed by linear regression) with carbon number, at four salt concentrations. The successive curves, from the bottom up, represent isocratic data at 0.21, 0.42, 0.63, and 0.84 M $(NH_4)_2SO_4$, respectively.

relationships (Fig. 4, $r > 0.997$ for all plots, $n = 4$). A linear dependence of the log of the capacity factor on the number of repeating molecular units in a homologous series is well established in reversed-phase chromatography[1,22,23].

Closer inspection of Fig. 4, however, reveals that the log k' values (including carbon numbers 2–4) are better related to carbon number by a second order polynomial. The first few members of a homologous series may not contribute equally to retention because of end effects caused by the spatial proximity of the first few methylene groups to the thioester moiety of the homolog[1]. Other examples of a non-linear relationship between retention and carbon number have been reported[15,24,25]. Studies in reversed-phase chromatography by Jandera[26] have shown that while linear fits are often quite good for a homologous series, more generality is achieved with a quadratic formulation. He derived the following equation relating log k' to carbon number from interaction index theory[9]:

$$\log k' = \log \beta + (\log \alpha)n_c + (\log \gamma)n_c^2 \quad (4)$$

The term α represents the retention ratio between two adjacent members of a homologous series. In a reversed-phase study of a homologous series, Colin *et al.*[5] defined log α as "solvophobicity" and found that this parameter varied almost linearly with the water content in a methanol–water mobile phase system. Analogously in this hydrophobic interaction study, "solvophobicity" is related to the salt concentration in the mobile phase. The intercept, log β, is a measure of the specific selectivity in a homologous series[26] and represents the interaction between the acyl CoA molecule ($n_c = 0$) with the stationary phase. The coefficient of the squared term, log γ, is usually insignificant enough to ignore in reversed-phase systems so that log k' is related to carbon number by the linear relationship[21]

$$\log k' = \log \beta + (\log \alpha)n_c \quad (5)$$

The curvature in the plot of log k' *versus* carbon number (Fig. 4) indicates that the slope (log α), which is a measure of methylene selectivity, increases with the carbon number of the series. Also, as the salt concentration in the mobile phase increases, log β increases (Table I), reflecting the increased retention contributed by the acyl CoA moiety ($n_c = 0$) at higher salt concentrations. It is evident from Fig. 4 that in HIC (for this homologous series) the coefficient of the squared term (log γ) of the empirically derived equation is not insignificant.

The relative contribution to retention of the CoA moiety of the homologues was assumed to be constant at a particular mobile phase composition. This assumption has been applied for molecules larger than the *n*-alkanes commonly used in a homologous series study. For example, cholesterol homologues have been extensively treated in this way in reversed-phase chromatography[2]. However, an analyte may be bound to the stationary phase in several orientations, each with a characteristic affinity. In a homologous series the distribution of binding orientations may change from one homologue to the next. This would result in the same fragment of the analyte contributing different amounts of binding energy in different homologues. Such effects may be responsible for the failure of strict additivity in slope and intercept along the homologous series (Figs. 2 and 3), as noted above.

Retention of structural fragments of CoA

Plots of log k' *versus* ammonium sulfate concentration for adenosine and adenosine 5'-monophosphate (AMP) are shown in Fig. 5 along with plots for CoA, propionyl and hexanoyl CoA. The experimental conditions are the same as for Fig. 1, except that a different lot of SynChropak propyl column packing was used. The line of best fit for hexanoyl CoA plot has a significantly greater slope and intercept than propionyl CoA, similar to the plots of Fig. 1. The line of best fit for CoA has a smaller slope and intercept than that of propionyl CoA, but the difference is markedly less than the same three-carbon compositional difference between propionyl and hexanoyl CoA. It is evident that retention for this homologous series is strongly influenced by the length of the alkyl chain. While the structural fragments of the CoA molecule represented by adenosine and AMP obviously contribute to retention of the acyl CoA homologues, their effect on retention is outweighed by that of the alkyl chain of the homologue as the carbon number of the chain increases.

Further inspection of Fig. 5 shows nearly the same slopes for adenosine and

TABLE I

SECOND ORDER POLYNOMIAL FITS OF LOG k' *versus* CARBON NUMBER, FOR SEVERAL $(NH_4)_2SO_4$ CONCENTRATIONS (M_s)

$\log k' = \log \beta + (\log \alpha) n_c + (\log \gamma)\, n_c^2$.

$(NH_4)_2SO_4$ M_s	*1st term* log β	*2nd term* log α	*3rd term* log γ	*Correlation coefficient* (r)
0.210	−0.84326	−0.054278	−0.018336	−0.9957
0.420	−0.81104	−0.018747	−0.016349	−0.9969
0.630	−0.77718	−0.016249	−0.014392	−0.9978
0.840	−0.74340	−0.051088	−0.012462	−0.9985

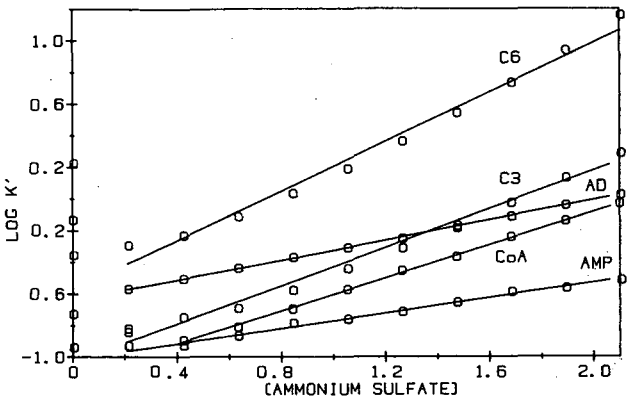

Fig. 5. Retention of various structural components of acyl CoA as a function of ammonium sulfate concentration. AD = Adenosine; AMP = adenosine 5'-monophosphate; CoA = coenzyme A, C_3 = propionyl CoA; C_6 = hexanoyl CoA. Experimental conditions are the same as Fig. 1, except that a different lot of SynChropak propyl column packing was used.

AMP, but a substantially lower intercept for AMP. In terms of the simple interpretation of eqn. 3 (above), the contact areas are nearly the same for the two compounds, and introduction of the hydrophilic phosphate ester into adenosine adds a repulsive component to the attractive hydrophobic interaction. Comparison of the retention curves of adenosine and CoA shows that while the slope for CoA is greater than for adenosine, CoA is less strongly bound over the range of salt concentration examined. Interpreting the slope of eqn. 3 as before, this result means that while CoA exhibits a greater contact area than adenosine, that additional contact area (the phosphopantetheine moiety) contributes a repulsive component to the hydrophobic binding.

Free-energy considerations

Since the log of the capacity factor is related to ΔG by the well-known equation

$$\log k' = \log \varphi - \Delta G/2.3\ RT \tag{6}$$

where φ is the phase ratio and is assumed to be constant, the free energy of transfer of a methylene moiety, ΔG_{CH_2}, can be calculated. From our data, ΔG_{CH_2} can be estimated from the slope of a linear fit of log k' *versus* carbon number[27], by using only log k' values for homologues of $n_c > 3$.

$$\Delta G_{CH_2} = -2.303\ RT\ (\Delta \log k'/n_c) \tag{7}$$

The slopes of log k' *versus* carbon number (Fig. 4, n_c = 4, 6, 8, 10) and the estimated free energies of transfer of a methylene group from the mobile phase to the stationary phase are given in Table II.

Each free energy listed in Table II is the free-energy contribution of a methylene group approximated from a linear fit. Confidence intervals are not reported because the calculation of the errors in the slopes determined by a linear fit are valid only if the

TABLE II
FREE ENERGY OF BINDING IN HIC, PER CH_2 GROUP

$(NH_4)_2SO_4$	Slope ($\Delta \log k'/n_c$)	ΔG_{CH_2} (cal/mol)
0.000[a]	0.1694[a]	−235[a]
0.210	0.2081	−288
0.420	0.2154	−299
0.630	0.2227	−309
0.840	0.2300	−319
0.000[b]	–	−278[b]

[a] These values were not included in determining the slope of Fig. 6 (see text).
[b] This pair of values was obtained from extrapolation of the data of Fig. 6.

data are linear, and clearly, the data are better fit to a quadratic equation for each isocratic mobile phase. Each additional methylene moiety contributes a little more to retention than the previous methylene group. Therefore, only an "average" free-energy contribution is estimated.

Since the slope values listed in Table II are linearly related to the concentration of ammonium sulfate in the mobile phase, it follows that the free energy of transfer of a methylene moiety is linearly related to salt concentration. A plot of the free energies (Table II) *versus* ammonium sulfate concentration (M_s) yields a slope of approximately -48.2 ± 0.7 (95% confidence interval) cal/mol per 1 *M* ammonium sulfate increase (Fig. 6). Because of probable electrostatic contributions, 0.0 *M* ammonium sulfate data are not included in this linear fit.

It is of interest to compare the experimentally derived free energy of transfer values in this study to values derived from liquid–liquid partition and reversed-phase chromatography experiments. Tomlinson *et al.*[24] have shown that a comparison of liquid–liquid partition to reversed-phase chromatography retention data is thermodynamically valid. For example, Zaslavsky *et al.*[28], calculated the free energy of transfer of the methylene moiety from water to hexane (using data acquired by Fendler *et al.*[29]) to be −692 cal/mol at 25°C. By reversed-phase chromatographic means,

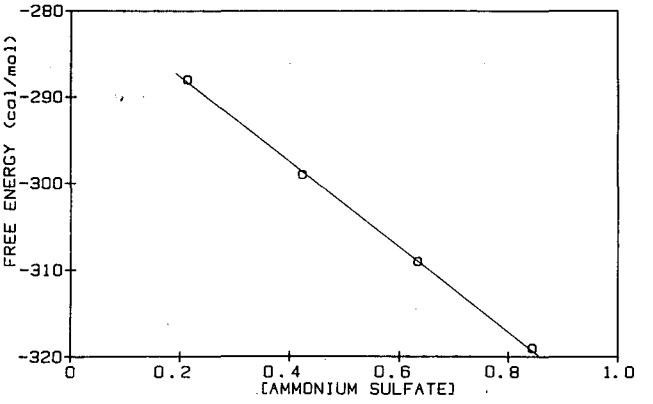

Fig. 6. "Average" free energy of transfer of a methylene group (range C_4 to C_{10}) from mobile to stationary phase, as a function of $[(NH_4)_2SO_4]$. Data from Table II.

Tanaka and Thornton[30] studied the partitioning of homologous series of alkanes and long-chain carboxylic acids between water and reversed-phase (C_{18}) chromatographic stationary phases and calculated a ΔG_{CH_2} on order of -700 cal/mol at 30°C.

The free-energy comparison given here is not meant to be used as a direct comparison of a C_3 hydrophobic interaction column with a C_{18} reversed-phase column, since a C_3 column has a lower percentage carbon loading (lower density, short alkyl chains) than a reversed-phase column (higher density, long alkyl chains). However, our calculation of $\Delta G_{CH_2} = -280$ cal/mol (at 30°C) does suggest that the alkyl moieties of the CoA derivatives are substantially less solvated by the hydrocarbon moieties on the C_3 stationary phase employed in our study than by the methylene group's partition from water into hexane ($\Delta G_{CH_2} = -692$ cal/mol at 25°C[28]) or partition from water into a C_{18} stationary phase ($\Delta G_{CH_2} = -700$ cal/mol at 30°C[30]). We suggest that geometric constraints must preclude total immersion of alkyl groups of the analyte in the thin and non-uniform layer of propyl moieties of the polymeric HIC stationary phase.

Stationary phase contributions

A relative change in selectivity of 11% resulted in our hydrophobic interaction study (Table I) within a change in mobile phase composition of approximately 0.6 *M* ammonium sulfate (the stationary phase remaining constant). The magnitude of this change in selectivity on the propyl HIC column (due to less than a 1 *M* change in salt concentration) can be better appreciated when realizing that a change in a reversed-phase column from C_8 to C_{18} would result analogously in a 10% relative selectivity change (since it is commonly accepted for the highly carbon-loaded reversed-phase column that there is an approximately 1% increase in selectivity per carbon atom[8]). The magnitude of the relative selectivity change in this HIC study is not surprising and supports the premise that the influence of the stationary phase on retention in HIC is outweighed by the influence of the mobile phase. This premise is generally accepted in RPC[23].

ACKNOWLEDGEMENTS

The authors thank the University of Delaware (Biomedical Research Grant) for financial support and SynChrom, Inc. for the gift of an HIC column. The final stages of this work were supported by the National Science Foundation (grant CHE-8707592).

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Journal of Chromatography, 477 (1989) 259–270
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 578

SEPARATION OF β-LACTAM ANTIBIOTICS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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SUMMARY

The retention behaviour of β-lactam antibiotics in micellar electrokinetic chromatography (EKC) was investigated. Sodium dodecyl sulphate (SDS) and sodium N-lauroyl-N-methyltaurate were used as anionic surfactants at concentrations of 0.05–0.3 *M*. It was found that the retention of ionic substances in micellar EKC is determined by the following three factors: the electrophoretic migration of the ionic substances, the interaction between the ionic substances and ionic surfactants and solubilization of the solute by the micellar phase. A difference in the retention behaviours of cationic substances was observed between the two anionic surfactants, which have different groups neighbouring the charge-bearing groups. The effect of an ion-pairing reagent was also investigated to make the effect of the micelle clearer. All test solutes were successfully separated by micellar EKC at SDS concentrations above 0.1 *M*, with theoretical plate numbers ranging from 70 000 to 260 000.

INTRODUCTION

Micellar electrokinetic chromatography (EKC) is a new type of liquid chromatography based on micellar solubilization and electrokinetic migration[1,2]. Micellar solubilization operates as the partition mechanism, that is, a solute is distributed between the micellar phase and the aqueous phase. A strong electroosmotic flow generated within the capillary tube causes all kinds of solutes to elute at one end of the tube with high column efficiency.

Micellar EKC using surfactants has been successfully applied to the separation of electrically neutral substances[3–5], and was shown to give high resolution within a shorter time than conventional high-performance liquid chromatography (HPLC).

In addition to neutral substances, micellar EKC has brought many advantages for the separation of ionic substances[6–11]. Selectivity and peak shapes have been

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much improved in comparison with capillary zone electrophoresis (CZE). As an example that demonstrates the high resolving power of EKC, we have previously reported the separation of water-soluble vitamins[12].

In this paper, we describe the separation of a mixture of seven penicillin antibiotics (PCs) and a mixture of nine cephalosporin antibiotics (CEPs) by micellar EKC using two different anionic surfactants, sodium dodecyl sulphate (SDS) and sodium N-lauroyl-N-methyltaurate (LMT), in comparison with CZE. We discuss the retention characteristics of the ionic substances, to which PCs and CEPs belong, by considering the electrophoretic effect of the solute and the ion-pair formation between cationic substances and anionic surfactants in addition to micellar solubilization. To make the effect of the micelle clearer, the effect of ion-pairing reagents that do not form a micelle was investigated using sodium pentane sulphonate and sodium octane sulphonate. The effect of an additive to the surfactant solution is also described.

EXPERIMENTAL

Apparatus and procedure

Micellar EKC was performed in a 650 mm × 0.05 mm I.D. fused-silica capillary tube (Scientific Glass Engineering, North Melbourne, Australia) with a Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Otsu, Japan) delivering up to +25 kV. The electric current was monitored between the negative electrode and the negative terminal of the power supply with an ammeter throughout the operation. Detection was carried out by the on-column measurement of UV absorption at 210 or 220 nm at a position 150 mm from the negative end of the tube. For data processing a Shimadzu Chromatopak C-R2AX was used.

A sample solution was siphoned from the positive end into a fused-silica tube filled in advance with a buffer solution, and each end of the tube was dipped into a separate reservoir containing the same buffer solution with which the tube was filled. A d.c. voltage was then applied between the two ends of the tube through platinum electrodes dipped in the reservoirs. Regarding the reproducibility of the system, the coefficient of variation for the retention time of the solute was 0.5–0.9% (n = 5–7) from run to run and 1.5–2.4% (n = 5) from day to day in micellar EKC. When the buffer solution was changed, the new solution was left in the tube for *ca.* 30 min to equilibrate with the capillary walls. The capillary tube was sometimes washed with the same buffer solution during the experiment. Other apparatus and experimental procedures were the same as those described previously[12].

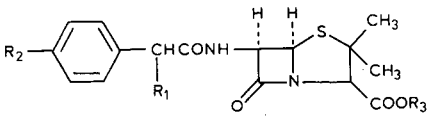
Reagents

Benzylpenicillin (PCG), ampicillin (ABPC), carbenicillin (CBPC), sulbenicillin (SBPC), piperacillin (PIPC), amoxicillin (AMPC), cefotaxime (CTX), cefoperazone (CPZ), cefmenoxime (CMX), cefpiramide (CPM), ceftriaxone (CTRX), cefpimizole (CPIZ) and cefminox (CMNX) were obtained from commercial sources or the National Institute of Hygienic Sciences. Aspoxicillin (ASPC), ceftazidime (CAZ) and C-TA were obtained from our laboratory. These test samples are summarized in Tables I and II. All samples were used as received and dissolved in water at concentrations of 0.5–2 mg/ml to give satisfactory peak heights.

SDS from Nacalai Tesque (Kyoto, Japan) and LMT from Nikko Chemicals

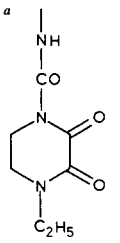
TABLE I

STRUCTURES OF THE SEVEN PENICILLIN ANTIBIOTICS



Penicillin (PC)	*Abbreviation*	*Symbol*	R_1	R_2	R_3
Benzylpenicillin	PCG	1	H	H	K
Ampicillin	ABPC	2	NH_2	H	Na
Carbenicillin	CBPC	3	COONa	H	Na
Sulbenicillin	SBPC	4	SO_3Na	H	Na
Piperacillin	PIPC	5	[a]	H	Na
Aspoxicillin	ASPC	6	[b]	OH	H
Amoxicillin	AMPC	7	NH_2	OH	H

[a]



[b] $-NH-CO-CH(NH_2)-CH_2CONHCH_3$.

(Tokyo, Japan) were used as anionic surfactants. Sodium pentanesulphonate and sodium octanesulphonate were purchased from Wako (Tokyo, Japan) as ion-pairing reagents. These were dissolved in a buffer solution prepared by mixing 0.02 *M* sodium dihydrogenphosphate solution with a 0.02 *M* sodium tetraborate solution to give appropriate pH values, and these solutions were filtered through a 0.45-μm membrane filter, degassed by sonication prior to use.

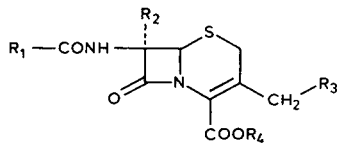
RESULTS AND DISCUSSION

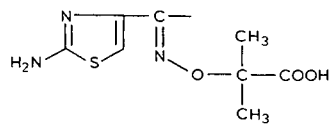
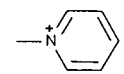
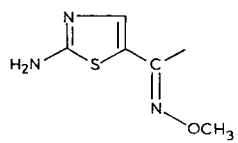
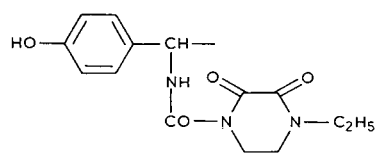
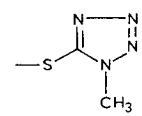
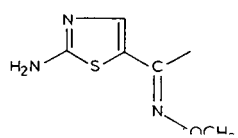
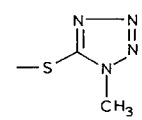
CZE separation

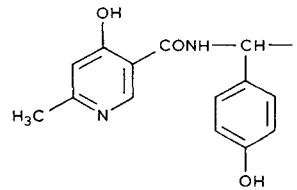
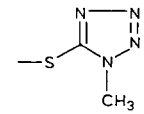
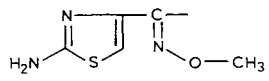
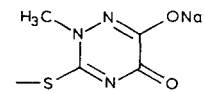
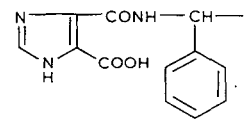
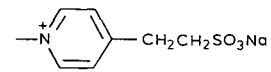
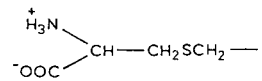
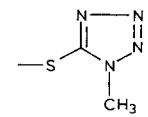
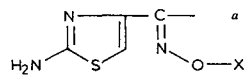
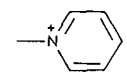
We first investigated the separation of a mixture of seven PCs and a mixture of nine CEPs with a buffer solution containing no surfactant. In this CZE mode, one of the experimental parameters for improving the resolution is the pH of the buffer solution, and we consequently examined the effect of pH in the range 6–9. The elution order in CZE is almost explained in terms of the electric charge and polarity of the solute. That is, under the experimental conditions mentioned above, cationic solutes elute first because both the electroosmotic flow and the electrophoretic migration of the solute are in the same direction to the negative end of the tube, electrically neutral solutes migrate after cationic solutes with the same velocity as the electroosmotic flow and negatively charged solutes migrate slowest because they are pulled back strongly by the electrophoretic effect.

TABLE II

STRUCTURES OF THE NINE CEPHALOSPORIN ANTIBIOTICS



Cephalosporin (CEP)	*Abbreviation*	*Symbol*	R_1	R_2	R_3	R_4
Ceftazidime	CAZ	A		H		–
Cefotaxime	CTX	B		H	—OCOCH$_3$	Na
Cefoperazone	CPZ	C		H		Na
Cefmenoxime	CMX	D		H		H

Cefpiramide	CPM	E		H		Na
Ceftriaxone	CTRX	F		H		Na
Cefpimizole	CPIZ	G		H		–
Cefminox	CMNX	H		OCH_3		Na
C-TA	– –	I		H		–

[a] X = pyrrolidone derivatives (no ionic substituent).

The separation of the mixtures of antibiotics in CZE with a buffer solution at pH 9.0 is shown in Fig. 1. Some solutes eluted very closely or overlapped. Complete separation was not achieved in the CZE mode over the experimental pH range, indicating insufficient selectivity in the CZE separation of these antibiotics.

The solutes having cationic groups, such as a dioxopiperazinyl group (PIPC), an N-methylasparaginyl group (ASPC) or a pyridinium group (CAZ, C-TA), migrated relatively faster than the others and the solutes that have two carboxyl groups (CBPC, CMNX) and a sulphonate group (SBPC, CPIZ) in addition to a carboxyl group eluted late. This retention behaviour agrees well with the above-mentioned explanation.

In addition to the elution order, column efficiency is also influenced by the pH or composition of the buffer solution[11]. Electrostatic interactions between solutes, in particular cationic solutes, and negatively charged capillary walls are indicated by peak tailing. An asymmetric peak appears when an unsuitable buffer solution is used. In the pH range examined above (6–9), the peak shapes were improved with increasing pH, particularly for the separation of the mixture of seven PCs. This improvement is probably a consequence of ion suppression of cationic groups of the solutes. From these observations, we decided to use a buffer solution of pH 9.0 in the subsequent experiments.

Micellar EKC with SDS solutions

We next investigated the effect of surfactant concentration on the retention times of solutes. The results obtained in micellar EKC with buffer solutions containing SDS are summarized in Fig. 2. Typical chromatograms for the seven PCs and nine CEPs obtained by micellar EKC with 0.15 and 0.3 *M* SDS solutions at pH 9.0 are

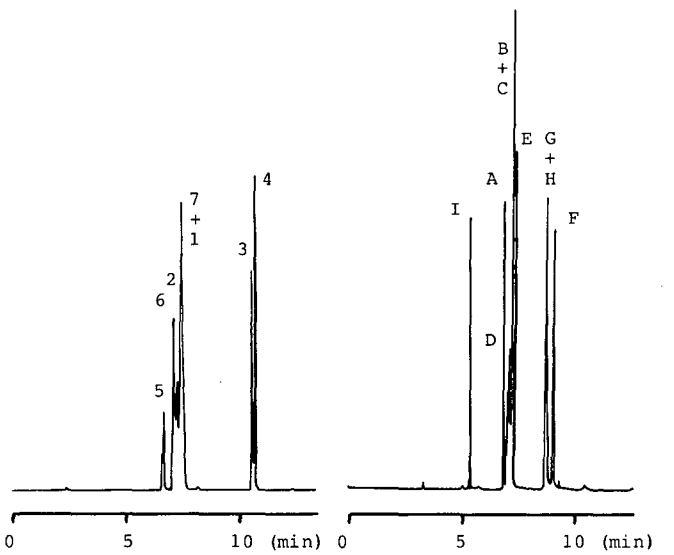


Fig. 1. Separation of seven PCs and nine CEPs by CZE. Conditions: buffer, 0.02 *M* phosphate–borate buffer (pH 9.0); separation tube, 650 mm × 0.05 mm I.D.; length of the tube used for separation, 500 mm; applied voltage, 20 kV; temperature, ambient; detection wavelength, 210 nm; attenuation, 0.04 a.u.f.s. Solutes are indicated by the symbols given in Tables I and II.

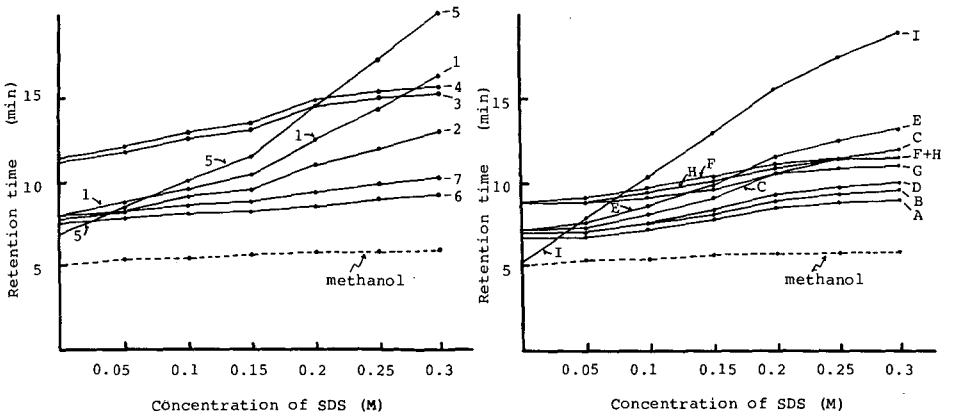


Fig. 2. Effect of SDS concentration on the retention times of PCs and CEPs. Applied voltage, 20 kV. Other conditions as in Fig. 1.

shown in Figs. 3 and 4. The retention times of the solutes increased with an increase in SDS concentration even if the solute was ionized, although the electroosmotic flow was not changed significantly over the whole SDS concentration range.

This trend of the retention times is especially remarkable with PCs. The retention times of PCG and PIPC were delayed more than those of CBPC and SBPC in 0.3 *M* SDS on increasing the SDS concentration, probably because PCG and PIPC were more easily incorporated into SDS micelles owing to their high lipophilicity. On the

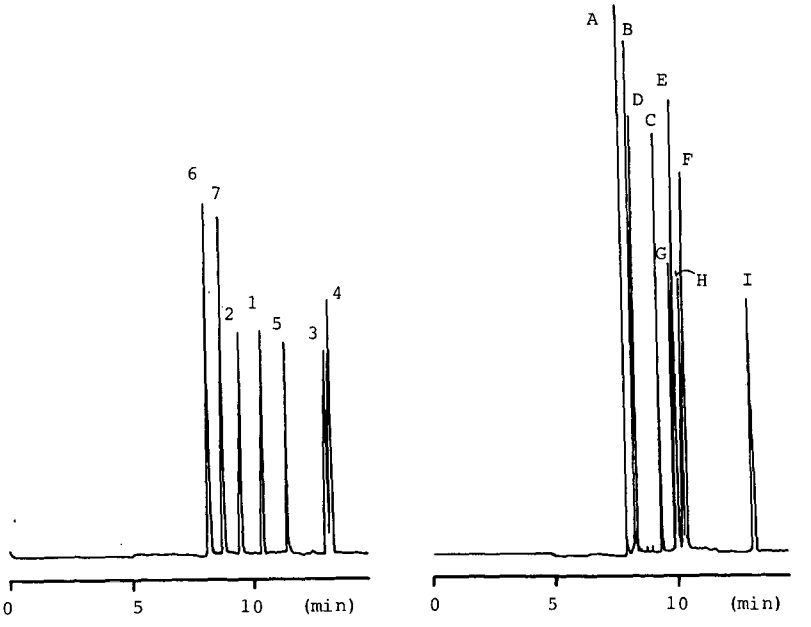


Fig. 3. Micellar EKC of PCs and CEPs. Conditions: applied voltage, 20 kV; SDS concentration, 0.15 *M*. Other conditions as in Fig. 1.

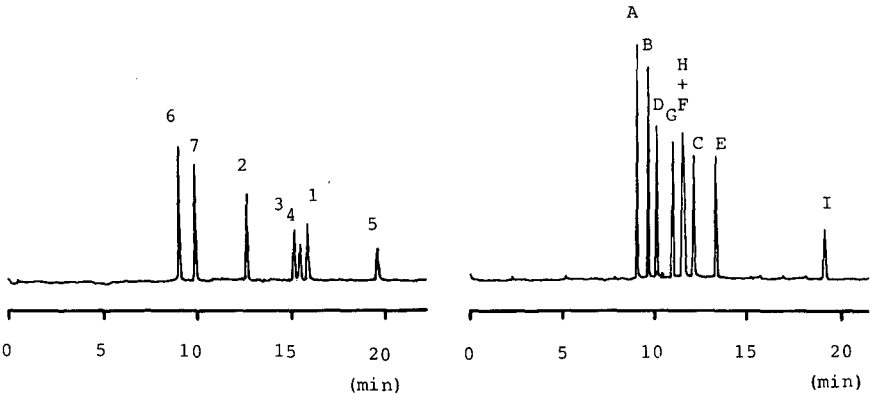


Fig. 4. Micellar EKC of PCs and CEPs. SDS concentration, 0.3 *M*. Other conditions as in Fig. 1.

other hand, the retention behaviour of CBPC and SBPC did not change as much as that of the others with variation in SDS concentration. As these solutes have an anionic group such as a sulphonic or a carboxyl group in their structure, the electrostatic repulsion between the ionized solutes and SDS micelles will suppress the micellar solubilization of the solutes.

The retention times of CEPs also gradually increased with increasing SDS concentration similarly to those of PCs. The migration velocities of CPM, CPZ and, in particular, C-TA were more retarded than those of the others with increasing SDS concentration. CPM and CPZ have relatively more lipophilic groups in their structure and the micellar solubilization effect will consequently act more strongly than with the others. The change in retention time was the largest for C-TA among the CEPs. This may be due to the presence of a pyridinium group, which has a positively charged nitrogen atom. Hence an ion pair may be formed between the cationic group of the solute and the polar group of the anionic surfactant and its formation in addition to the micellar solubilization effect may contribute to the large variation in the retention time. The retention time of CAZ, which also has a pyridinium group, however, did not change as much as that of C-TA. This result is explained in terms of the extra carboxyl group in CAZ compared with C-TA. The marked differences described above show that the electrophoretic mobility of the solute itself is still the most effective parameter in micellar EKC for the separation of ionic solutes.

The factors that affect the elution times of ionic solutes in micellar EKC in our system can be summarized as follows: (1) the number of negatively charged groups (a carboxyl group and a sulphonate group in the solute), (2) the distribution ratio of the solute in the micellar phase to that in the aqueous phase (lipophilicity of the solute) and (3) ion-pair formation between the cationic group of the solute and the polar group of the anionic surfactant. The elution of the solutes will be determined mainly by these three factors, depending on the physico-chemical properties of the solutes.

In addition to the improvement in selectivity, the peak shapes were also improved by addition of SDS even at lower pH, where asymmetric peaks were observed in the CZE mode for the separation of these antibiotics. The same result was observed in the separation of water-soluble vitamins, indicating one of the advantages of micellar EKC.

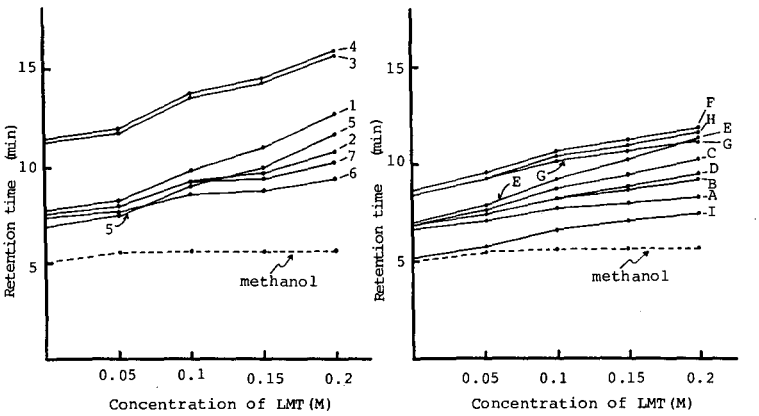


Fig. 5. Effect of LMT concentrations on the retention times of PCs and CEPs. Detection wavelength, 220 nm. Other conditions as in Fig. 1.

Micellar EKC with LMT solutions

LMT was also employed as an anionic surfactant and the effect of LMT concentration on the retention times of the PCs and CEPs were examined. The results are shown in Fig. 5. Typical chromatograms obtained with a 0.15 *M* LMT solution at pH 9.0 are shown in Fig. 6. The effect of LMT concentration was not as great as that of

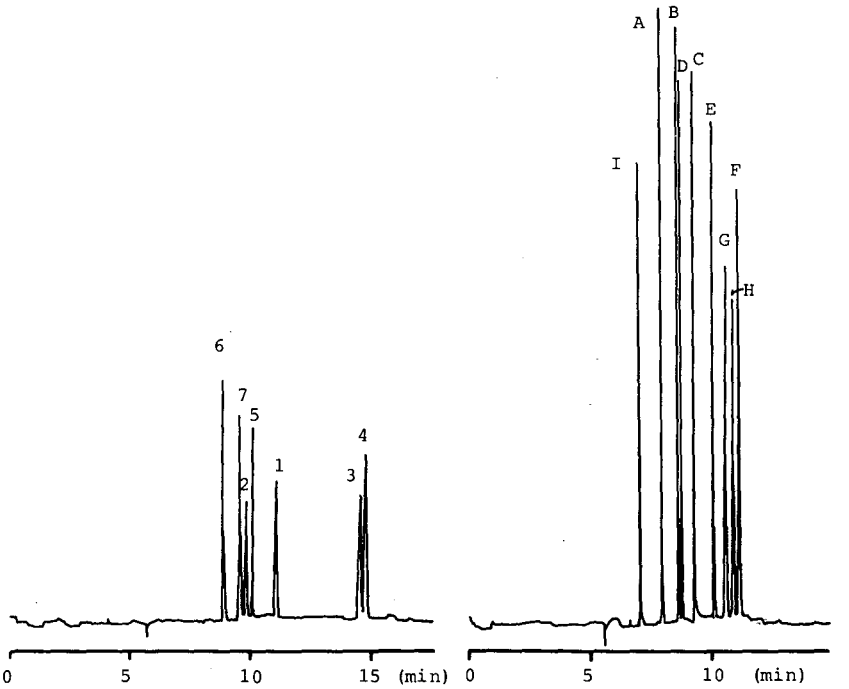


Fig. 6. Micellar EKC of the PCs and the CEPs. LMT concentration, 0.15 *M*; detection wavelength, 220 nm. Other conditions as in Fig. 1.

SDS concentration, although the elution behaviour of the solutes was almost the same as that obtained with SDS.

The variation of the retention time of PIPC was relatively large among the PCs. However, PIPC in a 0.2 *M* LMT solution eluted between PCG and ABPC, although its retention time was longer than that of PCG in a 0.2 *M* SDS solution.

The effect of LMT concentration on the retention times of CPM and CPZ was almost the same as that of SDS concentration. On the other hand, the retention time of C-TA in LMT solutions varied much less than that in SDS solutions.

These marked differences in the retention behaviour of PCs and CEPs, in particular of PIPC and C-TA, between in SDS and LMT solutions can be attributed to the differences in the physico-chemical properties of the two surfactants. We have already observed the same results in the separation of water-soluble vitamins[12]. These differences are probably due to differences in the ionic groups, *i.e.*, a sulphate group in SDS and a sulphonate group in LMT. In addition to the charge-bearing groups, the groups adjacent to them are also different. LMT has an $-N(CH_3)-C(=O)-$group near the ionic group in comparison with a linear hydrocarbon chain in SDS. Consequently, the ion-pair formation between the solutes and the ionic group of LMT might be blocked by steric hindrance. The physico-chemical properties of the two surfactants are given in Table III.

All solutes were successfully separated within 15 min by micellar EKC with SDS or LMT solutions at concentrations of 0.1–0.3 *M*. The numbers of theoretical plates observed in Figs. 3 and 6 are in the range 70 000 (ABPC)–260 000 (PIPC) for PCs and 120 000 (CMNX)–250 000 (CPM) for CEPs, as calculated according to the equation $N = 2\pi(t_R h/A)^2$, where t_R, h, and A are retention time, peak height and peak area, respectively.

Effect of the micelle and additives

To make the effect of the micelle clearer, we investigated the effect of ion-pairing reagents that are often used in reversed-phase HPLC, *i.e.*, sodium pentanesulphonate (C_5) and sodium octanesulphonate (C_8) in a buffer solution at pH 9.0. The effect of C_5 concentration on the retention times of PCs and CEPs was investigated with 0.1 and 0.2 *M* C_5 solutions. Typical chromatograms obtained with 0.2 *M* C_5 are shown in Fig. 7. The retention times of all the solutes increased with increasing concentration of the ion-pairing reagent because of the decrease in the electroosmotic flow (Fig. 8). The selectivity in separation was slightly improved in comparison with CZE. However, the change in the elution order observed in micellar EKC was not observed in this system. C_8 gave almost the same results as C_5, although the elution

TABLE III

PHYSICO-CHEMICAL PROPERTIES OF SDS AND LMT

Surfactant	*MW*	*Kraft point (°)*	*Critical micellar concentration (mmol/l)*
SDS	288.38	9–10	8.1
LMT	343.46	<0	8.7

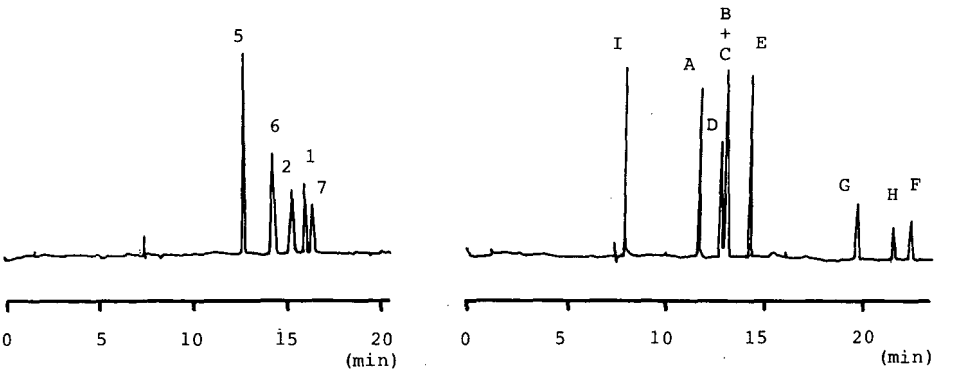


Fig. 7. Separation of PCs and CEPs with a buffer solution containing 0.2 *M* sodium pentanesulphonate. Other conditions as in Fig. 1.

orders of some solutes (PIPC and ASPC, CPIZ and CMNX) altered slightly in 0.1 and 0.2 *M* C_8 solutions. The addition of an ion-pairing reagent was not as effective as the addition of the micelle in improving the resolution, although the separation was slightly improved over the poor resolution given by CZE. The drastic change in the elution order of PCs and CEPs observed in micellar EKC was due to the effect of the micelle because the ion-pairing reagents used in this study do not form a micelle. The charge of the ionic solutes may be neutralized by the ion-pair formation between the solute and the surfactant, and this might consequently accelerate the micellar solubilization and cause the drastic change in the retention behaviour of the ionic solutes. The effect of ion-pair formation is probably superimposed on the effect of micellar solubilization in the presence of the micellar phase.

We would also expect an improvement in selectivity by mixing an additive with

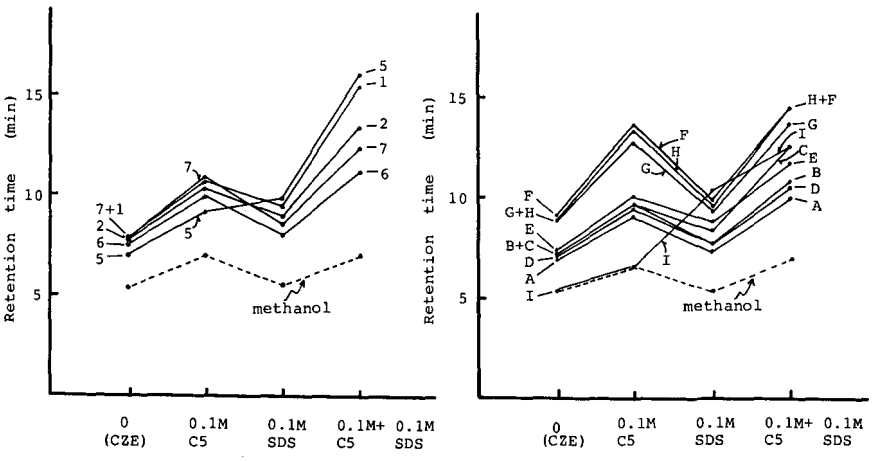


Fig. 8. Effect of the ion-pairing reagent on the separation of PCs and CEPs by mixing with a surfactant. 0, 0.02 *M* phosphate–borate buffer (pH 9.0), CZE separation; C_5, sodium pentanesulphonate. Applied voltage, 20 kV. Other conditions as in Fig. 1.

a surfactant to separate a complex mixture. The results obtained with a solution containing 0.1 *M* SDS and 0.1 *M* C_5 are compared in Fig. 8 with the results given by the other separation modes. Almost the same results were obtained with either C_8 or C_5. When a mixture of an ion-pairing reagent and a surfactant was used, a different retention behaviour was observed to that with either reagent alone. We intend to investigate separations by this mixing method.

We also intend to apply this micellar EKC to the determination of antibiotics in plasma and urine on the basis of above study. Recently, Nakagawa *et al.*[13] reported the determination of CPM in plasma by micellar EKC. A direct plasma injection method was applied and pretreatment of the plasma sample was not necessary. This also demonstrates the capability of micellar EKC.

In conclusion, micellar EKC has brought many advantages for the separation of ionic substances, including penicillin and cephalosporin antibiotics. Selectivity and peak shapes were much improved in comparison with CZE. Satisfactory separations of the seven PCs and the nine CEPs were achieved within 15 min with high theoretical plate values. As very small amounts of sample can also be detected by this method, in addition to the excellent separation capability, EKC will become a powerful analytical method, with further development of the detection system and the injection system, in clinical or biological analysis where ultra-small-scale separations are demanded.

ACKNOWLEDGEMENTS

We thank Professor Terumichi Nakagama, Faculty of Pharmaceutical Sciences, Kyoto University, for helpful advice and discussions. We also thank Mr. Reiji Shimizu, Head of the Analytical Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., for his encouragement throughout this study.

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Journal of Chromatography, 477 (1989) 271–276
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 600

EVALUATION OF ASSAY SPECIFICITY IN NON-SUPPRESSED ION CHROMATOGRAPHY

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(First received January 30th, 1989; revised manuscript received April 27th, 1989)

SUMMARY

The utilization of tandem conductivity and indirect photometric detectors for demonstrating the purity of peak responses in non-suppressed ion chromatography is discussed. The differing nature of the two detection mechanisms, both of which can be performed simultaneously with a phthalate buffer mobile phase, provides the means for species discrimination. Specificity can be evaluated by comparing peak response ratios (ratio of the detector response from a sample and standard) obtained from both detectors. The utility of the method is demonstrated by assessing the ability of the method to identify interferences in overlapping nitrate and sulfate peaks.

INTRODUCTION

The evaluation and confirmation of assay specificity (that is, the ability of the method to produce a response related to the presence of a single solute) is a vital part of any method development process. This is especially true in chromatography, where the specificity issue essentially reduces to a question of operational peak purity (that is, is the chromatographic peak produced solely by the detector responding to a single species). One approach used to assess chromatographic peak purity involves the utilization of two (or more) distinct detection strategies to identify peculiarities in terms of peak shape and magnitude of response. Such an approach is particularly powerful if the two detection strategies target vastly different properties of the solutes and is particularly convenient if the chromatographic system is such that the alternate detection processes can be accomplished simultaneously (either with the same or tandem detectors).

Such a scenario is realized in non-suppressed ion chromatography (IC) wherein the commonly employed phthalate buffer mobile phase allows for both conductivity and indirect photometric detection. Despite being classified as a bulk property detection mechanism, conductivity detection in IC actually deals with a solute-specific property (its equivalent ionic conductance). The indirect detection mechanism relies only on the solute-induced change in mobile phase counter-ion concentration to produce a response and thus is truly analyte independent (if the solute has no intrinsic

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UV absorbance at the wavelength of interest). Thus utilization of both strategies (in tandem) provides a means of assessing specificity in IC applications.

In this paper we discuss a quantitative mechanism for assessing specificity based on this tandem detector strategy and demonstrate its utility in a practical application.

THEORY

For a chromatographic detector operating within its linear dynamic range, the relationship between response and solute concentration is

$$r = mC \tag{1}$$

where m is the response factor and C is the solute concentration. The response ratio (R) between two samples containing different concentrations of the solute is simply their concentration ratio,

$$R = r_1/r_2 = C_1/C_2 \tag{2}$$

Since this ratio is independent of the response factor, it should be constant regardless of the nature of the detector. Therefore the selectivity factor (S), defined as the ratio of R in detectors A and B, is equal to 1,

$$S = R_A/R_B \tag{3}$$

However, if the chromatographic peak consists of two solutes (a and b), the response equation becomes (in the most simplistic case wherein no solute–solute interactions occur)

$$r = m_aC_a + m_bC_b \tag{4}$$

and the response ratio between a sample containing both solutes and a standard containing only one (a) becomes

$$R = 1 + (m_bC_b/m_aC_a) \tag{5}$$

In this case, the selectivity factor between the two detectors becomes

$$S = \frac{1 + (m_{b,A}C_b/m_{a,A}C_a)}{1 + (m_{b,B}C_b/m_{a,B}C_c)} \tag{6}$$

Only in the case where $(m_{b,A}/m_{a,A}) = (m_{b,B}/m_{a,A})$ will S be equal to one. Thus calculation of S for a peak using two different detectors can potentially provide specificity and/or identity related information. A calculated S value significantly different from one implies that either the peak is produced by two or more components or that the solute in the sample and the standard are not the same. Alternately, obtaining two different R values in the two different detectors indicates the same situations.

The successful application of this strategy to IC utilizing tandem conductivity and indirect photometric detectors requires that they meet the criterion that

$$(m_{b,cond.}/m_{a,cond.}) \neq (m_{b,indirect}/m_{a,indirect}) \quad (7)$$

For transparent analytes, $m_{b,indirect} = m_{a,indirect}$ if the concentrations of analyte a and b are expressed in normality[1]. In non-suppressed conductivity detection, the response (change in mobile phase conductance due to the presence of the solute) can be written

$$R = C_s(E_s - E_e)/1000K \quad (8)$$

where C_s is the solute concentration, E is the equivalent ionic conductance of the solute (s) or the eluent (e) and K is the cell constant[2]. Thus, the ratio m_a/m_b for conductivity is directly proportional to the ratio of the equivalent ionic conductances for species a and b. Since equivalent ionic conductances for most common inorganic anions are not the same (see for example, ref. 3), the criterion in eqn. 7 is met and the proposed specificity evaluation scheme is appropriate.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of an Applied Biosystem's Spectroflow 400 pump and 757 variable-wavelength UV detector, a Micromertics 728 autosampler coupled to an electronically actuated Rheodyne 7010 injection valve, a Waters Model 430 conductivity monitor and a Hewlett-Packard HP 3357 computer integrator. The chromatographic column was a Dionex AS-1 anion separator and the specificity evaluation was performed with a mobile phase containing $1.92 \cdot 10^{-3}$ M potassium hydrogen phthalate at pH 6.5. The UV detector was operated at a wavelength of 250 nm, mobile phase flow-rate was 1.5 ml/min and the sample size was 10 μl.

Procedure

Standard solutions containing either sulfate or nitrate in the concentration range 20–300 ppm were injected into the chromatographic system (in replicate) to evaluate response linearity over this range. Test articles containing known amounts of sulfate and nitrate were injected (in replicate) into the chromatographic system and the responses obtained from both detectors were recorded. In all cases, peak area was used for quantitation.

RESULTS AND DISCUSSION

In order to demonstrate the utility of the proposed specificity evaluation method, chromatographic conditions producing known peak overlap had to be identified. The different effect of mobile phase composition on the retention characteristics of dissimilarly charged analytes in IC is well documented[4–8] and peak overlap between nitrate and sulfate has been observed[9]. As noted previously, these species also have dissimilar specific ionic conductances (71 and 80 Ω^{-1} cm^2 equiv.$^{-1}$ at 25°C in aqueous solution for nitrate and sulfate, respectively[3]), and thus are

appropriate candidates for the demonstration. Using the elution models of Jenke and Pagenkopf[10–12], a mobile phase capable of producing sulfate–nitrate coelution was identified with only a few scouting experiments (Fig. 1). When this mobile phase ($1.92 \cdot 10^{-3}$ M potassium hydrogen phthalate at pH 6.5) was used, peak coelution was achieved; even in samples containing equimolar concentrations of sulfate and nitrate there is no visual indication that the resulting chromatographic peak is impure (Fig. 2). Peak area response for both detectors was linear over the concentration range of 20 to 300 ppm for both analytes; the sensitivity ratio calculated from the slopes of the calibration curves is 1.13 which agrees well with the predicted selectivity ratio (from equivalent conductances) of 1.14. This predicted selectivity ratio represents the product of the ratio of the specific ionic conductances of the analytes (1.14) and the ratio of the molar response ratios in the indirect photometric detector (1.0). Precision at the 100 ppm concentration level is on the order of 0.4% R.S.D. (n = 11) for both analytes and with both detectors. Thus the proposed specificity evaluation method is directly applicable and in this case should provide tight confidence intervals for the respective detector's response ratios.

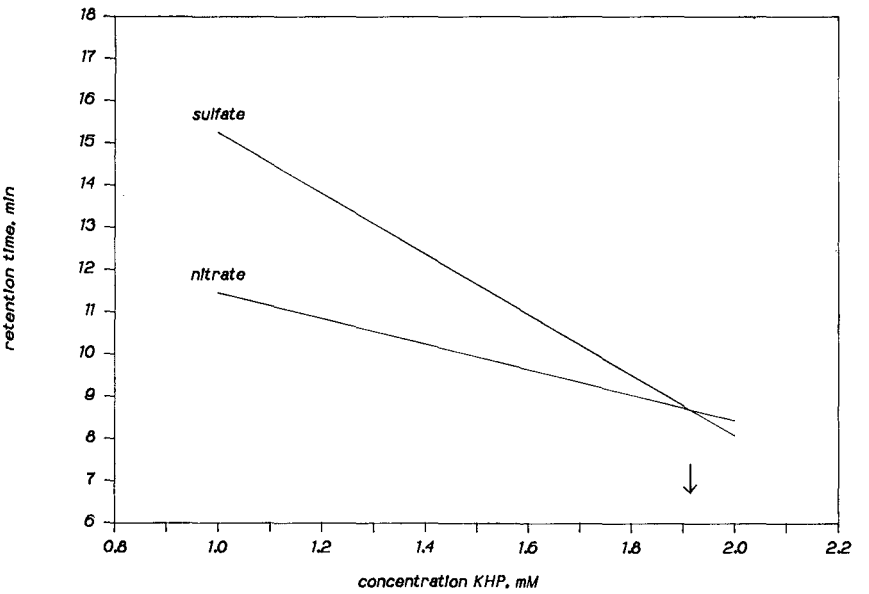

Fig. 1. Effect of mobile phase composition on the elution characteristics of nitrate and sulfate. The arrow indicates that mobile phase for which coelution occurs. KHP = potassium hydrogen phthalate.

The response ratio data obtained from the analysis of six nitrate–sulfate mixtures is shown in Table I. If the confidence interval (95% level) for the response ratios for a particular analyte overlap, then no interference is recognized and the peak is judged to be pure. For instances where the concentration ratio between the analyte and interferent is 10 or smaller, comparison of the response ratios effectively indicates the lack of specificity. However, for samples where the analyte–interferent ratio is

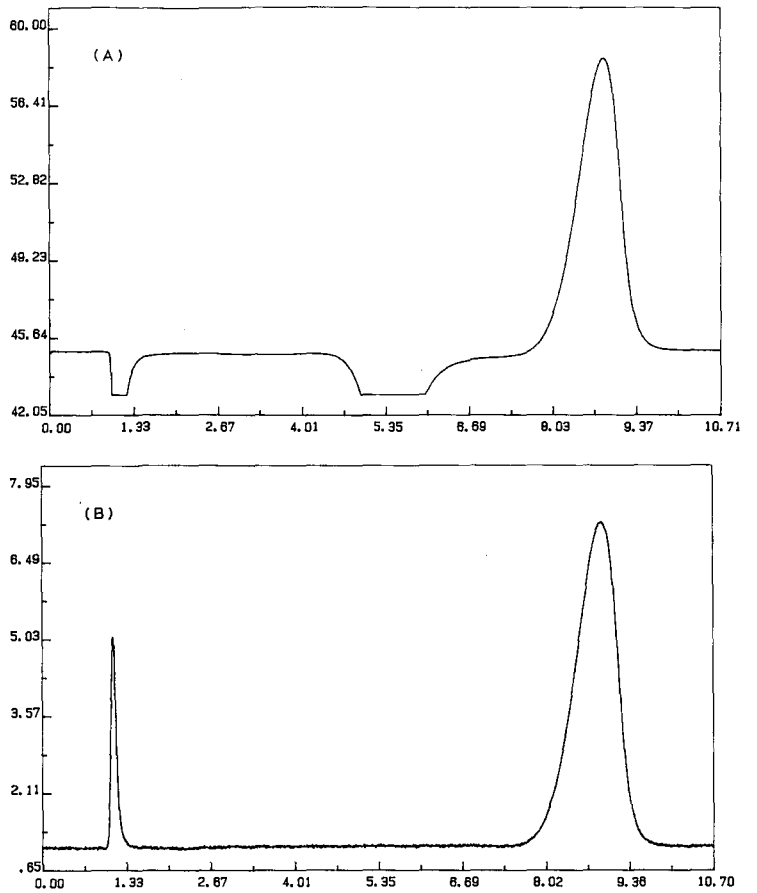

Fig. 2. Typical chromatograms obtained from a mixture containing equimolar amounts of sulfate and nitrate. Trace A is from the conductivity detector while trace B is from the UV detector.

TABLE I

SPECIFICITY EVALUATION OF THE ANALYTE PEAK

Mixture	*Conc. (ppm)*		*Response ratio*[a]			
	Sulfate	*Nitrate*	*Versus nitrate*		*Versus sulfate*	
			UV	*Cond.*	*UV*	*Cond.*
1	222[b]	132	3.24–3.31	3.47–3.54	2.96–3.02	2.76–2.81
2	222[b]	26	2.41–2.46	2.66–2.70	2.20–2.24	2.11–2.15
3	222[b]	13	2.28–2.32	2.60–2.65	2.08–2.12	2.07–2.11
4	111	264[b]	3.17–3.23	3.27–3.33	2.89–2.95	2.61–2.65
5	22	264[b]	2.28–2.33	2.26–2.30	2.09–2.13	1.80–1.83
6	11	264[b]	2.16–2.20	2.16–2.20	1.97–2.01	1.72–1.75
7	–	265	2.05–2.11	2.00–2.06	1.88–1.93	1.60–1.64
8	222	–	2.20–2.25	2.46–2.50	2.00–2.05	1.96–2.00

[a] 95% Confidence interval.
[b] Major analyte.

much greater than 10 (*e.g.* samples 3, 5 and 6), the methodology fails to identify that an interferent is present. Of course, the minimum relative concentration of interferent which the methodology is able to detect is influenced by the magnitude of the selectivity ratio (larger ratio enhances the ability to detect low level interferents) and the precision of the methodology (since the comparison is statistical). In point of fact, the somewhat small range of equivalent ionic conductances which are exhibited by many common ionic solutes limits the magnitude of the selectivity ratio and will commonly define the method's ability to establish specificity. Clearly, the effectiveness of the methodology requires the assay to be fairly precise and/or the database (number of injections) to be large.

In addition to allowing for a peak purity assessment, the method can be used to determine the "identity" of the species responsible for a given peak. For example, "Mixtures" 7 and 8, which in actuality contained only one of the analytes, illustrate the method's ability to identify the species responsible for producing a chromatographic peak. Clearly, in these cases the analyte producing the "unknown" response is identified as the one for which the confidence intervals of the response ratios overlap.

The authors note in passing that the case of dissimilarly charged interferent–analyte pairs (as per the example in this discussion) can also be addressed by using a second mobile phase to resolve the two. However, such an approach will not be effective in identifying a peak which is compromised by an interferent whose charge is the same as the analyte. The proposed method is applicable in both cases and is thus more general in scope.

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Journal of Chromatography, 477 (1989) 277–290
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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COMPARISON OF LIQUID CHROMATOGRAPHIC SELECTIVITY FOR POLYCYCLIC AROMATIC HYDROCARBONS ON CYCLODEXTRIN AND C_{18} BONDED PHASES

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(First received February 20th, 1989; revised manuscript April 20th, 1989)

SUMMARY

Selectivity towards polycyclic aromatic hydrocarbons (PAHs) was studied on cyclodextrin bonded phases and compared to selectivity observed on C_{18} phases. The study included the separation of eleven five-ring PAH isomers on each of three phase types; monomeric C_{18}, polymeric C_{18} and cyclodextrin. Retention of PAHs ranging in size from three to six condensed rings was also investigated. Retention on the cyclodextrin phase is based on inclusion complexing between the solute and cyclodextrin cavity, resulting in a strong shape dependence. However, the shape selectivity exhibited by the cyclodextrin phase is different from that exhibited by either the monomeric or polymeric C_{18} phases; retention on the cyclodextrin phase is strongly dependent on the shape and shows very little molecular weight dependence. Calculations of solute molecular widths were performed to predict the isomers' ability to enter the cyclodextrin cavity. The effect of sample solvent and injection volume was also investigated for the cyclodextrin phase. A retention model based on the solute shape is proposed for PAH isomers on β-cyclodextrin phase.

INTRODUCTION

Complex mixtures of polycyclic aromatic hydrocarbons (PAHs) are often encountered in environmental samples and their complexity is due to the numerous isomeric structures of PAHs. Considerable emphasis has been put into the separation of isomeric PAHs since certain isomers are more mutagenic and/or carcinogenic than others.

Differences in liquid chromatographic (LC) retention and selectivity towards PAH isomers among commercially available columns are well known, and although the retention mechanisms are not yet fully understood, models for retention have been presented for different types of stationary phases[1–5]. Sander and Wise have

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shown that the selectivity of C_{18} bonded phases for the separation of PAH is dependent on a number of parameters[2,6–8]. These parameters include phase type (monomeric or polymeric synthesis)[2,6], pore diameter and surface area of the silica substrate[7], and surface coverage or C_{18} ligand density[2,8]. Monomeric C_{18} phases are prepared by using monofunctional silanes whereas polymeric C_{18} phases are generally prepared using trifunctional silanes in the presence of water. The greatest selectivity for the separation of PAH isomers is achieved on polymeric C_{18} phases prepared on wide pore (>150 Å in diameter) silica substrates with low surface area (100 m^2/g)[2,6,7].

In investigations concerning the selectivity of PAH on different stationary phases, Wise and co-workers[1,2] found a relationship between retention on a polymeric C_{18} phase and the shape of the solute, defined as length-to-breadth ratio (L/B). The L/B value is determined by drawing the PAH molecule using the appropriate bond lengths and then constructing a box around the structure which provides the maximum length to breadth ratio. There is a high correlation between this ratio and solute retention for PAH isomers on polymeric C_{18} phases.

Armstrong and co-workers[9,10] suggested that cyclodextrin bonded phases could be an alternative to traditional C_{18} bonded phases for the separation of PAH isomers. Selectivity of the cyclodextrin bonded phase towards PAHs is based on inclusion complexing of the solute and the glucopyranose cavity and would thereby provide different selectivity than a C_{18} bonded phase. Armstrong and co-workers[9,10] suggested that cyclodextrin phases exhibit enhanced selectivity toward PAH isomers when compared to C_{18} phases. However, they showed the separation of only three pairs of isomers (benzo[*a*]- and benzo[*e*]pyrene, 1,2:3,4- and 1,2:5,6-dibenzanthracene, phenanthrene and anthracene) to illustrate this claim. Differences in selectivity of PAHs of different molecular weight on β- and γ-cyclodextrin have also been presented[9], although the observed changes in relative retention between the two phases were not explained.

In this work we compare PAH selectivity on monomeric and polymeric C_{18} bonded phase materials with the selectivity on a β-cyclodextrin bonded phase. PAH separations on a γ-cyclodextrin are also investigated briefly. Eleven PAH isomers of molecular weight 278 have been used by Wise and Sander[2] to illustrate the dependence of shape (L/B) for PAH retention on polymeric and monomeric C_{18} bonded phases. In this work, the same eleven isomers were used to compare retention mechanisms on cyclodextrin bonded phases with retention mechanisms on C_{18} bonded phases. PAHs of different molecular weights are studied, and a model of the retention of PAHs on cyclodextrin bonded phases is proposed.

EXPERIMENTAL[a]

Materials

Phenanthro[3,4-*c*]phenanthrene was obtained from Aldrich (Milwaukee, WI, U.S.A.), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene was obtained from Rütgers (Castrop-

[a] Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Rauxel, F.R.G.) and benzo[*a*]pyrene was obtained from BCR (Community Bureau of Reference, Brussels, Belgium). PAH isomers of molecular weight 178 and 228 (three and four aromatic rings) were all obtained from commercial sources. The five-ring PAH isomers of molecular weight 278 were obtained as reported previously[2]. The six-ring PAH isomers were obtained from W. Schmidt (Ahrensburg, F.R.G). Methanol, acetonitrile and water (all HPLC grade) were obtained from J. T. Baker (Whippany, NJ, U.S.A.).

Columns

Separations of the different PAH mixtures were performed on commercial columns: Vydac™ 201 TP (polymeric C_{18}), The Separations Group (Hesperia, CA, U.S.A.); Zorbax® ODS (monomeric C_{18}), MAC-MOD Analytical (Wilmington, DE, U.S.A.); and Cyclobond™ I (β-cyclodextrin) and Cyclobond™ II (γ-cyclodextrin), Astec (Whippany, NJ, U.S.A.). The four columns were all 250 × 4.6 mm I.D. with 5-μm packing material.

Chromatography

A liquid chromatograph consisting of a reciprocating piston pump, a solvent programming system, a 20-μl (where not otherwise stated) loop injector and a 254-nm fixed-wavelength detector was used throughout the studies. Retention data were collected on a chromatography data system. All samples were run isocratically with aqueous methanol mobile phases and the solutes were dissolved in methanol prior to injection. Temperature of the column was maintained at 30°C during all chromatographic runs.

The molecular widths and lengths of the PAH solutes were calculated with a XIRIS molecular modeling program (XIRIS Co., New Monmouth, NJ, U.S.A.) on a personal computer.

RESULTS AND DISCUSSION

Polymeric C_{18} phases on wide pore (*e.g.*, 300 Å) silica provide very high selectivity for the separation of PAH isomers[2,6,7]. The enhanced selectivity for isomeric PAHs observed with polymeric C_{18} phases, compared to monomeric C_{18} phases, can be attributed to a shape recognition ability of the polymeric phase. Relationships between shape and reversed-phase LC retention, as described previously by Wise *et al.*[1], have also been observed in this work with the polymeric C_{18} phase as well as with the cyclodextrin stationary phases. However, the shape recognition of the cyclodextrin phases is different from that of the polymeric C_{18} phase.

The Cyclobond™ stationary phases consist of cyclodextrins chemically bonded to 5-μm spherical silica gel. The cyclodextrins are arranged in the shape of a hollow truncated cone[11]. β-Cyclodextrin consists of seven glucopyranose units, which provide a cone with an inner diameter of 7.8 Å. γ-Cyclodextrin consists of eight glucopyranose units and yields a truncated cone with an inner diameter of 9.5 Å. The interior of the cyclodextrin cavity is relatively hydrophobic with a high electron density, and the exterior is hydrophilic. In this case, with an aqueous organic mobile phase, the proposed separation mechanism is based on an inclusion complex between the solute and the cone[11–14]. Any organic modifier present in the mobile phase will compete

with the solute for the preferred location in the hydrophobic cavity. In this way, the modifier will reduce solute-bonded phase interaction, and thus decrease retention. Acetonitrile has a stronger affinity for the cavity than methanol and is a stronger eluent.

The choice of sample solvent was observed to be critical for separations carried out on the cyclodextrin phases. A similar observation was made by Wilson[15]. In general for reversed-phase chromatography, the best column efficiency and solute peak shape results when solutes are dissolved in the mobile phase. Stronger solvents are often required, however, particularly for solutes of low solubility. On conventional C_{18} columns, detrimental solvent effects are usually minor, as long as the solvent strengths (sample solvent and mobile phase) are not too dissimilar and injection volume is small. For the cyclodextrin columns, the effect is readily seen. Attempts to chromatograph solutes dissolved in acetonitrile with an aqueous methanol mobile phase yielded extremely poor peak shape. For solutes dissolved in methanol and chromatographed in an aqueous acetonitrile mobile phase, peak shape was not affected. To study the cause of the poor peak shape, pure acetonitrile was injected onto the column immediately prior to injection of the sample (dissolved in methanol), using an aqueous methanol mobile phase. No degradation in peak shape was apparent. From this observation, it appears that interactions become significant only when the sample is dissolved in acetonitrile. This was further investigated by preparing two PAH sample solutions of the same concentration, one in methanol and one in acetonitrile. Three different volumes (5, 20 and 50 μl) of the solutions were injected onto the β-cyclodextrin column, using a 55% aqueous methanol mobile phase (Fig. 1). The sample dissolved in methanol showed only a slight band broadening as injection volume increased, whereas chromatographic performance with the acetonitrile solution was strongly dependent on injection volume. Peak splitting was apparent at 20-μl injection volume, and at 50-μl extremely poor peak shape resulted with two peaks present for each component. Acetonitrile disturbs the column partitioning equilibria by initially decreasing solute retention. When the kinetics of solute transfer between the mobile and the stationary phase is slow, only a portion of the retained band is affected by the stronger solvent "plug". Thus, a gaussian peak is changed into a bimodal distribution, and the result is peak broadening or, in extreme cases, peak splitting. This effect was also seen when 20 μl of the methanol sample solution was injected followed by an injection of 20 μl pure acetonitrile, as shown in Fig. 2. The acetonitrile molecules are essentially unretained and "catch up" with the retained solutes. The result is the same as if the sample was dissolved in acetonitrile. Peak splitting occurs even when the acetonitrile plug is injected as late as 3 min (the approximate column void volume) after the sample injection is performed.

Three PAH isomers of molecular weight 228 were run on both a β- and a γ-cyclodextrin stationary phase (see Fig. 3A and B, respectively). The elution order of the three components is different on the two phases; on both phases the bulky triphenylene elutes first, but the elution of benz[*a*]anthracene and chrysene are reversed. In the case of the β-cyclodextrin phase, chrysene elutes before benz[*a*]anthracene because of the difference in shape of the solutes. The long narrow portion of the benz[*a*]anthracene structure can enter the cavity more deeply and form a more stable inclusion complex than the bulkier chrysene. A comparison of the dimensions of the cyclodextrin cavity and solute widths reveals that the β-cavity is wide enough only for

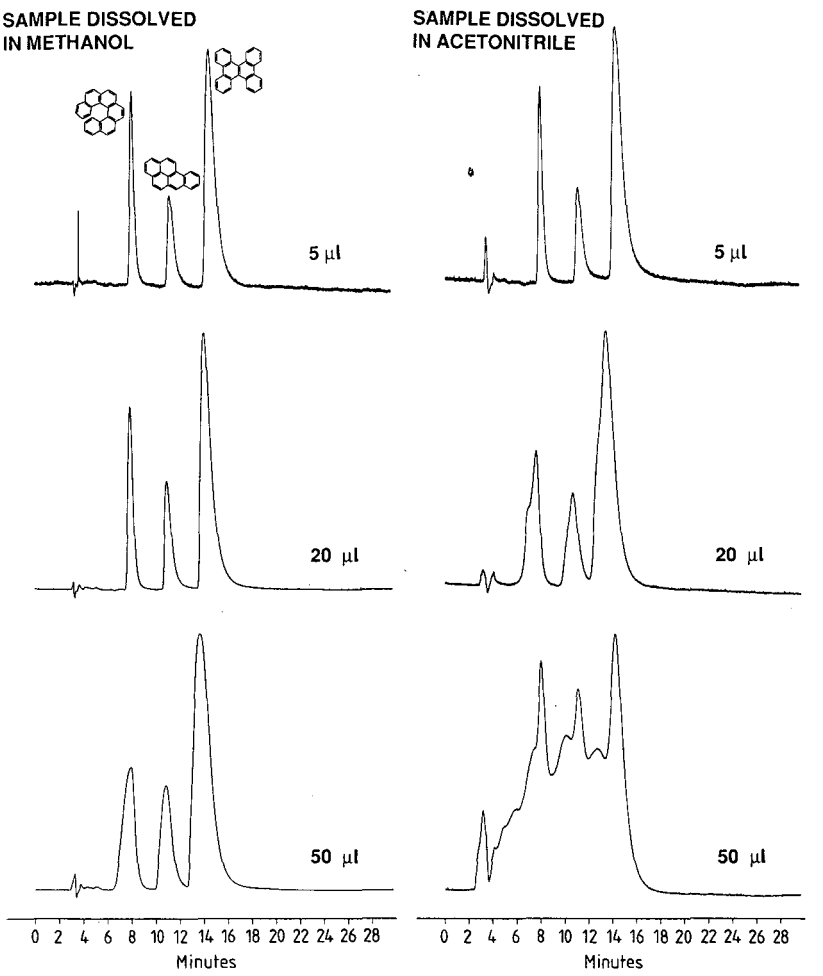

Fig. 1. Separation of phenanthro[3,4-*c*]phenanthrene, benzo[*a*]pyrene and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene on β-cyclodextrin (Cyclobond™ I). The mobile phase composition was methanol–water (55:45) and the flow-rate was 1 ml/min. Volumes indicate sample sizes injected.

a single chain of benzene rings to enter. In the case of the γ-cyclodextrin phase, the cavity is wide enough for chrysene to enter and thereby form a more stable complex (*i.e.*, resulting in longer retention) than the narrower benz[*a*]anthracene. On polymeric C_{18} columns these three PAH isomers elute in the same order as on the γ-cyclodextrin phase, but with much greater efficiency and resolution. This elution order is in agreement with increasing L/B ratios[1]. The low efficiency of the cyclodextrin phases for these solutes might be due to the low solubility of the solutes in the mobile phase. No increase in column efficiency has been observed using acetonitrile as the organic modifier. At comparable k' values of the solutes, the water concentration is higher compared to elution with an aqueous methanol mobile phase, and it seems likely that the solubility of the solutes is not enhanced.

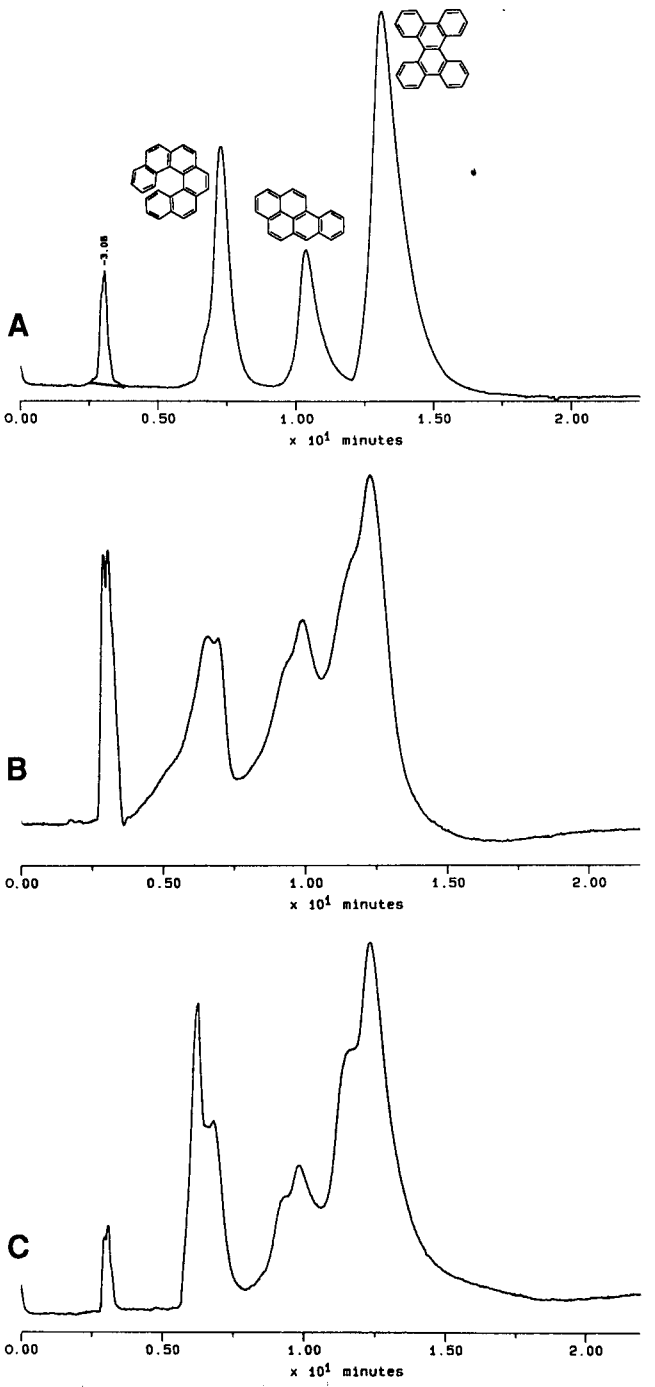

Fig. 2. Chromatographic conditions as in Fig. 1. The sample was dissolved in methanol and the injection volume was 20 μl. (A) Sample injected. (B) Sample injected followed by an immediate injection of 20 μl, pure acetonitrile. (C) Sample injected followed by an injection of 20 μl pure acetonitrile 3 min after sample injection.

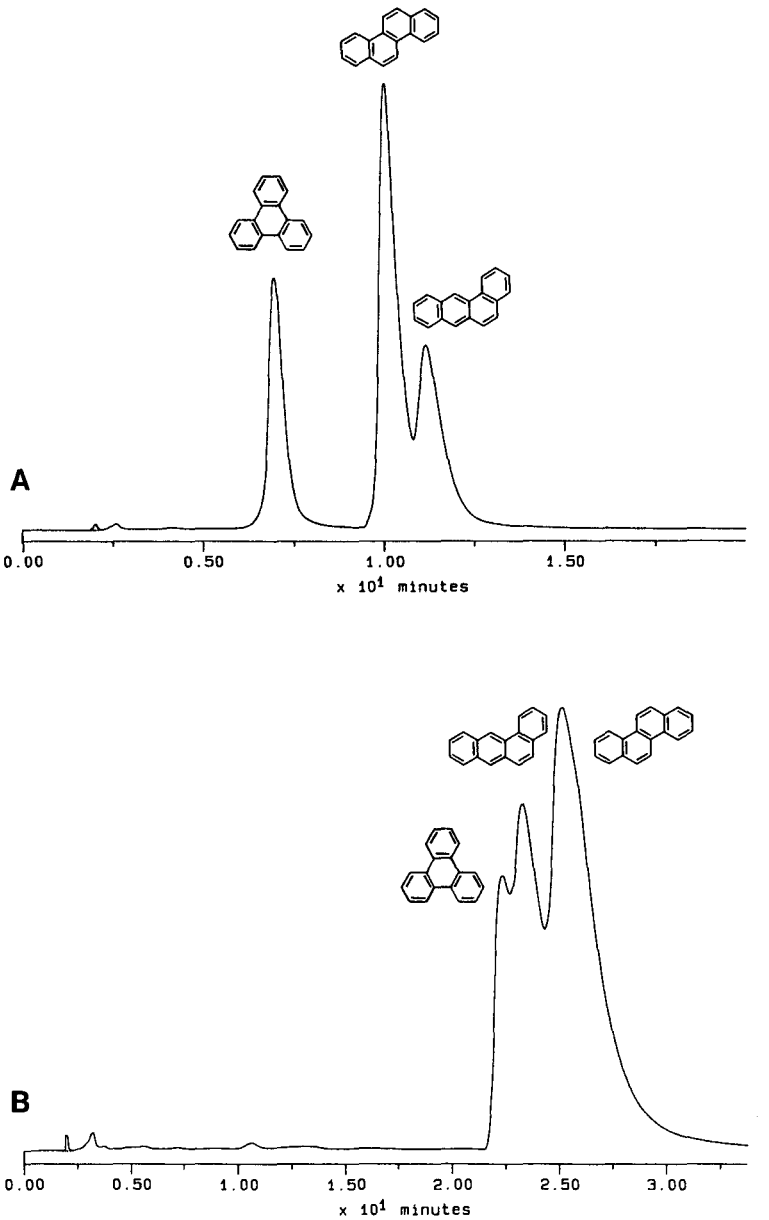

Fig. 3. Separation of PAH isomers on: (A) β-Cyclodextrin. The mixture was separated isocratically with methanol–water (50:50) at a flow-rate of 1.5 ml/min. (B) γ-Cyclodextrin. The mixture was separated isocratically with methanol–water (40:60) at a flow-rate of 1.5 ml/min.

The varying amounts of organic modifier needed to elute the components are indicative of the differences in binding energy of the inclusion complex for the two phases; with a mobile phase consisting of 50% aqueous methanol, chrysene elutes at 10 min on β- and at 7 min on γ-cyclodextrin. Less energy is involved in the interaction

of solute–γ-cyclodextrin than solute–β-cyclodextrin since these isomers are too small to interact strongly with the γ-cavity.

The efficiency and peak shape of the γ column is very poor for solutes of the size and shape discussed here. Six PAH isomers of molecular weight 278 (isomers 1, 3, 5, 8, 10 and 11, Fig. 4) that were completely resolved on the β-cyclodextrin phase gave only two peaks on the γ-cyclodextrin phase. This result is expected when widths of the solutes are calculated. The narrowest part of the molecules, a single chain of benzene rings, is about 7.3 Å wide. The widest part, which is 9.7 Å, is restricted from the 9.5-Å γ-cyclodextrin cavity. Only the narrow part of the molecule can enter. It seems likely that the inclusion complex formed with the narrow chain (7.3 Å) within the 9.5-Å cavity is relatively weak. It is obvious that the fit of the solute inside the cavity is crucial, and for the PAH molecules presented here, the β-cyclodextrin is more suitable than the γ-cyclodextrin.

The eleven five-ring PAH isomers of molecular weight 278 (listed in Fig. 4) showed the same retention behavior (*i.e.*, a molecule with a narrow shape will be

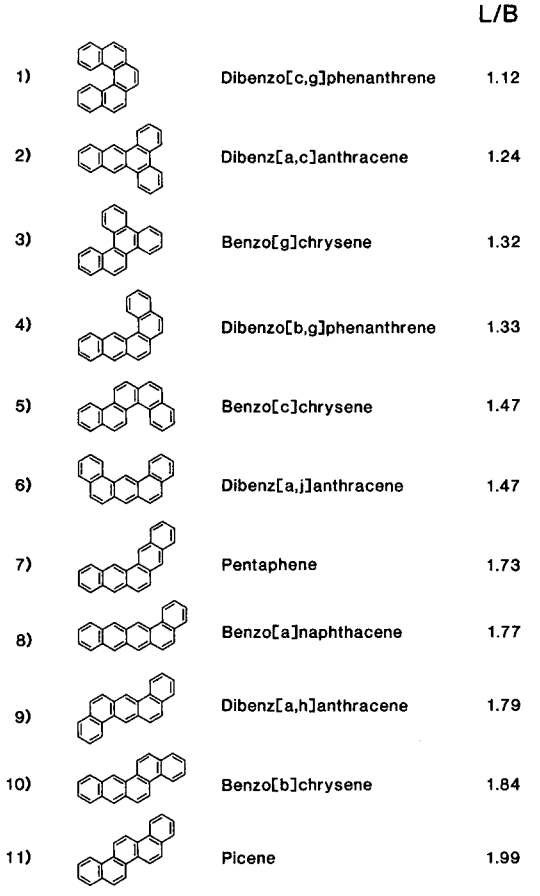

Fig. 4. Structures of five-ring PAH isomers used in this study. (L/B is the length-to-breadth ratio of the PAH).

TABLE I

RELATIVE RETENTION DATA; PAH OF MOLECULAR WEIGHT 278

Compound	*L/B*	*Relative retention*[a]		
		Polymeric C_{18}[b]	*Monomeric* C_{18}[c]	*Cyclodextrin*[d]
Dibenzo[*c,g*]phenanthrene	1.12	1.00	1.00	1.00
Dibenz[*a,c*]anthracene	1.24	3.37	1.53	2.57
Benzo[*g*]chrysene	1.32	1.98	1.40	1.91
Dibenzo[*b,g*]phenanthrene	1.33	2.41	1.57	1.77
Benzo[*c*]chrysene	1.47	3.37	1.67	2.30
Dibenz[*a,j*]anthracene	1.47	4.33	1.73	2.11
Pentaphene	1.73	5.50	1.92	3.57
Benzo[*a*]naphthacene	1.77	12.15	2.40	5.55
Dibenz[*a,h*]anthracene	1.79	7.01	1.87	2.99
Benzo[*b*]chrysene	1.84	12.62	2.22	4.10
Picene	1.99	16.84	2.26	3.82

[a] Retention relative to dibenzo[*c,g*]phenanthrene.
[b] Polymeric C_{18} (Vydac™ 201 TP) isocratic 95% aqueous methanol 1.5 ml/min 30°C.
[c] Monomeric C_{18} (Zorbax® ODS) isocratic 90% aqueous methanol 1.5 ml/min 30°C.
[d] β-Cyclodextrin (Cyclobond™) isocratic 50% aqueous methanol 1.5 ml/min 30°C.

retained longer than a bulky molecule) on the β-cyclodextrin as the four-ring PAHs above. Relative retention data of the isomers run on a polymeric C_{18}, a monomeric C_{18} and a β-cyclodextrin stationary phase are summarized in Table I. Relative retention data from Table I on each of the three phase types are also plotted *vs.* L/B ratios in Fig. 5. On the polymeric C_{18} column, the retention generally increases with increasing L/B ratio. In contrast, retention on the β-cyclodextrin is different than on either the polymeric or the monomeric C_{18} columns. Typical chromatograms of the isomer mixture run on each of the three columns are shown in Fig. 6. It is evident that a different retention mechanism is involved for the cyclodextrin phase when compared to traditional C_{18} phases.

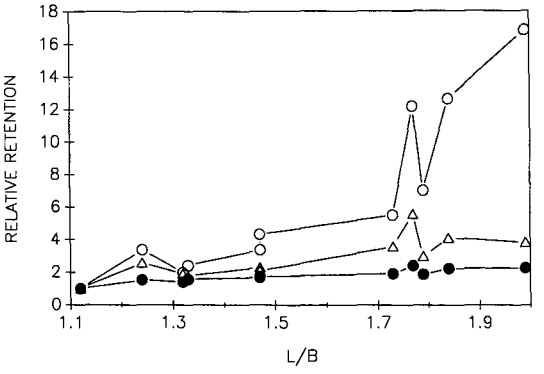

Fig. 5. Plots of relative retention *vs.* L/B ratios for five-ring PAH isomers on three different stationary phases. Retention is relative to dibenzo[*c,g*]phenanthrene. ○ = Polymeric C_{18}; ● = Monomeric C_{18}; △ = β-cyclodextrin.

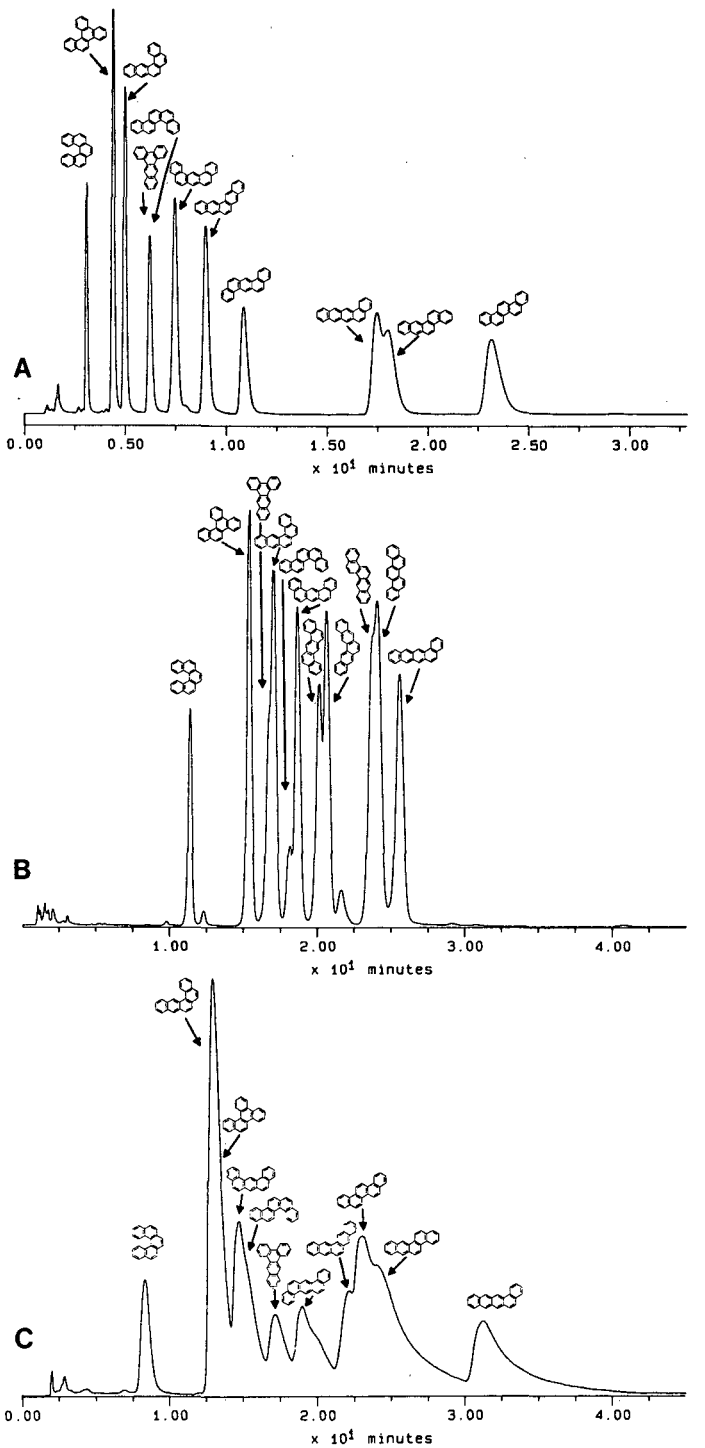

Fig. 6. Eleven five-ring PAH isomers separated on: (A) Polymeric C_{18} (Vydac™ 201 TP), isocratically methanol–water (95:5); (B) Monomeric C_{18} (Zorbax® ODS), isocratically methanol–water (90:10); (C) β-cyclodextrin (Cyclobond™ I), isocratically methanol–water (50:50). All chromatographic runs were performed at a flow-rate of 1.5 ml/min.

The retention of the pair benzo[*a*]naphthacene and picene clearly illustrates the difference between the mechanisms. The two molecules are narrow and well-retained on the polymeric C_{18}, monomeric C_{18} and cyclodextrin phases. Large differences do exist, however, in their relative retentions. Picene is the most rod-like of the eleven, as defined by the L/B ratio, and is thus retained the longest on the polymeric C_{18} phase. This trend has been observed by Wise and Sander[2] and is due to the high order of the polymeric phase, providing "slots" for long, narrow molecules to enter. On the cyclodextrin phase, picene cannot enter the β-cyclodextrin cavity longitudinally and therefore it will not be retained as long as benzo[*a*]naphthacene which has a single chain of four benzene rings in a row. The long narrow chain will enter deeply into the cavity and form a strong complex. This holds true for all of the eleven isomers; retention order is strongly dependent on how narrow the molecule is (*e.g.*, linear annelation of the benzene rings).

The elution order of these isomers (see Figs. 5 and 6) on the cyclodextrin column more closely resembles the elution order on the monomeric C_{18} than on the polymeric C_{18} column. This trend was observed primarily for solutes with L/B ratios of 1.7 and greater, *i.e.* later eluting solutes. However, elution order on the monomeric C_{18} of all eleven isomers is not identical to that on the cyclodextrin phase, and thus the mechanisms of retention for the two columns are different.

Differences in retention mechanisms are further illustrated by comparing the separation of PAHs of different molecular weights on the three types of columns. Retention data as k' values for several PAHs of varying molecular weight are summarized in Table II. On both monomeric and polymeric C_{18} columns, retention is largely dependent on the molecular weight of the solute. This trend is strongest for the monomeric C_{18} column where the three different molecular weights are divided into three distinct sections of the chromatogram. On the polymeric C_{18} column, retention is also dependent on the shape of the solute and the molecular weight dependence is less pronounced.

Retention on the cyclodextrin phase shows very little molecular weight dependence. The bulky five-ring benzo[*g*]chrysene elutes earlier than the long, narrow three-

TABLE II

k' VALUES OF THREE-, FOUR- AND FIVE-RING PAH ISOMERS

Compound	*Molecular weight*	k'		
		Monomeric C_{18}[a]	*Polymeric* C_{18}[b]	*Cyclodextrin*[c]
Phenanthrene	178	1.91	0.95	2.79
Anthracene	178	2.16	1.26	6.15
Triphenylene	228	3.77	2.69	3.06
Chrysene	228	4.57	4.82	6.07
Benzo[*g*]chrysene	278	7.78	5.94	5.34
Dibenz[*a,j*]anthracene	278	9.65	13.08	6.25

[a] Monomeric C_{18} (Zorbax® ODS) isocratic 90% aqueous methanol 1.5 ml/min 30°C.
[b] Polymeric C_{18} (Vydac™ 201 TP) isocratic 85% aqueous methanol 1.5 ml/min 30°C.
[c] β-Cyclodextrin (Cyclobond™ I) isocratic 50% aqueous methanol 1.5 ml/min 30°C.

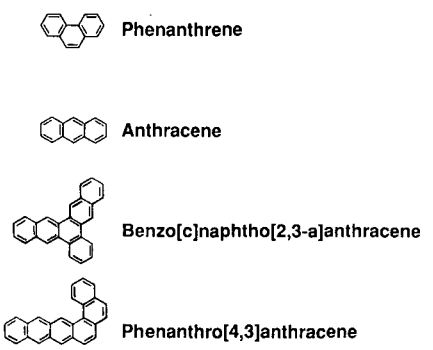

Fig. 7. Structures of PAHs listed in tables II and III.

ring anthracene. For the other PAHs in Table II, retention is dependent more on the ability of the solute to enter deeply into the cyclodextrin cavity than it is on the molecular weight of the solute. Only in cases of PAH molecules with similar shape and the same number of benzene rings in a row, will the heavier of the two molecules elute later (*e.g.*, three-ring phenanthrene elutes just before four-ring triphenylene). Several five- and six-ring PAH (for structures of six-ring isomers see Fig. 7) were also studied and the results are listed in Table III. The monomeric C_{18} column provides a separation based primarily on molecular weight with little differentiation between isomers. In contrast, the polymeric C_{18} is influenced profoundly by shape (L/B ratio) within an isomer group (*e.g.*, picene is the longest retained). For the cyclodextrin, the number of rings in a row acts as the dominant factor in determining retention, *e.g.*, benzo[*a*]naphthacene with four rings in a row is retained longer than the higher molecular weight six-ring isomers, whereas picene is the earliest eluting compound of the PAH in Table III.

An undesirable aspect of the cyclodextrin columns studied is the change in retention that occurs with time. Column equilibration times were found to be exces-

TABLE III

k' VALUES OF FIVE- AND SIX-RING PAH ISOMERS

For structures see Figs. 4 and 7.

Compound	*Molecular weight*	*k'*		
		Monomeric C_{18}[a]	*Polymeric* C_{18}[b]	*Cyclodextrin*[c]
Pentaphene	278	4.57	2.12	3.92
Picene	278	5.66	6.66	3.62
Benzo[*a*]naphthacene	278	5.84	4.65	5.64
Benzo[*c*]naphtho[2,3-*a*]anthracene	328	7.39	3.60	4.73
Phenanthro[4,3-*b*]anthracene	328	8.66	3.92	4.94

[a] Monomeric C_{18} (Zorbax® ODS) isocratic 97% aqueous methanol 1.5 ml/min 30°C.
[b] Polymeric C_{18} (Vydac™ 201 TP) isocratic 100% aqueous methanol 1.5 ml/min 30°C.
[c] β-cyclodextrin (Cyclobond™) isocratic 55% aqueous methanol 1.5 ml/min 30°C.

sive and frequent regenerations of the column were required to maintain column efficiency. Regeneration was accomplished by passing several column volumes of pure ethanol through the column, followed by pure water and methanol. Regeneration was found to be necessary even when aqueous organic mobile phases (no buffers) and pure standard solutions were used.

It can be concluded that in routine work, C_{18} columns provide better separation of PAH mixtures than cyclodextrin columns. Among C_{18} columns, the polymeric C_{18} column provides the highest selectivity, particularly for the separation of PAH isomers. However, cyclodextrin columns can offer unique selectivity and may be applied as an alternative method for separation of PAHs. The lack of retention dependence on molecular weight on the cyclodextrin phase may be advantageous for the separation of PAHs of different molecular weights. Chromatographic runs may be shortened and the use of solvent programming may be unnecessary in many cases. Another observation of using a cyclodextrin column is the lower amount of organic solvent needed for PAH separations compared to either monomeric or polymeric C_{18} phases.

RETENTION MODEL

Possible mechanisms of selectivity towards PAH on cyclodextrin bonded phases have been proposed by the column manufacturer[11]. Their results included retention data for a limited set of PAH isomers on the three types of columns, α- (6-glucopyranose units), β- and γ-cyclodextrin. In our work the mechanisms have been further investigated and the retention model for PAH isomers on β-cyclodextrin bonded phases has been extended. By using calculated widths of the different PAH isomers, we can determine whether the molecule is able to enter into and form a stable complex with the hydrophobic cavity. Width calculations were performed by adding two times the Van der Waal radius of hydrogen (1.2 Å) to the distance between hydrogen nuclei.

The inclusion complexing is illustrated in Fig. 8 using benz[*a*]anthracene as a model solute. The narrow part of the molecule is 7.3 Å. This portion of the molecule

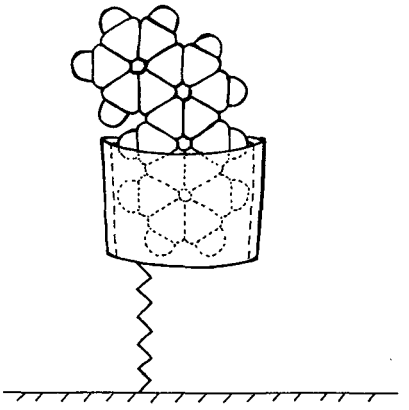

Fig. 8. PAH isomer benz[*a*]anthracene illustrates inclusion complexing with the β-cyclodextrin cavity. The narrow part of the molecule is 7.3 Å and able to enter the cavity, while the broader part (9.7 Å) is excluded.

will fit into the β-cavity of 7.8 Å while the broader part of the molecule, 9.7 Å, is excluded. A clear example of such exclusion results from molecules with an angular annelation of the benzene rings. As most PAHs are non-linear, only a portion of the molecule can participate in the complex. Molecules with linear annelation of the benzene rings ("rod-like" structures) will be able to enter deeper into the cavity.

This retention model takes into account only the shape of the molecule. Other interactions may also take place and affect solute retention. In this work it has been shown that the shape (the "rod-like" nature of the molecule) plays the most important role in solute retention. The longer the chain of single benzene rings (more narrow), the longer the PAH is retained on the cyclodextrin. Similar retention behavior was observed for larger PAH isomers with up to six condensed rings. For long narrow solutes, the molecule is able to enter the cavity completely. However, because the depth of the cavity is only 7.8 Å[13], the cavity is not deep enough for interaction along the full length of the solute. For example, the three-ring PAH anthracene has a molecular length of 11.5 Å and will not be completely covered by the cavity. Larger isomers containing such linear structures (*e.g.*, with the same stereochemistry as anthracene) will protrude, either at the mouth or at the bottom, from the cavity. Since retention is observed to increase with the addition of more aromatic rings in a row, the retention mechanism must include a solute-bonded phase interaction that occurs outside the cavity. Hydrophobic and electrostatic interactions may occur simultaneously with the inclusion complex, thus enhancing its stability.

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Journal of Chromatography, 477 (1989) 291–304
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 588

NEW METHOD FOR SURFACE CHARACTERIZATION BY GAS CHROMATOGRAPHY

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(First received January 31st, 1989; revised manuscript received April 20th, 1989)

SUMMARY

We report the development of a new chromatographic method for surface characterization, based on the principle of gas chromatographic intermediate surface testing. In this method, the chromatograms are obtained in parallel in a dual-oven chromatograph with a pneumatic switching valve. This is an unusual application of a dual-oven gas chromatograph and column switching, not previously reported. The method is fast, produces data that are more readily interpretable, and can provide information difficult or impossible to obtain with the previously developed single-oven, unswitched, sequential test. These new attributes make intermediate surface testing a simple, straightforward, and generally applicable method for studying chemical interactions at the gas-solid interface of low-surface-area materials.

INTRODUCTION

Dual-oven gas chromatographs and column flow switching devices are typically used to enhance the separation of complex mixtures through multidimensional chromatography[1–5]. We have developed an additional, novel application for these instruments by using them for intermediate surface testing[6]. This work is a result of the combination of successes and failures we experienced in using conventional intermediate surface testing to evaluate capillary tubing surfaces with a wide range of properties[7,8]. While the successes clearly demonstrated the power of intermediate surface testing for characterizing the surface chemistry of low surface area materials (which often can be studied only indirectly from analogous high surface area powders), the failures pointed out the several circumstances in which it is difficult or impossible to obtain meaningful information from a conventional intermediate surface test. In this paper we first describe the new two-oven, column-switched method we have developed, and we then present examples to illustrate the limitations of the conventional method and how the new one overcomes them.

THE NEW TWO-OVEN, COLUMN-SWITCHED METHOD

Basic principles

The new, dual-oven, column-switched method is shown schematically in Fig. 1a, and the conventional single-oven method[6] is shown for comparison in Fig. 1b. The upstream capillary (A) is a conventional gas–liquid chromatography column, the downstream capillary (B) is the material whose surface properties are to be characterized. Although the experimental procedures differ in the two methods, the basic principles are the same: The test consists of comparing the chromatograms obtained on column A alone and on A and B connected in series.

In both the conventional and the new methods, the function of upstream column A is simply to separate the components of a test mixture into a series of elution

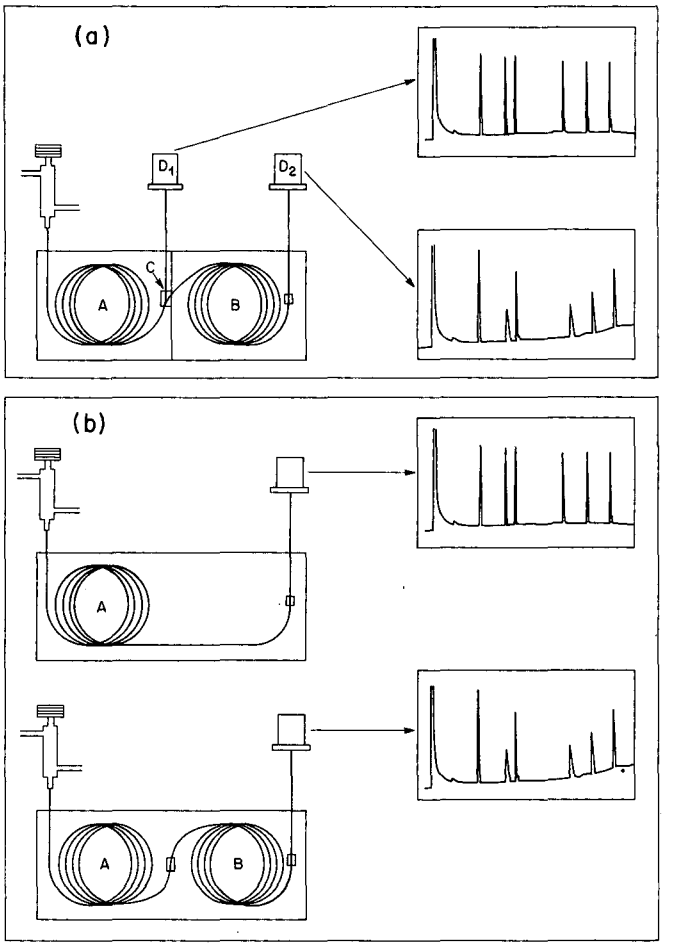

Fig. 1. (a) New dual-oven, column-switched method of intermediate surface testing. (b) Conventional method of intermediate surface testing. A = reference column; B = column to be tested; C = pneumatic switch; D_1, D_2 = detectors.

bands that have symmetrical peak profiles and are well-separated from the solvent band. (These "perfect" reference peaks could not be produced if the test mixture were injected directly onto the uncoated capillary to be tested.) If downstream capillary B is inert, these peaks elute from the coupled capillaries unchanged except for some symmetric band broadening and with an increased retention time corresponding to the dead time of the downstream capillary. However, if the downstream capillary is not inert, then changes in the shapes, areas, and relative retention times of the peaks provide information about both the nature and the strength of the components' interactions with the surface.

In the new method, the two capillaries are placed in different ovens of a dual-oven gas chromatograph and are connected to the six-port coupling device (C) of a pneumatic switch[9]. The switch diverts a small portion of the effluent of upstream capillary A to a detector (D_1), while transferring most of the material onto downstream capillary B, which is monitored by a second detector (D_2). The test mixture is injected onto the upstream column, and both the reference and test chromatograms are generated in the same experiment. The two ovens may be operated under different temperature conditions.

In contrast, in the original method, since columns switching is not employed, the reference and test chromatograms must be obtained in separate, sequential experiments. After the reference chromatogram is obtained on the upstream capillary alone, the capillary to be tested is installed between it and the detector, and the test mixture is chromatographed again. In addition, since only one oven is used, temperature conditions obviously must be the same for the two experiments.

Rationale for developing the new method

As the results below will show, the dual-oven, column-switched method has distinct advantages and, in retrospect, would seem to be the best approach to intermediate surface testing whenever the objective is to obtain specific information about capillary surface properties. However, it should be emphasized at the outset that the original single-oven method is quite adequate for the purpose for which it was originally developed, that is, as a quick and simple screening test to distinguish "good" from "bad" capillaries in the course of multi-step surface deactivation treatments[6]. For this purpose, interactions of simple one- or two-component test mixtures can be studied under isothermal conditions (see, for example, ref. 10).

However, when the objective of intermediate surface testing is to study capillary surface properties, test mixtures contain several components with a range of polarities and functional groups, and information about the surface chemistry of the downstream capillary is deduced from the behaviour of specific components[11]. With conventional single-oven testing, one is always constrained to choose between conflicting optimal temperature conditions for the upstream and downstream capillaries. For studying the downstream capillary, isothermal conditions are preferred so that all interactions of test components with the surface occur under identical conditions. In contrast, for generating the upstream reference chromatogram, temperature programming is preferred for two reasons: (1) so that test mixtures with several components encompassing a range of volatilities/polarities can be separated in a reasonable time in a single experiment, and (2) so that peaks elute from the upstream capillary with approximately constant widths. This makes the reference chromatogram a dis-

tinctive "fingerprint", so that additional band broadening and peak tailing caused by interactions with downstream column are easy to detect. (The most distinctive fingerprints can be obtained when the amount of each compound is adjusted for its detector response so that all peaks have equal areas. Under temperature programming, all peaks in the reference chromatogram will then have equal heights as well[12,13].)

In practice, for some applications, appropriate compromises can be made, and either isothermal or temperature-programmed conditions can be successfully applied[7,11,14,15]. However, this was not the case in our research program on the development and characterization of new capillary surfaces with a wider range of surface properties[7,8]. Two inherent limitations of the single-oven unswitched method became significant disadvantages: First, for a number of studies, it was impossible to find even a "compromise" temperature condition with which the test components of interest could be effectively separated on the upstream capillary and studied on the downstream one as well. Second, because it is necessary to disconnect the downstream capillary and generate a new reference chromatogram whenever either a test mixture or the temperature conditions are modified, the testing procedure became increasingly inefficient and time consuming.

It became clear that both of these problems could be overcome by using gas chromatographic instruments that have been developed for multidimensional techniques. Specifically, a dual-oven instrument would permit separate temperature control for the upstream and downstream column, and a column flow switch would enable test and reference chromatograms to be generated in the same experiment rather than sequentially.

EXPERIMENTAL

The chromatograph, a Sichromat 2 (Siemens, Karlsruhe, F.R.G.), was equipped with two flame ionization detectors, two split/splitless capillary inlets, and the requisite pneumatic components so that each oven can be operated separately as well as with column switching. Both ovens were equipped with auxiliary cryogenic cooling (liquid nitrogen) to assist in the maintenance of sub- and near-ambient temperatures. The upstream oven was equipped with a solenoid and needle valve for backflushing, which were not used for this application.

The six-port coupling piece of the pneumatic switch was located in the upstream oven and was used without modification. For this application, the switch was maintained in the "open" state, and the pressure drop across the switch was adjusted to divert about 10% of the effluent of the upstream column to the upstream detector, while transferring the remaining 90% to the downstream column. The principles of pneumatic switching are more fully described elsewhere[4,9,16].

Carrier gas (helium) flow was pressure-controlled at the inlet to the upstream column and at the midpoint of the system (pneumatic switch). The column dead time (retention time for an unretained peak[a]) was approximated from the retention time

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[a] Clearly, the concept of a non-retained peak is meaningless for a highly adsorptive capillary. If such a capillary is to be used in the downstream position, the necessary pressure drop must be calculated, or the linear velocity established experimentally by first substituting a non-adsorbing capillary (*e.g.* high-purity fused silica) of identical dimensions.

for *n*-butane. Inlet and mid-point pressures were adjusted to provide a linear velocity of 28 cm/s in both columns. The upstream oven was temperature-programmed from 40°C to 150°C at rates of 2.5°C, 5.0°C or 25°C/min. The downstream oven was operated isothermally at temperatures ranging from 40°C to 150°C. The injector and detector temperatures were 200°C and 250°C, respectively.

The upstream column was a standard 15 or 25 m × 250 μm I.D. capillary with a 0.2-μm film of immobilized methyl silicone stationary phase [*e.g.* Ultra 1 (Hewlett-Packard, Avondale, PA, U.S.A.) or SPB-1 (Supelco, Bellefonte, PA, U.S.A.)]. Uncoated capillary tubing tested in the downstream position was also 15 m × 200–250 μm I.D. Conventional fused-silica capillary tubing was obtained from several suppliers, and had been manufactured by Polymicro Technologies (Phoenix, AZ, U.S.A.) or Spectran (Sturbridge, MA, U.S.A.). Other capillary tubing, both pure and specifically doped fused silica, was manufactured in our laboratory, using the modified chemical vapor deposition process to prepare the preforms from which the capillaries were drawn[8].

The Grobs' "comprehensive standardized quality test" mix[12,13] and a "nonpolar test mix"[7] were purchased pre-mixed from Supelco. Other test mixtures of alkanes and ketones were prepared in-house from reagent-grade chemicals purchased from Aldrich (Milwaukee, WI, U.S.A.) and Burdick and Jackson (Muskegon, MI, U.S.A.). All samples contained 0.1–0.5 mg/ml per component in methylene chloride. Sample volumes were 1–2 μl, and the splitting ratio was 50:1–100:1 to deliver on-column approximately 2–5 ng per component. Sample components and their abbreviations are listed in relevant figure captions.

Signals from the detectors were recorded simultaneously on a dual-channel strip chart recorder (Model 7100B; Hewlett-Packard, Palo Alto, CA, U.S.A.) and either computing integrators (Model 3393; Hewlett-Packard, Avondale, PA, U.S.A.) or a PC-based (AT&T PC6300) chromatographic data system (System 2600, PE-Nelson, Cupertino, CA, U.S.A.).

RESULTS

The following four examples illustrate the limitations of the conventional method and the advantages of the new dual-oven column-switched method.

Studying adsorptive surfaces

When a downstream capillary surface is strongly adsorptive, high temperatures may be required to elute components of interest. However, these temperatures may exceed those at which good peak separations can be maintained on the upstream capillary. Conversely, if the test is performed at the lower temperatures or slower programming rates necessary to separate a test mix on the upstream capillary, components of interest may be totally adsorbed on the downstream capillary, and the intermediate surface test will provide little useful information.

This situation occurred in our studies of neodymia-doped fused silica, as illustrated in Fig. 2. Fig. 2a–c shows results for two different pure fused-silica capillaries (a, b) and a neodymia-doped capillary (c); Fig. 2d is the upstream reference chromatogram. All four chromatograms were obtained under identical temperature programmed conditions[7,8]. Note that three key test components, 2,6-dimethylphenol

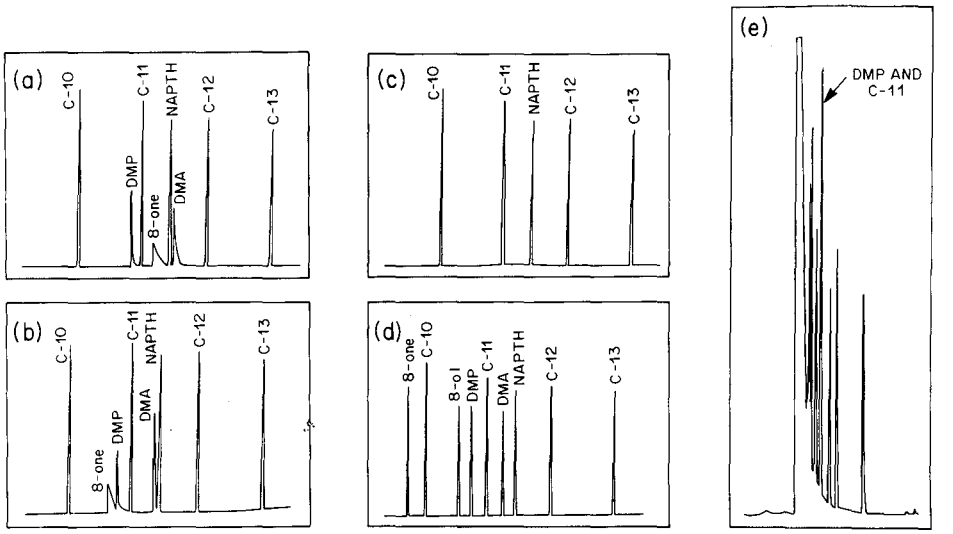

Fig. 2. (a–c) Conventional single-oven, unswitched intermediate surface tests of fused-silica capillaries, obtained under temperature-programming conditions as in refs. 7 and 8; a, b, two different samples of commercially available fused silica; c, neodymia-doped fused silica. (d) Upstream (reference) chromatogram obtained under same conditions as a–c. (e) Upstream (reference) chromatogram obtained isothermally at 150°C. Abbreviations: C-10 = *n*-decane; C-11 = *n*-undecane; C-12 = *n*-dodecane; C-13 = *n*-tridecane; DMP = 2,6-dimethylphenol; DMA = 2,6-dimethylaniline; 8-ol = *n*-octanol; 8-one = 2-octanone; NAPTH = naphthalene.

(DMP), 2,6-dimethylaniline (DMA), and 2-octanone (8-one), were totally adsorbed on the neodymia-doped capillary. Without peaks for these components, information cannot be deduced about acid/base properties (*e.g.* DMP/DMA ratios) and surface heterogeneity (peak shapes, esp. 8-one). Hence very little specific information is available about the neodymia-doped material.

Although the three compounds of interest could be eluted from the neodymia-doped capillary isothermally at 150°C, the results could not be easily interpreted because the upstream reference chromatogram (Fig. 2e) was poor. All test components eluted on the shoulder of the solvent peak, and C-11 and DMP co-eluted. To continue to study neodymia-doped surfaces with single-oven testing would have required the development of adequate reference chromatograms, for example by using longer and/or thicker-film columns and/or changing the components of the test mixture.

With the dual-oven, column-switched method, it was possible to raise the temperature of the neodymia-doped fused-silica capillary *without* changing either the composition of the test mix or the temperature program for the upstream capillary. Results are shown in Fig. 3. The upstream reference column was temperature programmed from 40°C to 150°C at 2.5°C/min, and the downstream column was held isothermally at 150°C. Peaks for DMP, DMA and 8-one can now be seen in the chromatogram. Since the reference chromatogram is unchanged (*cf.* Fig. 2d), the results may be compared with those obtained for the undoped fused silicas in Fig. 2a and b.

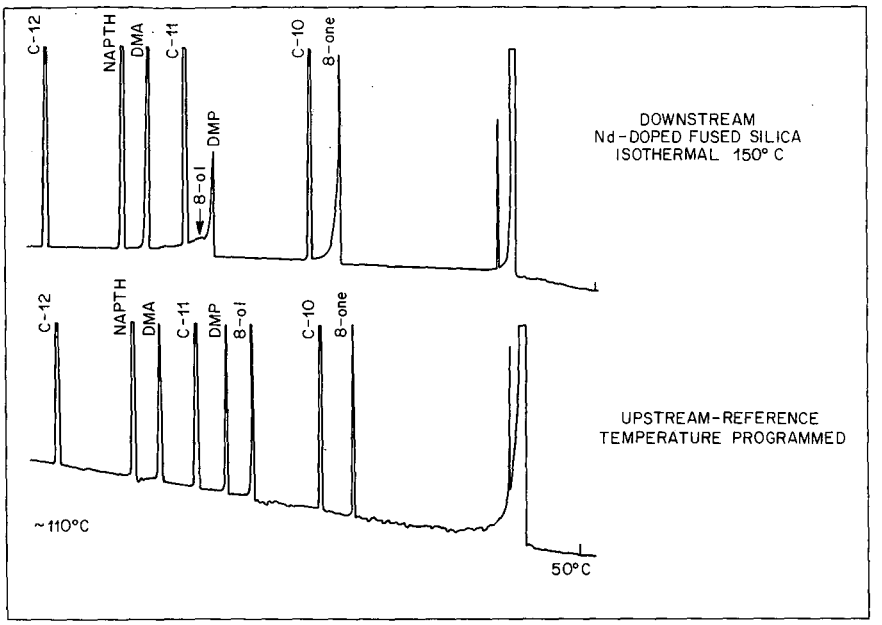

Fig. 3. Dual-oven, column-switched intermediate surface test of neodymia-doped capillary obtained isothermally at 150°C; upstream (reference) chromatogram temperature programmed as in Fig. 2c. Abbreviations as in Fig. 2.

Studying inert surfaces; studies at near-ambient temperatures

Single-oven intermediate surface testing also suffers from limitations when the downstream capillary must be studied at relatively modest temperatures —for example to study an inert surface, a weak adsorbate, or an adsorbent intended for operation at ambient conditions. When the downstream capillary imposes a relatively low upper temperature limit, low initial temperatures and/or slow programming rates must be used. Since the upstream capillary is constrained to the same conditions, either the reference chromatogram will be inordinately long, or the choice of test compounds will be limited to those which can be eluted from the upstream capillary in a reasonable time.

With two separately controlled ovens, significant time savings can be realized because the reference chromatogram can now be obtained at temperatures that exceed those permitted for the downstream capillary. For example, Fig. 4a and b shows chromatograms of the standard "non-polar test mix" obtained at programming rates of 25°C and 2.5°C/min. The corresponding temperature profiles are compared in Fig. 4c, with the elution temperatures of each component marked with a vertical tick-mark. For studies in which the temperature of the downstream capillary should not exceed *ca.* 100°C, only the slower temperature program can be used in a single-oven test. With two ovens, at least a fivefold savings in time can be achieved by using the faster programming rate to obtain the reference chromatogram, while still examining the downstream capillary at temperatures of 100°C or less.

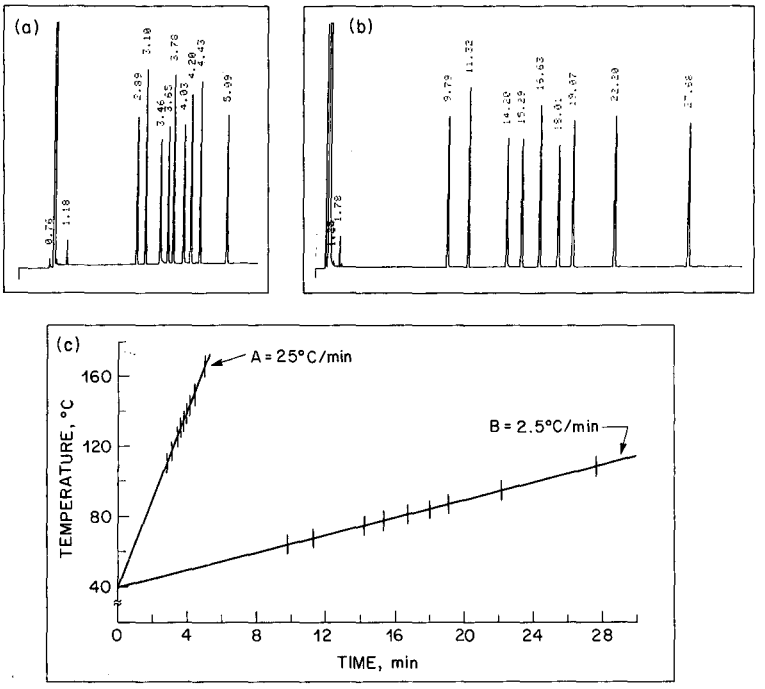

Fig. 4. (a, b) Upstream (reference) chromatogram obtained with initial temperature of 40°C and temperature-programming rate of 25°C/min (a) and 2.5°C/min (b). Numbers at the peaks indicate retention times in min. (c) Oven temperature profiles for the above programming rates, with elution time of each peak designated by vertical tick-mark.

Characterizing a surface at more than one temperature

In single-oven testing, it is difficult to study the temperature-dependence of surface properties in the downstream capillary because the upstream (reference) chromatogram also changes whenever the temperature and/or temperature program are changed. Even if one can account for the changing reference chromatograms, the study is time consuming, because one must repeatedly disconnect and reconnect the two columns to obtain a pair of reference (upstream alone) and test (upstream and downstream) chromatograms at each temperature. The dual-oven, column-switched method overcomes both of these problems. The temperature dependence of surface properties can be studied simply by changing the temperature of the downstream oven, without continually disconnecting and reconnecting the columns. Since the reference chromatogram remains constant, both qualitative and semi-quantitative data can be easily obtained from simple visual comparison of the chromatograms.

For example, Fig. 5 compares the intermediate surface tests of the neodymia-doped capillary at 100°C (a) and 150°C (b) obtained with the dual-oven, column-switched method. The reference chromatogram, obtained with temperature programming, is identical in both chromatograms. Differences in the downstream chromatograms at the two temperatures are immediately obvious: Both the retention time and peak shape improve much more for 8-one than for DMP and DMA when the

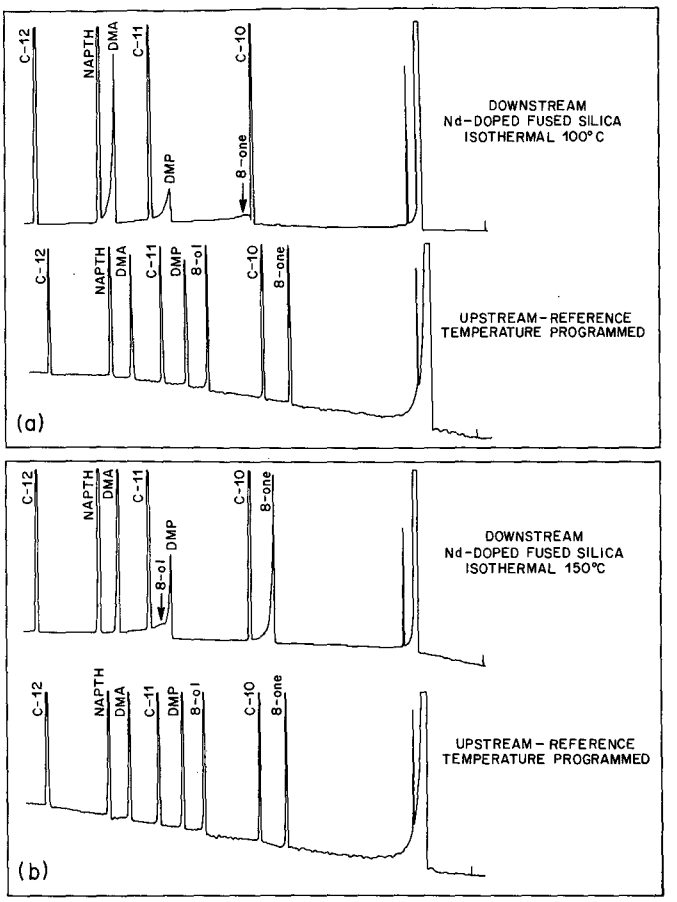

Fig. 5. Dual-oven, column-switched intermediate surface tests of neodymia-doped fused-silica capillary obtained at 100°C (a) and 150°C (b).

temperature is raised. For comparison, Fig. 6 shows the two significantly different reference chromatograms that would have been generated at 100°C and 150°C if the study had been done in one oven. Note that the pronounced tailing of the 8-one at 150°C would have been obscured in the solvent peak in the single-oven test although it is clearly visible in the dual-oven test.

Developing new test mixtures and optimizing test conditions

Obviously, if one wishes to change a test mixture or develop a new one, one must obtain a new reference chromatogram as well. In the old method, to do this requires repeatedly disconnecting and reconnecting the downstream capillary to obtain pairs of reference and test chromatograms. In the new method, the use of the column switch to couple the columns means that the reference and test chromatograms can be obtained in parallel in the same experiment. This results, in a minimum 50% reduction in the time required to complete the testing. Moreover, since the

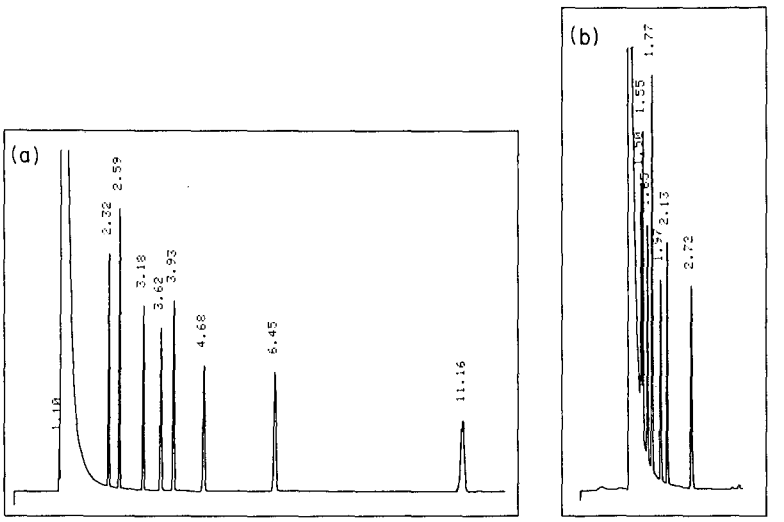

Fig. 6. Upstream (reference) chromatograms obtained isothermally at 100°C (a) and 150°C (b).

two columns remain coupled throughout testing, the possibility of making a "bad" butt-connection, with either excess dead volume or active sites, is virtually eliminated.

This feature of the new method was advantageous in our study of the adsorption of ketones on fused silica. It was of interest to determine if steric hindrance around the carbonyl group influences the adsorption. Fig. 7 compares results for 4-methyl-2-pentanone (a) and 2,4-dimethyl-3-pentanone (b) on a fused-silica capillary at 75°C. The stronger adsorption of the less substituted ketone (a) is immediately

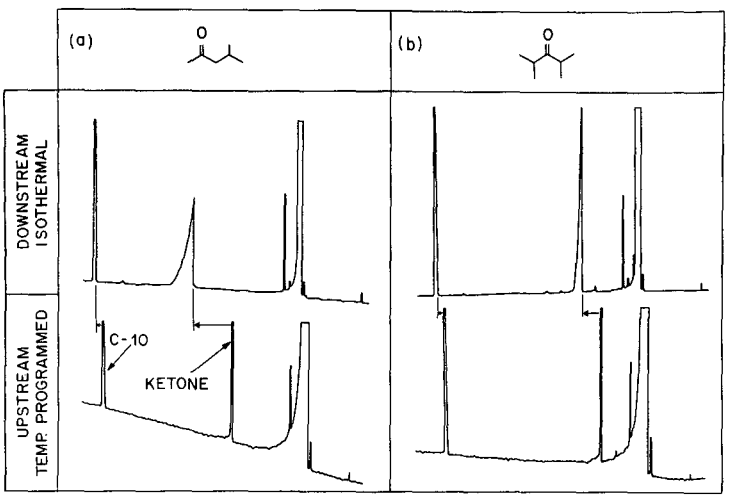

Fig. 7. Dual-oven, column-switched intermediate surface tests of 4-methyl-2-pentanone (a) and 2,4-dimethyl-3-pentanone (b) on commercially available fused-silica capillary at 75°C; *n*-decane included in test mixture; upstream (reference) chromatogram temperature programmed as in Fig. 2.

obvious in the longer retention time and greater broadening of the peak. (The rising baseline in the reference chromatogram of (a) is an artefact, as noted under *Instrumental performance.*) These results were obtained in two sequential experiments without disconnecting the columns. In contrast, to do this in a single-oven instrument would have required a minimum of four experiments, as well as the disconnecting and reconnecting of the two columns. Thus, a greater than 50% savings of time and effort was realized.

DISCUSSION

Instrumental performance

Just as in the original method, the new method requires that whatever device is used to couple the upstream and downstream capillary must not contribute either dead volume or adsorptive sites. The six-port coupling piece is significantly more complex than the simple butt connectors that can be used in the original method, and we were therefore concerned that it might be a source of significant adsorption. However, our experience is consistent with previously published reports[17,18]. Specifically, the coupling piece does not contribute to band broadening and adsorption or solvent tailing unless the upstream capillary is improperly installed. When this problem occurs, it affects the upstream chromatogram much more than the downstream one. Consequently, it is immediately obvious on the reference chromatogram and cannot be mistaken for a real result on the downstream column. For example, the rising baseline in the reference chromatogram in Fig. 7a was caused by organic contamination at the inlet to the coupling piece. Note that the downstream chromatogram is not similarly affected.

In the particular chromatograph we chose to use, the two ovens share a common raisable mantle. Oven temperature control is accomplished by raising and lowering the mantle, and the control mechanism always operates with the values of the upstream oven. For studies of adsorptive surfaces, in which the downstream oven is warmer than the upstream, problems can arise if the temperature difference between the ovens is greater than about 150°C. Heat conduction from the downstream oven overheats the upstream oven, causing the mantle to open and close periodically to keep the upstream temperature as close as possible to its set-point. The periodic changes in the temperature of the downstream oven cause corresponding fluctuations in the baseline signal, in which small peaks can be lost.

In principle, the problem can be circumvented by exchanging the electronics modules so that the temperature control mechanism is directed by the downstream oven. However, the analogous problem will then occur for applications in which the downstream oven is significantly cooler than the upstream. In either event, auxiliary cryogenic cooling can minimize the problem. Clearly, the problem can be eliminated by using an instrument in which each oven is equipped with a separate venting mechanism and temperature control. (Such an instrument was not commercially available when this study was undertaken.)

Since the primary function of the column switch is to serve as an effluent splitter, and no flow switching takes place during the chromatogram itself, it may appear that the use of the pneumatic switch is an overly complex approach. Although an effluent splitter could be implemented using a simple tee-piece and restriction capil-

lary, such an approach would be practical only if one were always investigating capillaries of identical dimensions. The pneumatic switch offers the advantage of being able to control the pressure at the midpoint of the system, and hence affords a simpler and more flexible means of studying capillaries with different dimensions.

Future directions; physico-chemical measurements

The capability of separate temperature control in the upstream and downstream ovens now provides the possibility of using intermediate surface testing to generate the data for physico-chemical measurements, such as isotherms, adsorption rates, and heats of adsorption. Our initial results show that this approach offers several advantages over existing gas–solid chromatographic methods in which the adsorbing surface is studied directly in a one-column experiment[19].

In a conventional one-column experiment, test compounds (and solvent, if any) are injected directly onto the surface to be tested. With intermediate surface testing, the upstream capillary separates test components from each other and from the solvent before they interact with the downstream capillary. Consequently, a number of compounds can be studied in the same experiment even if the adsorbing surface does not have sufficient selectivity to separate them itself. Additionally, the effects of solvent–adsorbate interactions or adsorbate–adsorbate interactions are minimized. Also, because the solvent peak passes down the capillary to be tested well in advance of all test components, the possibility that the solvent is perturbing the surface is minimized. (In addition, one could in principle use the pneumatic switch to dump the solvent to the upstream detector so that it would not influence the downstream column at all.)

With intermediate surface testing, information is obtained from the perturbation of an ideal or near-ideal peak by the surface of the downstream capillary. Therefore, changes in the peak profile arise only from interactions with the downstream capillary, and not from extra-column effects such as injection problems, which cannot be ruled out in one-column tests. In addition, small changes arising from weak interactions are easier to detect and quantitate on symmetric peaks than on the non-ideal peaks often obtained in one-column gas–solid chromatograms.

An obvious potential problem arises because when the column flows are pressure controlled, as is the case in most instruments. Because flow-rate varies inversely with temperature, retention time measurements obtained at different temperatures must be corrected for the changing dead-times. To overcome the problem, flow-controllers could be incorporated in an instrument, or the dead times could be measured, if a suitable non-adsorbing component were used.

With respect to the scope of the method, any material which can be drawn into capillary tubing can be studied. This can be an advantage: because of the large surface area of the capillary, low-surface-area materials such as glasses can be studied directly, rather than indirectly from analogous powdered samples (*e.g.* fused silica *vs.* silica gel). For the method to be as widely useful as possible, it should be applicable also to materials that are typically powders. Hence, the possibility of using packed micro-columns rather than capillaries in the downstream position is also being explored[20].

CONCLUSION

Intermediate surface testing is an unusual application for a dual-oven gas chromatograph and column switching, not previously reported. The new method has several significant advantages over single-column unswitched methods:

(i) Reference and test chromatograms are obtained in parallel rather than sequentially, thereby making possible the continuous, on-line monitoring of the upstream column. Consequently, results of true adsorption on the downstream column can be immediately distinguished from artefacts caused by a bad injection, sample degradation, etc.

(ii) When it is necessary to develop new test mixtures or to change chromatographic conditions, the new reference chromatogram is readily obtained without disconnecting the columns and reinstalling the upstream column alone.

(iii) Temperatures and temperature programs for the upstream and downstream capillaries can be set independently. The upstream capillary can be operated with temperature programming to generate a distinctive reference chromatogram in a reasonable amount of time. The downstream capillary can be operated isothermally, so that all components of the test mixture interact with it under identical conditions. Thus, both capillaries can be operated under optimal conditions, whereas with single-oven testing, the chosen conditions are always a compromise.

(iv) The downstream capillary can be studied as a function of temperature, with conditions for the upstream reference chromatogram held constant. This opens up the possibility of using intermediate surface testing to obtain physico-chemical data such as isotherms, heats of adsorption, etc.

In short, the new dual-oven, column-switched method is fast, produces data that are more readily interpretable, and can provide information difficult or impossible to obtain with a single-oven unswitched test. These new attributes make intermediate surface testing, originally developed solely to follow deactivation and coating steps in glass capillary chromatography columns, a simple, straightforward, and more generally applicable method for studying chemical interactions at gas–solid interfaces.

ACKNOWLEDGEMENTS

We thank Robert Slack and Rick Dougherty of ES Industries, Marlton, NJ, U.S.A., and Aachim Hein of Siemens AG for their assistance in optimizing the pneumatic switching system for our particular application.

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Journal of Chromatography, 477 (1989) 305–314
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE FERREDOXIN–THIOREDOXIN SYSTEM PROTEINS

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(First received January 20th, 1989; revised manuscript received April 17th, 1989)

SUMMARY

In order to separate the four proteins of the ferredoxin–thioredoxin system, their behaviour was studied on different high-performance liquid chromatographic columns. When the proteins were not activated, the mixture could be totally resolved by gel filtration chromatography on a TSK 3000 SW column, using a phosphate buffer containing 0.3 *M* sodium chloride, or by anion-exchange chromatography. Hydrophobic interaction chromatography did not allow a one-step separation. When the proteins were light activated and their cysteinyl residues derivatized with iodoacetate or iodoacetamide, filtration on the TSK 3000 SW column was found to be the only efficient method for separating the four proteins in a one-step process.

INTRODUCTION

Several plant chloroplast enzymes require activation by light in order to be catalytically active[1,2]. One mechanism responsible for the light regulation consists in a cascade of thiol-disulphide interchanges through the so-called ferredoxin–thioredoxin system. This system is composed of three different proteins: ferredoxin (Fd), ferredoxin–thioredoxin reductase (FTR) and thioredoxin (TRX)[3]. Among the activated target enzymes (fructose-1,6-bisphosphatase, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase, etc.) we have more closely studied the corn NADP-dependent malate dehydrogenase (NADP–MDH). In our laboratory, all the proteins involved in the light regulatory process are routinely purified, and a chloroplastic reconstituted system functioning *in vitro* has been developed, including photosynthetic membranes, Fd, FTR, TRX and NADP–MDH[4].

It has been shown that, during the light modulation process, NADP–MDH undergoes a post-translational modification, consisting in the reduction of one disulphide bridge on each subunit of the enzyme[5,6]. However, very little information is available about the intramolecular modification of the other proteins of the ferredoxin–thioredoxin system in the light-activated state, or about the redox regulation of this system. In order to obtain this information, it is necessary to isolate each of the

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four proteins of the system after reconstitution, light activation and thiol group derivatization by iodoacetic acid or iodoacetamide. Preliminary experiments have shown that NADP–MDH was unstable under these conditions, which excluded traditional purification procedures (1 week). Moreover, these procedures did not allow good yields with analytical injections. Hence a rapid and nearly quantitative procedure was necessary, which led us to develop a high-performance liquid chromatographic (HPLC) method. For this purpose we tested the separability of the four proteins of the ferredoxin–thioredoxin system by hydrophobic interaction, gel filtration and ion-exchange chromatography. In this paper, we report the results obtained in each instance and describe a method for separating the four proteins in one step.

EXPERIMENTAL

Proteins

Corn NADP–MDH was purified as described previously[7] and ferredoxin, ferredoxin–thioredoxin reductase and thioredoxin *m* were purified from spinach leaves as described[8–10]. Pea thylakoids were purified and stabilized as reported earlier[11].

HPLC

HPLC was performed at 20°C on a Laboratory Data Control system equipped with two minimetric pumps and a variable-wavelength UV detector monitored by a microcomputer (CCM). The following columns were used: TSK Phenyl 5PW, 75 × 7.5 mm I.D., (LKB) for hydrophobic interaction HPLC; TSK 2000 SW and TSK 3000 SW, 300 × 7.5 mm I.D., (Beckman) for gel-filtration (size-exclusion) HPLC; and poly(N-vinyldiimidazole) (PVDI), 100 × 4.6 mm I.D., (Société Française de Chromato Colonne) for anion-exchange HPLC. Proteins were detected at 280 nm.

Light-activation medium

The incubation medium (150 μl final volume) contained Tris–HCl, pH 8 (15 μmol), ferredoxin (23 μg), FTR (15 μg), thioredoxin (31 μg), NADP–MDH (32 μg) and chloroplast thylakoids (equivalent to 50 μg of chlorophyll). The activation was carried out for 15 min under nitrogen at 25°C under constant illumination at 300 W/m^2. Derivatization of new thiols was performed with [^{14}C]iodoacetic acid or [^{14}C]iodoacetamide for 15 min in the dark. Thylakoid membranes were removed by centrifugation and the supernatant injected directly on to the HPLC column.

Enzyme activity determination

After chromatographic separation, NADP–MDH was identified by its activity after activation in the presence of pure thioredoxin reduced by DTT[7]. TRX was identified by its ability to activate NADP–MDH in the presence of DTT[10]. FTR activity was determined in a complete reconstituted light-activation system[5] and Fd activity by measuring cytochrome *c* reduction in the presence of NADPH and pure ferredoxin–NADP reductase[12].

RESULTS AND DISCUSSION

Non-activated proteins

In a preliminary step, we studied the separation of the four chloroplastic pro-

TABLE I

RETENTION TIMES OF THE FOUR CHLOROPLASTIC PROTEINS AT TWO DIFFERENT FLOW-RATES ON A TSK PHENYL 5PW COLUMN

A linear 20-min gradient was used.

Flow-rate (ml/min)	*Retention time (min)*			
	NADP–MDH	*TRX*	*FTR*	*Fd*
1	20.0	20.0	4.2	9.4
2	17.2	17.8	4.2	6.3

teins without activation or subsequent derivatization. Several HPLC column supports were tested.

Hydrophobic interaction. A TSK Phenyl 5 PW column was used, and elution was performed with decreasing ammonium sulphate concentration from 1.8 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.2) using a linear gradient.

In Table I are reported the retention times (t_R) of the four proteins (Fd, FTR, TRX and NADP–MDH). Two flow-rates were tested with a 20-min gradient time. In this chromatographic system, FTR and Fd were well resolved, whereas NADP–MDH and TRX had similar retention times. As shown in Table I, increasing the flow-rate did not improve the separation significantly.

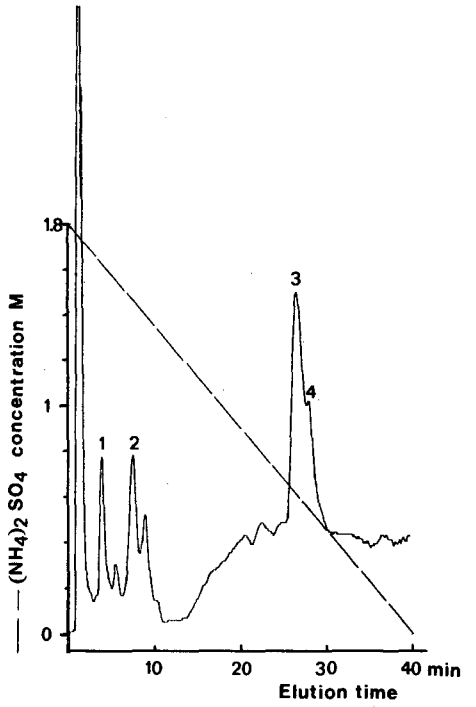

Fig. 1. Chromatogram of a protein mixture containing FTR (peak 1), Fd (peak 2), NADP-MDH (peak 3) and TRX (peak 4), obtained on a TSK Phenyl 5PW column at a flow-rate of 2 ml/min with a 40 min-linear gradient.

Variation of t_R with the gradient time, from 20 to 90 min, was investigated: TRX and NADP–MDH always coeluted (data not shown). Fig. 1 shows a typical chromatogram of the four-protein mixture, obtained with a 40-min gradient time and a flow-rate of 2 ml/min. As checked by absorbance measurement at 280 nm, the recovery of each protein ranged from 80 to 90%.

Gel filtration. As the molecular masses of NADP–MDH and TRX are different (80 and 12 kDa, respectively), the mixture of these two proteins should be resolved by gel filtration. We therefore tested two filtration columns, TSK 2000 SW and TSK 3000 SW.

Fig. 2 shows the separation of NADP–MDH from TRX on a TSK 2000 SW column, eluted with 30 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 0.5 ml/min. The two proteins eluted as sharp peaks; NADP–MDH eluted first at 15 min, followed by TRX, which was retained for 19 min. On this matrix, NADP–MDH and FTR (30 kDa) were not completely separated (data not shown).

Hence hydrophobic interaction chromatography followed by gel filtration on TSK 2000 SW allows the complete resolution of the four-protein mixture. If gel filtration columns are not available, separation of NADP–MDH from TRX can also be achieved rapidly be several ultrafiltrations on a Centricon 30 microconcentrator, which selectively retains proteins having a molecular mass >30 kDa. NADP–MDH is recovered in the retentate in a concentrated form and TRX is recovered in the filtrate and can be concentrated on a Centricon 10 microconcentrator.

Table II shows the molecular masses of the four proteins and their corresponding retention times when each protein was injected separately onto a TSK 3000 SW

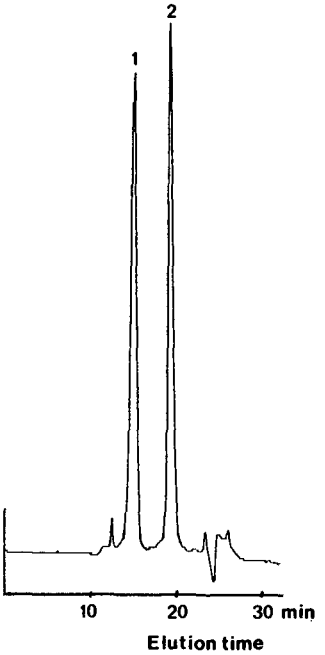

Fig. 2. Chromatogram of a protein mixture containing NADP–MDH (peak 1) and TRX (peak 2), obtained on a TSK 2000 SW column at a flow-rate of 0.5 ml/min.

TABLE II

MOLECULAR MASSES (MM) AND RETENTION TIMES OF THE FOUR CHLOROPLASTIC PROTEINS AFTER INJECTION OF EACH PROTEIN SEPARATELY ON A TSK SW 3000 COLUMN

The column was equilibrated with 30 m*M* Tris–HCl (pH 7.9) and the elution was performed with the same buffer at a flow-rate of 0.5 ml/min.

Protein	*MM (kDa)*	t_R *(min)*
NADP–MDH	80	12.6
Fd	11	13.8
FTR	32–34	14.7
TRX	12	16.9

column. Fd and TRX, having the same molecular mass, were expected to coelute on this column. However, Fd presents an abnormal retention time, as it eluted before FTR and TRX, hence with an apparently higher molecular mass than expected. With respect to this phenomenon, it is interesting that in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Fd migrates atypically as a 20-KDa protein[13].

Although the four proteins exhibited different retention times when injected individually, the elution profile obtained when the mixture was injected showed only three peaks (Fig. 3A), which means that two proteins coeluted. In order to determine which proteins coeluted, different mixtures of three proteins were tested: NADP–MDH + Fd + TRX (Fig. 3B) and NADP–MDH + FTR + TRX (Fig. 3C). In both instances three peaks were obtained, with the expected retention times. However, NADP–MDH + FTR + Fd (with Fd in excess over FTR) showed only two peaks with t_R = 12.6 and 13.7 min, corresponding to NADP-MDH and Fd, respectively (data not shown). On the other hand, the mixture of Fd and FTR with a 1:1 stoichiometry showed only one peak with a shorter t_R (12.4 min) (data not shown). This set of results strongly suggests that Fd and FTR form a complex that is eluted with the same t_R as NADP–MDH. This is not surprising as FTR is routinely purified by affinity chromatography on a ferredoxin – Sepharose column[9]. This hypothesis was confirmed by performing the same experiment in the presence of 0.3 *M* sodium chloride in order to dissociate the complex: two peaks were observed, which corresponded to FTR (t_R = 19.7 min) and Fd (t_R = 21 min), these values being identical with those observed with separate injections of each protein in the presence of sodium chloride (data not shown). Remarkably, under these conditions Fd was retained for a longer time than FTR, in contrast with its behaviour in the absence of sodium chloride.

These results are in agreement with those recently reported by Hirasawa *et al.*[14], who showed that FTR formed an electrostatically stabilized 1:1 complex with Fd.

Following these observations, the mixture of the four proteins was then chromatographed on a TSK 3000 SW column in the presence of 0.3 *M* sodium chloride. Four peaks were obtained (Fig. 4); each was tested for its enzymatic activity and its purity checked by SDS-PAGE. The results are summarized in Table III. As checked by absorbance measurement at 280 nm, the recovery of each protein was about 90%.

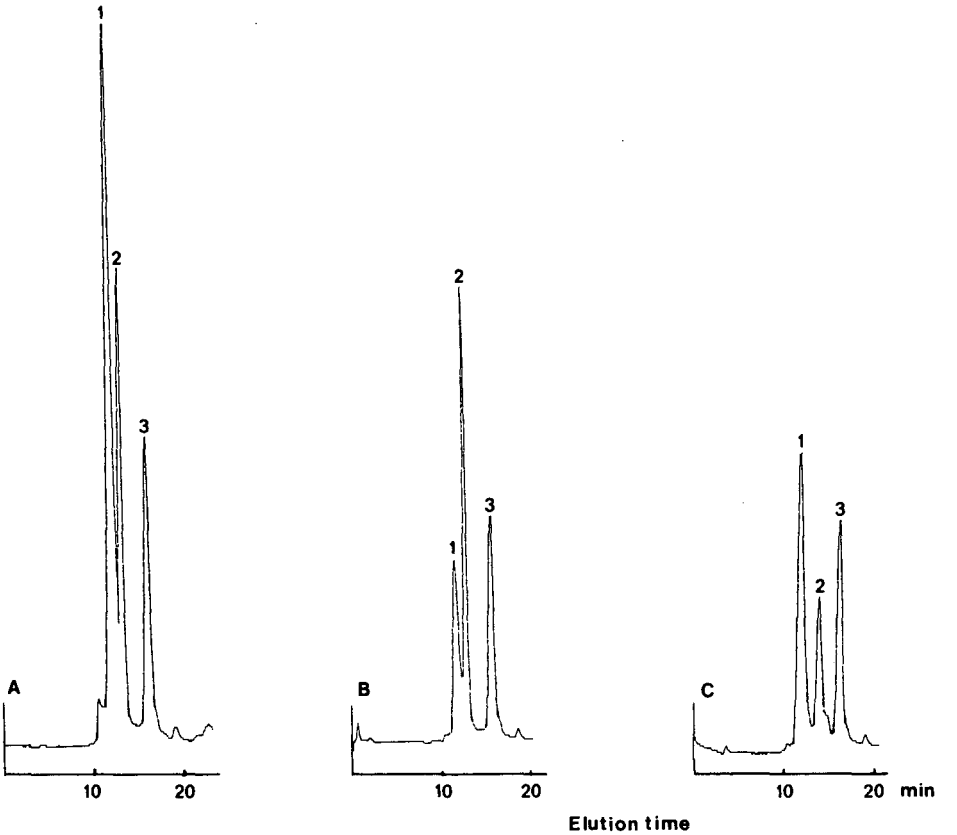

Fig. 3. Chromatograms of protein mixtures containing (A) NADP–MDH, FTR, Fd (in excess over FTR) and TRX, (B) NADP–MDH (peak 1), Fd (peak 2) and TRX (peak 3) and (C) NADP–MDH, (peak 1), FTR (peak 2) and TRX (peak 3), obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min.

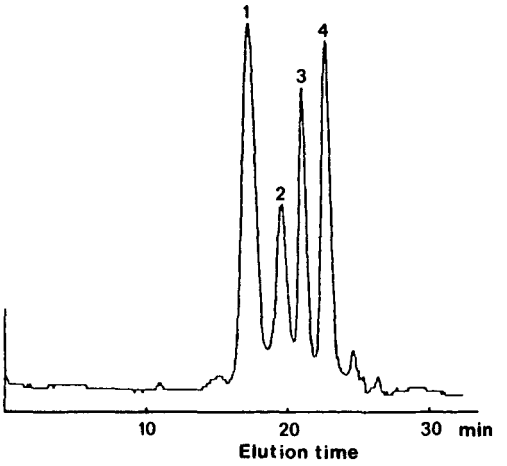

Fig. 4. Chromatogram of a protein mixture containing NADP–MDH (peak 1), FTR (peak 2), Fd (peak 3) and TRX (peak 4), obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min in the presence of 0.3 *M* sodium chloride.

TABLE III

RETENTION TIMES, ENZYMATIC ACTIVITIES AND ELECTROPHORETIC ASSIGNMENTS OF THE FOUR PEAKS OBTAINED AFTER CHROMATOGRAPHY OF THE PROTEIN MIXTURE ON A TSK 3000 SW COLUMN IN THE PRESENCE OF 0.3 *M* SODIUM CHLORIDE

The column was equilibrated with 30 m*M* Tris–HCl (pH 7.9) containing 0.3 *M* sodium chloride and elution was performed with the same buffer at a flow-rate of 0.5 ml/min. The enzymatic activities were checked as described under Experimental.

Peak	t_R *(min)*	*Enzymatic activity*	*Electrophoretic assignment*
1	17.4	NADP–MDH	NADP–MDH
2	19.6	FTR	FTR
3	21	Fd	Fd
4	22.6	TRX	TRX

Hence, chromatography on TSK 3000 SW in the presence of 0.3 *M* sodium chloride allowed the rapid separation of the four proteins in only one step.

Ion exchange. The third matrix tested was an anion-exchange support, PVDI. Fig. 5 shows an elution pattern of the four-protein mixture obtained with a 30-min

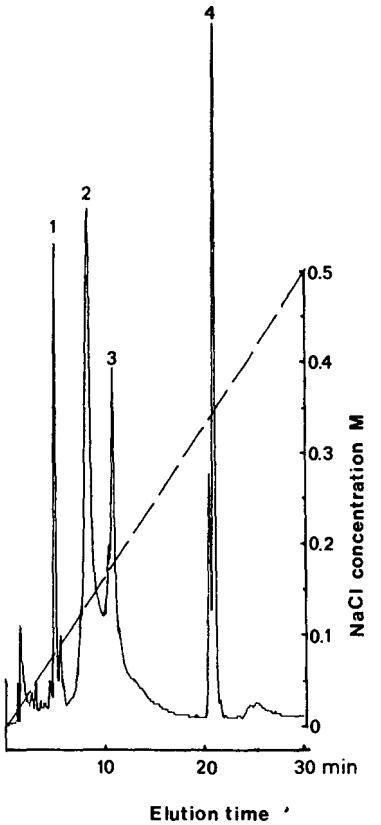

Fig. 5. Chromatogram of a protein mixture containing TRX (peak 1), NADP–MDH (peak 2), FTR (peak 3) and Fd (peak 4), obtained on a PVDI column at a flow-rate of 1 ml/min using a 30-min linear 0–0.5 *M* sodium chloride gradient in 30 m*M* Tris–HCl buffer (pH 7.5).

linear gradient from 0 to 0.5 *M* sodium chloride in Tris–HCl buffer. TRX eluted first, as a sharp peak, followed by NADP–MDH and FTR, and finally Fd at the end of the gradient. A minor contaminant of this Fd preparation, which eluted slightly before the major peak, was separated.

The variation of the t_R with the gradient time (from 20 to 60 min) was investigated. In this range, the four proteins were well resolved (data not shown). Therefore, a 30-min gradient can be used routinely to separate these four proteins and the recovery of each was *ca.* 70–80%. Similar results were obtained with a Mono-Q column (Pharmacia).

Hence a one-step separation was also achieved by ion-exchange chromatography on a PVDI column.

Light-activated proteins

In a second step, we studied the separation of the same proteins after light activation and thiol group derivatization with [^{14}C]iodoacetate. After such a treatment, FTR, TRX and NADP–MDH were recovered as ^{14}C-carboxymethylated proteins whereas Fd, which does not contain any derivatizable thiol group, was not modified[5]. The same columns were tested.

Hydrophobic interaction. Identical results were obtained as for non-derivatized proteins (data not shown).

Gel filtration. As the molecular masses are not significantly modified after derivatization, the same chromatographic behaviour was expected as for non-activated proteins. In fact, after activation and thiol derivatization performed with [^{14}C]iodoacetamide, the mixture was totally resolved on TSK 3000 SW in the presence of 0.3 *M* sodium chloride, although the peak containing derivatized NADP–MDH (peak 1) was slightly broadened (Fig. 6). SDS-PAGE revealed (data not shown) that each fraction had the same assignment when the proteins were derivatized, *i.e.*, NADP–MDH eluted first, followed by FTR (peak 2), Fd (peak 3) and TRX (peak 4). Derivatization with iodoacetamide did not lead to significantly different t_R in comparison with non-derivatized proteins. However, when derivatization was performed with iodoacetate, TRX showed a shorter t_R of 21.8 min instead of 22.6 min (data not shown). This unexpected behaviour could be explained by ionic interactions between the matrix of the column and the protein.

Ion exchange. Fig. 7 shows the elution profile obtained after injection of the mixture of light-activated proteins on a PVDI column using a 40-min linear gradient. Derivatization was performed with [^{14}C]iodoacetate, and each fraction was analysed by SDS-PAGE and its radioactivity counted. [1-^{14}C]carboxymethyl-TRX was recovered in a purified form in peak 1. Peak 2 (t_R = 14.3 min) was not radioactive and SDS-PAGE revealed that it contained NADP–MDH (i.e., the fraction of protein that had not been activated and hence had no thiol group available for derivatization). Peak 3 contained a mixture of [1-^{14}C]carboxymethyl-NADP–MDH and [1-^{14}C]carboxymethyl-FTR and, as expected, Fd was recovered in peak 4. In this assay, NADP–MDH was not totally reduced. In the cases where it was, peak 2 was not present in the elution pattern. The different retention time of carboxymethylated NADP–MDH, compared with underivatized NADP–MDH, could be explained by the presence of several new negative charges. Nevertheless, when iodoacetamide was used instead of iodoacetate, similar results were unexpectedly obtained.

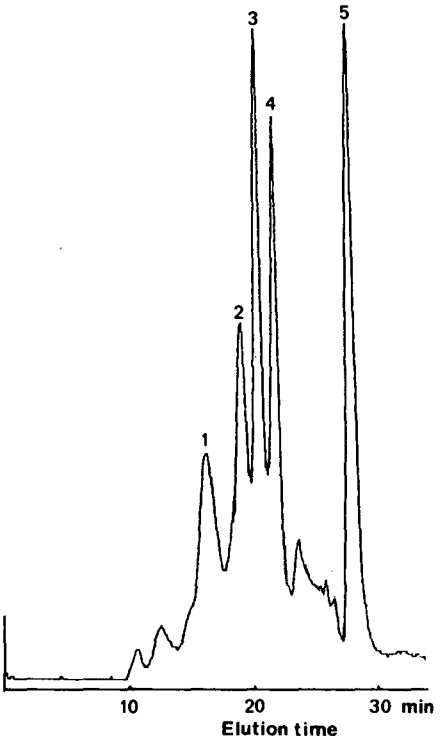

Fig. 6. Chromatogram of the light-activated protein mixture after derivatization with iodoacetamide, obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min in the presence of 0.3 *M* sodium chloride. Peak 5: excess iodoacetamide.

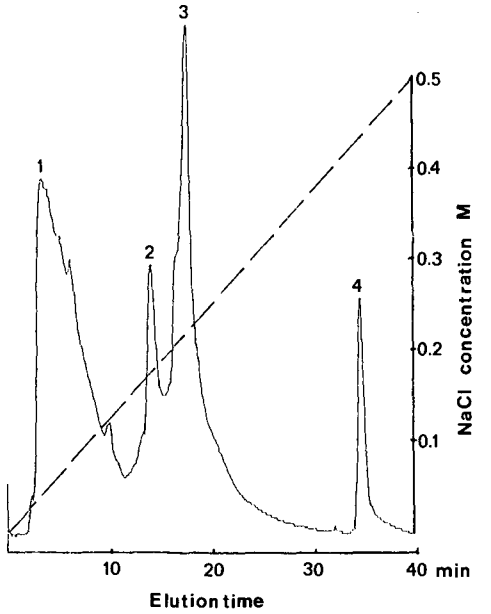

Fig. 7. Chromatogram of the light-activated protein mixture after derivatization with iodoacetate, obtained on a PVDI column at a flow-rate of 1 ml/min using a 40-min linear gradient.

Therefore, ion-exchange chromatography is less efficient when the protein mixture is activated and derivatized, as FTR and NADP–MDH cannot be separated.

CONCLUSION

Several HPLC column matrices (gel filtration, ion-exchange, hydrophobic interaction) have been tested in order to separate rapidly the four proteins involved in the *in vitro* enzyme light-activation system: thioredoxin, ferredoxin, ferredoxin–thioredoxinreductase and NADP–malate dehydrogenase. When proteins were not activated, hydrophobic interaction on a TSK Phenyl 5PW column allowed a good separation between FTR, Fd and a mixture containing TRX and NADP–MDH to be obtained. TRX was further resolved from NADP–MDH by gel filtration on a TSK 2000 SW column or by ultrafiltration on a Centricon 30 microconcentrator. Alternatively, the four-protein mixture could be completely resolved on a TSK 3000 SW column using a buffer containing 0.3 *M* sodium chloride.

Another attractive chromatographic system is anion-exchange chromatography on a PVDI or Mono-Q column, which allowed a one-step separation of each protein.

When proteins were light activated and then carboxymethylated, ion-exchange chromatography could not be used satisfactorily. However, gel filtration remained a rapid and efficient method for resolving the mixture, provided that sodium chloride is present in the filtration medium in order to dissociate the Fd–FTR complex.

ACKNOWLEDGEMENTS

We thank Professor P. Gadal and Dr. M. Hodges for reading the manuscript.

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Journal of Chromatography, 477 (1989) 315–325
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 569

NON-AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY COUPLED ON-LINE TO REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INTERFACE DEVELOPMENT AND APPLICATIONS TO THE ANALYSIS OF LOW-MOLECULAR-WEIGHT CONTAMINANTS AND ADDITIVES IN FOODS[a]

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(First received May 6th, 1988; revised manuscript received April 14th, 1989)

SUMMARY

An interface has been developed which permits the on-line coupling of size-exclusion chromatography in tetrahydrofuran with aqueous reversed-phase high-performance liquid chromatography. The interface isolates the required size exclusion chromatography fraction and dilutes it with water to ensure reconcentration of analytes on the reversed-phase column prior to gradient elution. Operational parameters and the influence of analyte polarity have been examined in detail. A predictive system is presented for determining the applicability of the system to any analyte, based on solute retention times on an ODS phase eluted with a methanol–water gradient. The method is illustrated with examples of direct analyses of crude lipid extracts from a snack product for 2,6-di-*tert.*-4-methylphenol and from chocolate for dibutyl phthalate. Detection limits of *ca.* 0.5 mg/kg have been achieved.

INTRODUCTION

Few trace-level contaminants may be determined directly in foodstuffs. They must usually be separated from the bulk of the food prior to analysis by relatively non-selective methods such as solvent extraction. The majority of measurements are made using chromatographic techniques and the complexity of food extracts places severe demands upon the performance of chromatography columns. Even where

[a] This article is based in part on work presented as a poster at the *3rd Symposium on Handling of Environmental and Biological Samples in Chromatography, Palma de Mallorca, October 1986.*

immunological methods are employed, sample preparation requirements are often stringent in order to avoid matrix effects.

Davis and Giddings[1] have estimated that a column efficiency of *ca.* 200 000 theoretical plates is required to give a 90% probability (at a resolution, R_s, of 1.0) that on chromatography of a sample containing 20 randomly chosen compounds, individual peaks will be due to a single component. Martin *et al.*[2,3] have presented an analysis which indicates that the separation problem is even more severe. In contrast, the efficiency of a typical high-performance liquid chromatography (HPLC) column is 10 000–20 000 plates and for capillary gas chromatographic (GC) columns, 50 000–100 000 plates. Some analytes possess properties permitting selective detection (by electron capture or fluorescence for example), while proper choice of chromatographic phase selectivity may also alleviate the problem. Nevertheless, in most cases extensive cleanup of food extracts is necessary in order to achieve an acceptable degree of confidence in the identification of the target compound.

Current methods of cleanup often require a laborious series of solvent partition and low-resolution chromatography stages. The time assigned to cleanup in many cases accounts for 80–90% of the entire analysis and this is an unacceptable diversion of manpower from other more productive work. It is not unusual for a batch of six samples to represent one day's throughput for a skilled analyst. In addition, manual processing of samples is a tedious task which however requires constant attention to detail in order to achieve consistent results. Thus there is a demand for the automation of sample cleanup.

The application of sequential chromatographic stages (multi-dimensional chromatography) to resolve components of complex mixtures is well known. The method is often carried out off-line but this is not desirable in routine analysis. On-line multi-dimensional separation is in principle simple, but practical considerations have limited its utility. Two (or more) columns of differing selectivity are connected via a switching valve. The analyte peak from the first column is diverted onto the second under conditions chosen such that the analyte is strongly retained. Step or gradient elution of the second column separates the analyte from compounds co-eluting from the initial column. Many HPLC instruments provide timed events capable of controlling external devices, facilitating the automation of column switching methods.

The choice of chromatographic modes to couple depends to some extent upon the nature of the sample and in particular the solubility of target compounds. However, if optimum results are to be obtained, it is important to select modes which have different underlying mechanisms. Reversed-phase HPLC and size-exclusion chromatography (SEC) offer complementary advantages. Reversed-phase chromatography depends upon enthalpic differences between solutes. Its selectivity may be varied between fairly wide limits by suitable choice of conditions but column capacity is relatively limited. SEC is highly predictable —all solutes elute within one column volume — and thus automation is facilitated. Because it is non-enthalpic and does not involve interactions of solutes with column packings, sample loadings can be relatively high. However, separation efficiency is limited. The closely related technique of gel permeation chromatography (GPC) has been applied in selected areas of lipid cleanup[4,5], notably for organochlorine and organophosphorus pesticides. An automated system was devised by Tindle and Stalling[6] and this is commercially available. Recently Hopper and Griffitt described[7] an accessory to this system which automat-

ically concentrates the eluent fraction containing the desired compound into a GC autosampler vial ready for immediate injection.

On-line column switching is facile if the eluent employed with the first column is non-eluting for the analyte when on the second column. Thus it is a simple matter to combine aqueous SEC with reversed phase HPLC[8–12]. The only interface required is a standard high-pressure valve; most common injection valves may be employed and electric or pneumatic actuators are readily obtainable. However, many analytes and residues have limited solubility in water, restricting the utility of this method.

The critical aspect of any column switching method is the refocusing of analyte at the head of the second column. HPLC peaks are typically 0.5–1.0 ml in volume, which, unless reconcentrated on transfer, would give rise to an inferior performance on the second column. When the two modes of chromatography employed are incompatible in the sense that the mobile phase for the primary separation is a strong eluent in the second, the only general method available for their on-line combination entails heart-cutting. In this approach, the volume of analyte peak transferred is restricted in order to limit degradation of second column performance. However, sensitivity is often unacceptably reduced. Johnson *et al.*[13] employed heart cutting between SEC in tetrahydrofuran (THF) and reversed-phase chromatography to determine the pesticide malathion on tomato plants, but with a detection limit of 200 mg/kg.

This communication describes a system for combining on-line non-aqueous SEC with reversed-phase chromatography which achieves detection limits (with phthalates, for example) of less than 1 mg/kg.

EXPERIMENTAL

Apparatus and materials

The system consisted of a Waters (Millipore, Harlow, U.K.) Model 6000A pump; a Gilson (Anachem, Luton, U.K.) Model 231–401 diluter and autosampler fitted with a 500-μl loop; an LKB (Milton Keynes, U.K.) Model 2150 pump; a Rheodyne Model 7010 valve injector fitted with a 5-ml loop and pneumatically operated actuator; a Gilson gradient chromatograph, Model 702, controlled by an Apple IIe microcomputer with external events module 501; a Spectra-Physics Model SP8773 UV detector or Perkin-Elmer (Beaconsfield, U.K.) LS 4 fluorescence detector; and a Trivector (Sandy, U.K.) Trilab 2000 data station. Other components used included a zero dead volume "T" (Valco); a precolumn filter containing a 2-μm frit (Upchurch); and a detector outlet pressure restrictor set at about 2 bar (Upchurch).

A high-performance poly(styrene–divinylbenzene) size-exclusion column, 100 Å PLgel, (5 μm, 300 × 7.7 mm), was obtained from Polymer Labs. (Church Stretton, U.K.). Spherisorb ODS reversed-phase columns (250 × 4.9 mm, 5 μm), were from Hichrom (Reading, U.K.). All organic solvents used were of HPLC quality, from Rathburn Chemicals (Walkerburn, U.K.). HPLC grade water was purchased from Fisons (Loughborough, U.K.). Food samples were purchased locally from normal retail outlets.

Lipid extraction

A weighed amount of food was homogenised in 100 ml acetone–hexane (1:1

v/v). Where required, samples were spiked before homogenisation and left to stand overnight. The residue was filtered and re-extracted with a further 100-ml aliquot of the same solvent. The combined filtrates were dried over sodium sulphate and solvent removed by rotary evaporation. The mass of fat obtained was recorded and the lipid stored at −18°C until required. Lipid residues were dissolved in toluene for coupled column analysis.

Chromatography

Size exclusion. Samples dissolved in toluene were placed in the autosampler and 100-μl aliquots injected by partial loop fill. For all analytes an autosampler cycle time of 50 min was selected to allow for completion of the remainder of the analysis. As mobile phase THF was used at 1.0 ml/min.

Interface conditions. During SEC fractionation of the crude lipid extract, water was pumped into the "tee" union at the outlet of the SEC column (Fig. 1) at a constant 4.0 ml/min. At a time dependant upon the SEC retention of the target compound, the interface valve was switched to load the trapping loop (initially filled

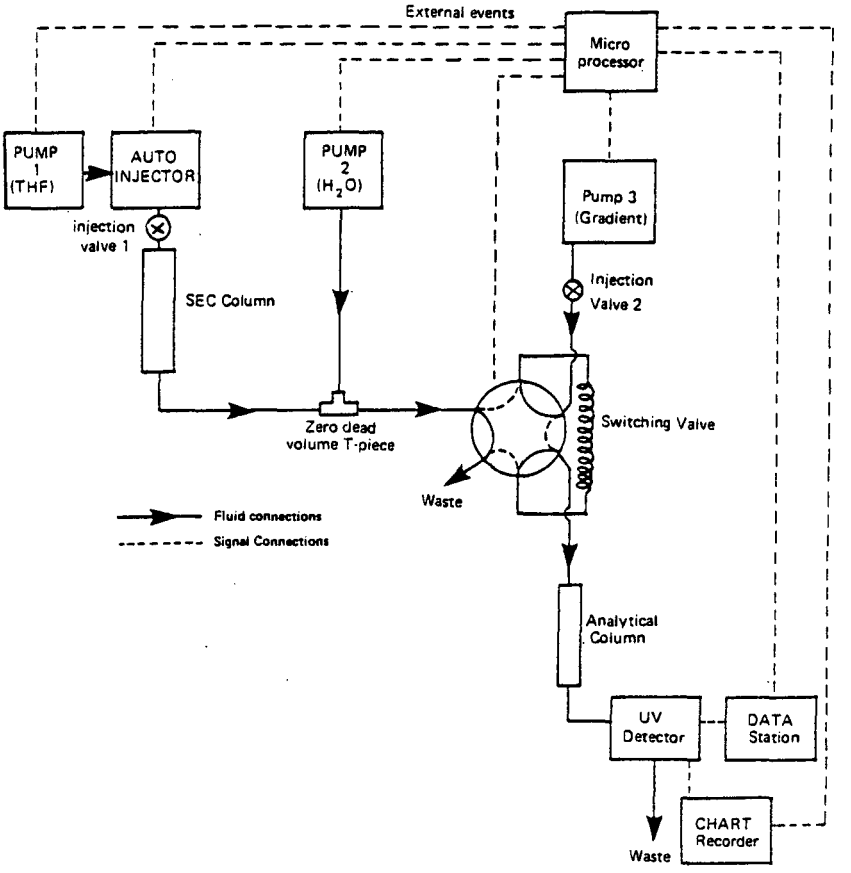

Fig. 1. Diagrammatic representation of the coupled LC–LC system.

with HPLC quality water) with the premixed analyte peak, now in THF–water. After a set collection time (1.00 min for all applications reported) the interface valve was actuated to inject the trapped diluted SEC peak onto the reversed-phase column, which had been pre-equilibrated with water during the SEC separation.

Reversed phase. Four loop volumes of water were used to flush the sample onto the reversed-phase column. The loop was then switched out of line and a linear gradient initiated, running up to 100% acetonitrile over 20 min. After 5 min hold at 100% acetonitrile, a rapid (2 min) reversed gradient was carried out, and the column re-equilibrated with water for a further 5 min.

RESULTS AND DISCUSSION

System development

The optimum eluents for non-aqueous SEC have solubility parameters close to that of the gel employed. Although surface-modified silica may be used, it has several drawbacks; incomplete surface coverage, or loss of bonded material on use, will lead to exposure of active silanols with consequent potential selective adsorption of sample components. The restricted range of pore sizes, and particularly the low pore volumes available with narrow pore silicas, further limits work with small molecules. Thus cross-linked polystyrene is preferred for the separation of a wide range of lipophilic organic compounds and for this gel effective solvents include toluene, chloroform and THF. All these are strong eluents for reversed-phase chromatography and cannot be injected directly in volumes of 0.5–1.0 ml, typical of peak widths on standard 7.7-mm internal diameter high-performance SEC columns. Non-aqueous SEC cannot therefore be coupled directly with reversed-phase chromatography.

One possible on-line interface methold would utilise some form of transport device to remove the SEC mobile phase and replace it with water or other non-eluting solvent before injection onto the reversed-phase column. This would be essential with an SEC eluent immiscible with water, but mechanical devices of this kind are expensive and often present difficulties in operation. An alternative approach involves diluting the SEC peak with a weak solvent, prior to injection onto a reversed-phase column, so that the mixture is non-eluting for the analyte on the chosen packing. The analyte may then be reconcentrated from the diluted peak by adsorption onto the head of the reversed-phase column and subsequently efficiently separated by gradient elution. In this case the SEC mobile phase must be miscible with water, and THF is the only practicable candidate. The major problem with THF as eluent is its poor stability. This was overcome by not transferring THF from the containers it was supplied in and by maintaining it in darkness under a constant low purge of helium. In addition samples for analysis were dissolved in toluene, rather than THF. It was found that interferences accumulated rapidly in samples stored in THF. Toluene is a good solvent for lipids, is highly compatible with the SEC gel and because of its small size is well resolved from all common analytes.

The potential of the proposed method of interfacing was shown in earlier work[4], where phthalates in 400 μl acetonitrile were reconcentrated after passive dilution of the sample during loading into a 2.0-ml sample loop filled with water. The valve was plumbed so that during injection the sample passed through the loop in the same direction as on loading, instead of being swept backwards as in the standard

configuration. Sufficient mixing was achieved simply by the axial dispersion caused by injection. However, with THF as eluent and analytes more polar than phthalates, poor peak profiles were obtained. This arose as a direct result of inadequate mixing on-line. Similar samples mixed off-line gave excellent peak shapes. Preliminary experiments with loops filled with ballotini to promote mixing were abandoned because of the low free volume fraction of the loop; a packed column of 690 × 4.6 mm would have been required to obtain a 5-ml sample volume.

Thus the interface shown in Fig. 1 was devised. The required SEC peak is diluted with water introduced from another pump via a "tee" immediately following the SEC column outlet and the mixture is trapped in a switching valve with a suitable large volume loop. The SEC peak dilution ratio may be altered as required by varying the THF and water pump flow-rates. The trapping loop was employed to avoid putting the SEC column under excessive pressure when loading the diluted analyte peak on to the reversed-phase column. One problem with polystyrene SEC columns is the relative softness of the gel, which restricts the maximum acceptable pressure drop across the type of column employed in this work to about 100 bar. Direct coupling of the columns would be more flexible, permitting microprocessor control over the volume of SEC peak transferred simply by altering the valve switching times. To achieve this at a constant dilution ratio in the current system, it is necessary to install a trapping loop of a different size. This was not considered a significant problem because the automated apparatus is designed for long runs of analyses of the same kind.

Water quality is a major constraint on the sensitivity achievable with this method. A considerable volume of water (up to 30 ml) is pumped through the reversed phase column between analyses, and many impurities will be concentrated at the head of the column in a manner similar to that required for the analyte. Early experiments using water from a commercial purification system showed an unacceptable level of impurities. HPLC grade water from several commercial suppliers was evaluated by inspection of a blank gradient chromatogram with detection at 254 nm. Water quality varied significantly between suppliers, but the water selected for use was essentially free from interferences.

Pump flow-rate stability is critical to successful implementation of a column switching method based on timed events. The performance of the HPLC pumps employed was monitored and shown to be satisfactory. At least 50 measurements were taken during each test period (1 h) at regular intervals over two years. Typical coefficients of variation (C.V.) for THF and water pumps (1 ml/min and 4 ml/min respectively) were 0.7 and 0.9%; there was little change with time. This gives an indication of short-term fluctuations of pump flow, over time periods of less than 1 min. A more important indication of pump stability was obtained by monitoring analyte retention times. The C.V.s for the peak maxima retention times of dibutyl phthalate, diethylstilboestrol and 1-phenylpropan-2-ol on the SEC column were found to fall within the range 0.14–0.31% (n = 12).

SEC columns containing packings with pore sizes of 50 and 100 Å were compared for their resolution from lipids of representative small solutes. The 50-Å column gave inadequate separation and was therefore not evaluated further. A single 300-mm, 100-Å column was used for all the work reported here. One potential limitation of the proposed method is the high resolution of these columns, which may result in resolution of individual members of analyte families thereby restricting group

analyses. However, analyte homologues varying by 2–3 methylene groups would co-elute within the 60-s trapping period for SEC peaks employed during the evaluation of the technique. This is an unnecessarily wide window for single compounds, as shown by peak area data. Peaks from dibutyl phthalate standards were integrated over selected intervals and the collection times required to obtain 95% and 98% recoveries found to be 23 and 28 s. Thus on this basis the collection window could usefully be reduced to 40 s. Obviously the narrower this window, the fewer potential interferences will be transferred to the reversed phase column. However, some allowance has to be made for pump flow rate variations and for sample viscosity effects.

One major application of the reported system is the direct analysis of lipophilic analytes in co-extracted fat. The SEC retention times of small molecules decrease in the presence of high concentrations of lipids through the effect of viscous drag[4]. Both the concentration and type of lipid has an effect on viscosity, as shown in Fig. 2, which plots phthalate recovery against sample dilution ratio for a range of spiked fats and oils.

For any given analyte, the detection limit attainable is a function of mass loading onto the SEC column and of the extent of interferences in the final reversed-phase chromatogram. Increasing the injection volume for a given mass of lipid reduces the effect of viscosity on analyte elution times but broadens the analyte SEC peak by the additional volume. Fig. 3 shows dibutyl phthalate peak areas against volume of injected spiked vegetable oil, diluted 1:16 with toluene, with constant switching times. No more than 400 μl of this sample was acceptable, equivalent to a lipid injection of *ca.* 25 mg. Additional experiments using direct fluorescence detection of the eluent from the SEC column (to overcome the high UV background from olive oil) with a sample consisting of zearalenone spiked into diluted olive oil also indicated that a maximum of 25 mg oil (100 μl of a 1:4 solution) could be injected without a reduction in retention time for co-injected analytes. In neat olive oil the retention time was reduced by 18 s, representing a 30% loss of solute with the peak collection window of

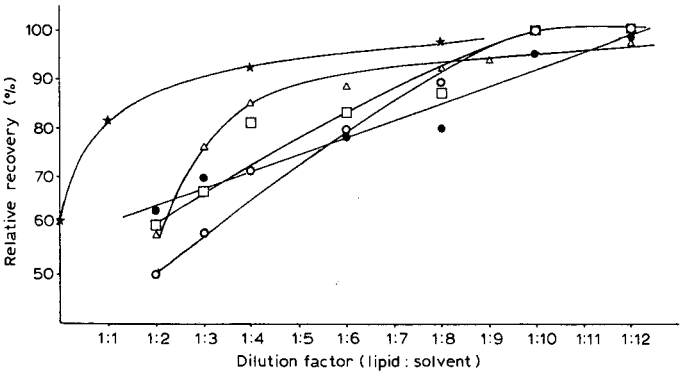

Fig. 2. Comparison of recovery of DBP with lipid concentration for a range of lipid types. (□) Crisp, (○) chicken, (●) beef, (△) chocolate, (★) olive oil. Where required lipid was extracted as described in Experimental. Analysis was carried out as described in Experimental. Chromatographic conditions: columns, PLgel, 100 Å, 5 μm, 300 × 7.9 mm and Spherisorb ODS, 5 μm, 250 × 4.9 mm. Detection at 254 nm. SEC eluent, THF at 1 ml min^{-1}. Dilution water, 4 ml min^{-1}. RPC mobile phase at 1 ml min^{-1}; 0–20 min, 100% H_2O; 20–30 min, 0–100% acetonitrile; 30–50 min, 100% acetonitrile.

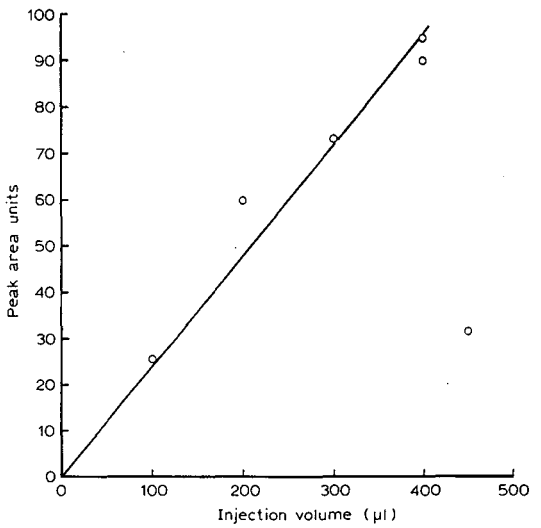

Fig. 3. Effect of sample volume on recovery of dibutyl phthalate in vegetable oil. Chromatographic conditions as in Fig. 2.

60 s set according to the SEC retention time for zearalenone standards injected in the absence of oil. For dibutyl phthalate added to vegetable oil a detection limit of *ca.* 0.2 mg/kg could be attained at a signal-to-noise ratio of 3:1. The sensitivity achievable with samples will be discussed below.

Prediction of analysis conditions

The extent of dilution with water required for the SEC peak to be retained on the reversed-phase column is an important system parameter. The holding loop flushing time is a major element of the overall analysis time and the volume of water required for purging could be a constraint upon achievable sensitivity depending upon its purity. Thus an attempt was made to predict the dilution ratio required for any given analyte.

The important parameter for solute reconcentration is its retention on the reversed phase column in a mobile phase consisting of water and THF. The problem reduces to calculation of the water–THF composition just permitting acceptable reconcentration. It was anticipated that for any given reversed phase column, there would be a constant limiting analyte capacity ratio, below which reconcentration would not be possible. Determination of this unknown minimum capacity ratio was carried out empirically. Three test solutes covering a moderate polarity range (dibutyl phthalate, diethylstilboestrol and 1-phenylpropan-2-ol) were chromatographed on the reversed-phase column in a number of isocratic acetonitrile–water mobile phases giving rise to capacity ratios of between 0.5 and 20 and graphs of capacity ratio *versus* mobile phase acetonitrile content prepared. The same compounds were also chromatographed on the coupled column system using interface loops varying in size from 2.5 to 5 ml. Comparison of the coupled column chromatograms obtained under these conditions with those from standards injected directly onto the Spherisorb column

showed whether reconcentration had taken place. This indicated the maximum THF concentration permitting concentration. The simple solvent transfer rule[14]:

$$\varphi_{CH_3CN} = \varphi_{THF} \cdot \delta_{CH_3CN}/\delta_{THF} \quad (1)$$

where φ_{THF}, δ_{THF}, φ_{CH_3CN}, δ_{CH_3CN} are the mole fractions and solubility parameters of THF and acetonitrile respectively, was then applied to calculate the acetonitrile content of a water–acetonitrile mixture having the same solvent strength. Interpolation of this value on the graph previously prepared gave the equivalent isocratic acetonitrile–water capacity ratio. For all three test compounds it was found that a capacity ratio of *ca.* 2.5 indicated reconcentration. It is therefore necessary only to determine experimentally the composition of the isocratic acetonitrile–water mixture giving a capacity ratio of 2.5 for any analyte, and, using the solvent transfer rules, to translate this into an equivalent THF concentration and thus to a water dilution factor.

The process of prediction was taken one step further to avoid the need to carry out repetitive isocratic retention experiments for each required analyte. Berridge[15] has published a BASIC computer program (based on the calculations of Dolan *et al.*[16]) which calculates the isocratic mobile phase composition yielding the same retention time as that found experimentally when the same column is eluted with a gradient of $6.6/t_0$ % methanol per min.

Application of this program and eqn. 1 indicated that any solute giving under the specified conditions a gradient retention time of greater than 19 min should have on the same column a capacity factor of >2.5 in an aqueous mobile phase containing 20% THF. Reconcentration on the coupled column system would occur with a loop volume no greater than 5 ml. This result was tested by comparison of retention data on the specified methanol gradient with the effectiveness of reconcentration on the coupled column system for a further ten compounds. These included phenol (adequate reconcentration) and sulphadimidine, orcinol and caffeine (inadequate). It was concluded that a retention time in excess of 22 min on the standard gradient was a more reliable predictor of reconcentration. The three compounds for which reconcentration failed are readily water soluble and are therefore good candidates for coupled aqueous SEC–reversed-phase chromatography. It is probable therefore that any analyte of limited water solubility would be suitable for separation on the coupled column system described here.

Applications

Samples of extruded potato snack products containing 2,6-di-*tert.*-butyl-4-methylphenol (BHT) and of chocolate confectionary products containing dibutyl phthalate were analysed by the method described. Coupled column chromatograms of standards and samples are shown in Figs. 4 and 5. It can be seen that the cleanup achieved is adequate for determination of these compounds at about 0.5 mg/kg (blank samples were not available) and that the major limitation on sensitivity is the presence of co-extractives. Approaches to achieving better resolution could be to use a longer SEC column of the same or different pore size, to add a flushable pre-column between the SEC and reversed-phase columns, or to switch the analyte from the reversed-phase column onto a third column with different selectivity. A more realistic alternative may be to enhance overall selectivity by incorporating post-column deri-

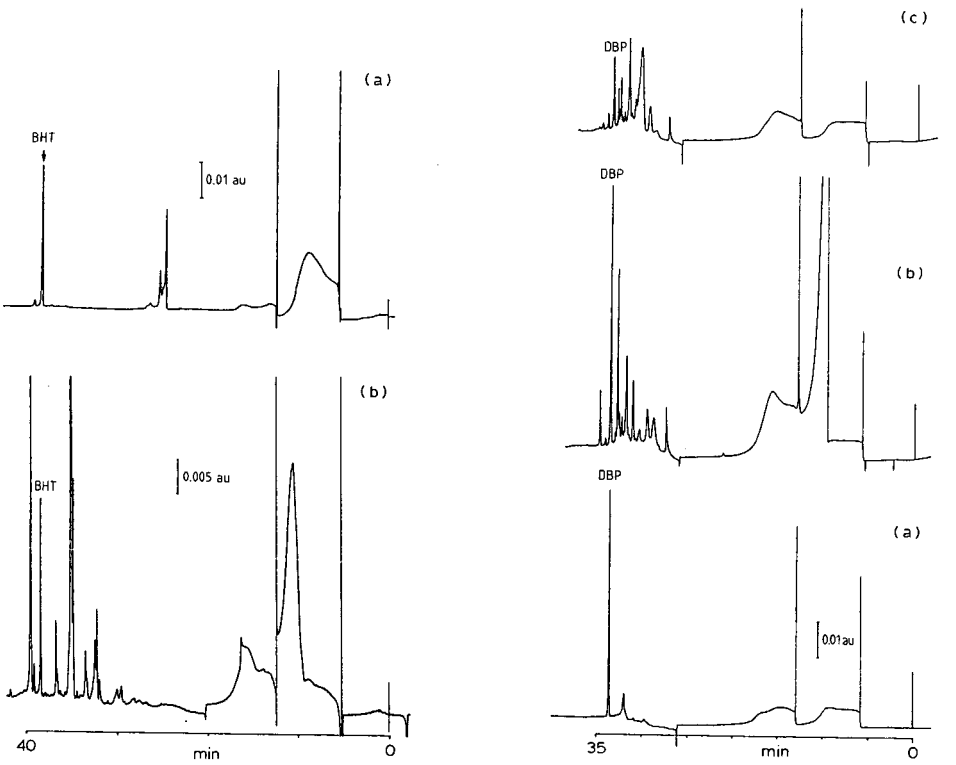

Fig. 4. Analysis of BHT in solvent and in a reformed snack product on the coupled LC–LC system. Sample: (a) 1.0 μg BHT in 100 μl toluene, (b) 25 μl extracted lipid and 75 μl toluene. Analysis was carried out as described in Experimental. Columns as in Fig. 2, detection at 280 nm. Gradient: 0–15 min, 100% H_2O; 15–17 min, 0–20% acetonitrile; 17–30 min, 20–80% acetonitrile; 30–35 min, 80–100% acetonitrile; 35–40 min, 100% acetonitrile.

Fig. 5. Analysis of DBP in solvent and in chocolate extract on the coupled LC–LC system. Sample: (a) 0.15 μg DBP in 100 μl toluene, (b) chocolate bar extract (25 μl) in 100 μl toluene, (c) chocolate sweet extract (25 μl) in 100 μl toluene. Chocolate extractions were carried out as described in Experimental. Analysis was carried out as described in Experimental. Chromatographic conditions as in Fig. 2.

vatisation for fluorescence detection. Pre-column derivatisation could create SEC resolution problems due to the increased size of the analyte.

CONCLUSIONS

It has been shown that non-aqueous SEC and reversed-phase chromatography may be coupled for the determination in crude lipid extracts of analytes having a polarity equal or greater to that of phenol with, for the examples shown, detection limits of about 0.5 mg/kg. Maximum lipid loadings on the SEC column employed were examined in detail and found to be *ca.* 25 mg. There was a dependency of the preferred sample concentration/volume conditions on the nature of the lipid.

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Journal of Chromatography, 477 (1989) 327-336
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 570

GAS–LIQUID CHROMATOGRAPHIC ANALYSES

L[a]. RETENTION, DISPERSION AND SELECTIVITY INDICES OF POLYCHLORINATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS

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SUMMARY

The retention (I), dispersion (I_M) and selectivity (I^*) indices of sixteen polychlorinated dibenzo-*p*-dioxins and fourteen polychlorinated dibenzofurans were determined on a low-polarity HP-5 capillary column using a gas chromatograph connected with an ion-selective detector. I_M and I^* values were also calculated for all 73 dibenzo-*p*-dioxins from the di- to the octachloro isomer and for all possible 135 chlorinated dibenzofurans based on the predicted retention index data reported earlier. The effect of the position of chlorination is shown and the results are compared with those for several series of chlorinated aromatics.

INTRODUCTION

Previously, the gas chromatographic (GC) retention behaviours of the following series of aromatics were studied in our laboratory: chlorinated benzenes[1–3], phenols [1,3–7], catechols (1,2-dihydroxybenzenes)[4], salicylaldehydes (2-hydroxybenzaldehydes)[7,8], 4-hydroxybenzaldehydes[7,9], anisoles (methoxybenzenes)[3,6,10,11] and veratroles (1,2-dimethoxybenzenes)[11,12]; the acetate esters of chlorinated phenols[3,6,10,13–16], catechols[14] and guaiacols (1-hydroxy-2-methoxybenzenes)[14]; several esters of monochloro-[16–22], pentafluoro-[21–24] and nitro-[21,22,25–27] benzoic acids; nitrated polynuclear aromatic hydrocarbons (PAHs)[28]; and polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)[29]. Most frequently these studies concerned GC separations of complex mixtures on capillary columns with different polarities and the relative retention data, most also the retention indices together with the retention index increments due to substitution and some the lipophilicity of the compounds of interest. Later the dispersion and selectivity indices in the GC of various groups of the above compounds based on our retention index data were determined and reported by Evans and Haken[30,31].

[a] For Part XLIX, see ref. 29.

0021-9673/89/$03.50

As a continuation of our GC studies on harmful organochlorine compounds occuring in the environment[32], this investigation deals with the GC retention, dispersion and selectivity indices of PCDDs and PCDFs obtained on a low-polarity HP-5 capillary column with suitable temperature programming. The dispersion and selectivity indices were also determined for almost all possible chlorinated congeners of interest based on the predicted retention index data reported earlier[33–35]. The effect of the position of chlorination is shown, and the results are compared with those for several series of chlorinated aromatics.

EXPERIMENTAL

Materials

The PCDDs and PCDFs investigated were obtained from Wellington Labs. (Guelph, Canada) or CIL (Cambridge Isotope Labs., Woburn, MA, U.S.A.)[29], the ^{13}C-labelled isomers originating from CIL. Mixtures of *n*-alkanes were obtained from SGE (North Melbourne, Australia).

Methods

A Hewlett-Packard 5890/5970 gas chromatograph–mass spectrometer system with an HP-5 fused-silica wall-coated open-tubular (WCOT) column (25 m × 0.20 mm I.D., film thickness 0.11 μm), operated in the splitless injection mode was used. The HP-5 stationary phase (5% diphenyl–95% dimethylpolysiloxane) is very similar to DB-5, BP-5, SPB-5, GC-5, CP-SIL 8, 007-2, RSL-200, SE-52, OV-73 and SE-54 based on the McReynolds' constants. The temperatures of the injector and transfer-line were 250 and 300°C, respectively. The column temperature was held at 100°C for 1 min, then programmed to 180°C at 20°C min^{-1} and to 290°C at 5°C min^{-1} and held at 290°C until elution of peaks had ceased. Helium was used as the carrier gas at 7.0 p.s.i. head pressure, the column being coupled directly to the ion source of the mass spectrometer. The selected-ion monitoring mode was used to follow ions characteristic of PCDDs, PCDFs and *n*-alkanes.

The Kováts retention indices were calculated off-line as described earlier[36] and the dispersion and selectivity indices as reported previously by Evans *et al.*[37].

RESULTS AND DISCUSSION

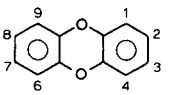
Tables I and II show the retention indices for PCDDs and PCDFs, respectively, obtained on the HP-5 capillary column. The total ion chromatograms of these two groups of components are illustrated in Figs. 1 and 2, respectively. Data for some ^{13}C-labelled substances are given in Table III.

Our earlier studies[29] showed the GC separation of the title compounds on low-polarity NB-54 and NB-1701 capillary columns using a gas chromatograph with electron-capture detection (ECD). As the substances of interest are very insensitive to flame ionization detection (FID), particularly with the low concentrations used, and as the *n*-alkanes used as reference compounds are not suitable for analysis with ECD, the Kováts retention indices could not be determined by that method. However, by using the technique given above, the retention indices can be determined without problems.

TABLE I

RETENTION (I), DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES FOR PCDDs ON AN HP-5 CAPILLARY COLUMN

Conditions as in Fig. 1.



Systematic No.[a]	*Compound*[b]	I	I_M	I^*
42	1,3,6,8-	2262.3	2281.2	−18.9
48	2,3,7,8-	2353.1	2281.2	71.9
41	1,2,8,9-	2393.8	2281.2	112.6
58	1,2,4,6,8-	2464.3	2526.8	−62.5
61	1,2,4,7,9-	2464.3	2526.8	−62.5
54	1,2,3,7,8-	2554.8	2526.8	28.0
56	1,2,3,8,9-	2579.5	2526.8	52.7
71	1,2,4,6,7,9-	2668.3	2772.3	−104.0
72	1,2,4,6,8,9-	2668.3	2772.3	−104.0
66	1,2,3,4,7,8-	2741.8	2772.3	−30.5
67	1,2,3,6,7,8-	2746.9	2772.3	−25.4
70	1,2,3,7,8,9-	2762.0	2772.3	−10.3
63	1,2,3,4,6,7-	2762.0	2772.3	−10.3
74	1,2,3,4,6,7,9-	2893.9	3017.9	−124.0
73	1,2,3,4,6,7,8-	2937.2	3017.9	−80.7
75	1,2,3,4,6,7,8,9-	3132.6	3263.5	−130.9

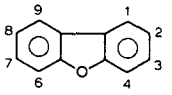
[a] From ref. 38.

[b] Numbers indicate the chlorinated positions.

TABLE II

RETENTION (I), DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES FOR PCDFs ON AN HP-5 CAPILLARY COLUMN

Conditions as in Fig. 2.



Systematic No.[a]	*Compound*[b]	I	I_M	I^*
69	1,3,6,8-	2216.1	2167.1	49.0
83	2,3,7,8-	2324.0	2167.1	156.9
63	1,2,8,9-	2391.0	2167.1	223.9
106	1,3,4,6,8-	2404.0	2412.7	−8.7
94	1,2,3,7,8-	2496.3	2412.7	83.6
114	2,3,4,7,8-	2535.7	2412.7	123.0
96	1,2,3,8,9-	2583.4	2412.7	170.7
116	1,2,3,4,6,8-	2635.6	2658.3	−22.7
118	1,2,3,4,7,8-	2692.8	2658.3	34.5
121	1,2,3,6,7,8-	2699.2	2658.3	40.9
130	2,3,4,6,7,8-	2732.4	2658.3	70.1
120	1,2,3,4,8,9-	2778.8	2658.3	120.5
131	1,2,3,4,6,7,8-	2873.9	2903.8	−29.9
134	1,2,3,4,7,8,9-	2956.7	2903.8	52.9

[a] From ref. 38.

[b] Numbers indicate the chlorinated positions.

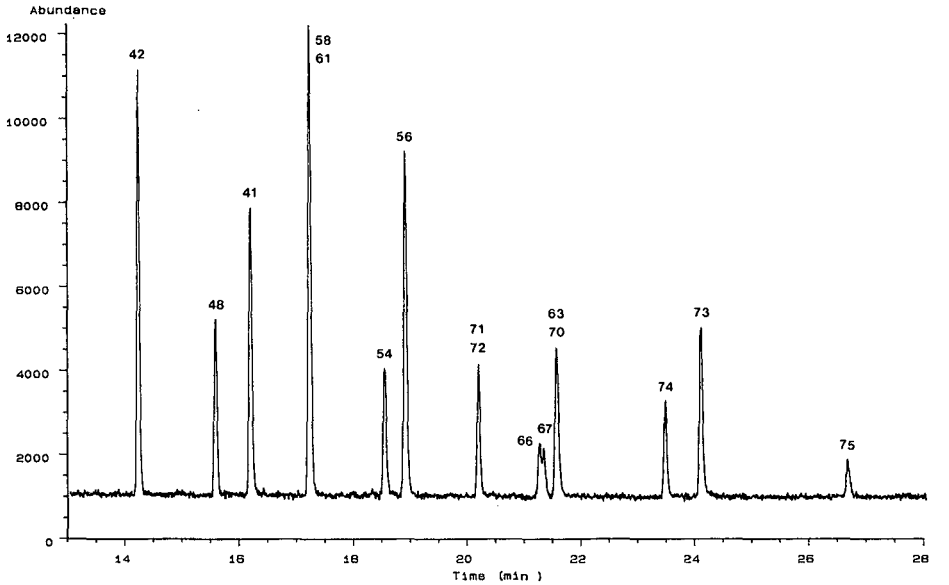


Fig. 1. Total ion chromatogram of a mixture of PCDDs, obtained under the conditions given under Experimental section. For numbering of peaks, see Table I.

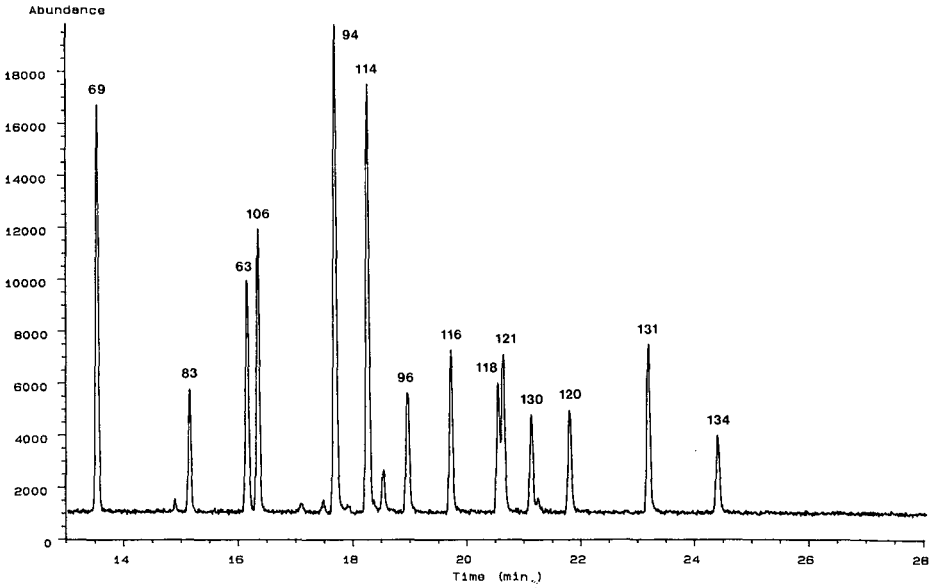


Fig. 2. Total ion chromatogram of a mixture of PCDFs. Conditions as in Fig. 1. For numbering of peaks, see Table II.

A separation of the individual compounds in two mixtures almost identical with that obtained earlier on NB-54[29] capillary column was obtained on HP-5 (Figs. 1 and 2). The retention indices in Tables I and II seem te be lower than those obtained earlier on DB-5 capillary columns[33–35]. This is due mainly to the different stationary phases, but also to the different operating conditions used. From an environmental analysis point of view, important ^{13}C-labelled reference compounds are eluted on HP-5 earlier than the corresponding unlabelled substances, showing a difference from -0.2 to -0.5 retention index units (i.u.) (Table III). It should be noted that deuterium labelling causes a considerable reduction in retention so that, for example, perdeuterated aliphatic long-chain hydrocarbons and esters can be completely separated from their non-deuterated homologue on a low-polarity OV-1 capillary column[39,40]. Our previous studies with partially deuterated chlorinated esters[41–43] and some aromatic compounds, *viz.*, chlorinated guaiacols[44] and veratroles[45,46], also showed a slightly earlier elution of a deuterated congener. The difference observed is negligible, however, even on highly polar stationary phases, where generally the structural effects are maximized. Hence it seems evident that the differences in Table III would be greater on analysing the components on a polar column.

The dispersion (I_M) and selectivity (I^*) indices developed recently by Evans and co-workers[30,31,37,47,48] are given in Tables I and II for PCDDs and PCDFs on HP-5, respectively. For comparison, the corresponding data for almost all possible chlorinated congeners are given in Tables IV and V, based on the predicted retention indices reported earlier[33,34]. These predicted indices had to be used as measured indices were not available for all isomers. However, the results show that these predicted indices are very close to the experimental values obtained on DB-5 capillary columns, *i.e.*, the difference varies from -11 to $+12$ i.u. for chlorinated dibenzo-*p*-dioxins[33] and from -15 to $+19$ i.u. for chlorinated dibenzofurans[34]. The former contains 41 measured values (73) and the latter 115 (135) (calculated values in parentheses).

As is evident, the I_M values increase with an increase in the degree of chlorination, *i.e.*, the molecular weight. As a consequence of the lower retention indices, the selectivity indices presented in Tables I and II are lower than those in Tables IV

TABLE III

RETENTION INDICES FOR SOME ^{13}C-PERLABELLED PCDDs AND PCDFs ON AN HP-5 CAPILLARY COLUMN

Conditions as in Figs. 1 and 2.

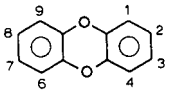
Compound[a]	*Retention index, I*	*Difference,* $I(^{13}C) - I(^{12}C)$[b]
2,3,7,8-D	2352.7	−0.4
1,2,3,7,8-D	2554.5	−0.3
1,2,3,4,7,8-D	2741.3	−0.5
1,2,3,4,6,7,8,9-D	3132.4	−0.2
2,3,7,8-F	2323.6	−0.4
1,2,3,7,8-F	2495.9	−0.4

[a] Numbers indicate the chlorinated positions; D = Dioxin, F = furan.

[b] Retention indices for parent substances given in Tables I and II.

TABLE IV

RETENTION (I), DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES FOR CHLORINATED DIBENZO-*p*-DIOXINS



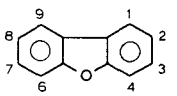
Isomer[a]	I[b]	I_M	I^*	*Isomer*[a]	I[b]	I_M	I^*
1,3-	1936	1790	146	1,2,6,9-	2378	2281	97
1,4-	1951	1790	161	1,2,3,4-	2379	2281	98
1,6-	1968	1790	178	1,2,3,7-	2382	2281	101
1,7-	1972	1790	182	1,2,3,8-	2382	2281	101
1,8-	1972	1790	182	2,3,7,8-	2386	2281	105
2,7-	1976	1790	186	1,2,3,9-	2392	2281	111
2,8-	1976	1790	186	1,2,7,8-	2397	2281	116
1,9-	1982	1790	192	1,2,6,7-	2408	2281	127
2,3-	1984	1790	194	1,2,8,9-	2422	2281	141
1,2-	1995	1790	205	1,2,4,7,9-	2511	2527	−16
1,3,6-	2129	2036	93	1,2,4,6,8-	2511	2527	−16
1,3,7-	2133	2036	97	1,2,4,6,9-	2533	2527	6
1,3,8-	2133	2036	97	1,2,3,6,8-	2539	2527	12
1,3,9-	2143	2036	107	1,2,4,7,8-	2545	2527	18
1,2,4-	2143	2036	107	1,2,3,7,9-	2553	2527	26
1,4,8-	2148	2036	112	1,2,3,6,9-	2568	2527	41
1,4,9-	2158	2036	122	1,2,4,6,7-	2570	2527	43
1,7,8-	2177	2036	141	1,2,4,8,9-	2570	2527	43
2,3,7-	2181	2036	145	1,2,3,4,7-	2576	2527	49
1,2,3-	2185	2036	149	1,2,3,4,6-	2586	2527	59
1,2,6-	2188	2036	152	1,2,3,7,8-	2587	2527	60
1,2,7-	2192	2036	156	1,2,3,6,7-	2598	2527	71
1,2,8-	2192	2036	156	1,2,3,8,9-	2612	2527	85
1,2,9-	2202	2036	166	1,2,4,6,8,9-	2725	2772	−47
1,3,6,8-	2290	2281	9	1,2,4,6,7,9-	2725	2772	−47
1,3,7,9-	2304	2281	23	1,2,3,4,6,8-	2747	2772	−25
1,3,6,9-	2319	2281	38	1,2,3,6,8,9-	2760	2772	−12
1,3,7,8-	2338	2281	57	1,2,3,6,7,9-	2760	2772	−12
1,2,4,7-	2340	2281	59	1,2,3,4,6,9-	2769	2772	−3
1,2,4,8-	2340	2281	59	1,2,3,4,7,8-	2781	2772	9
1,4,6,9-	2341	2281	60	1,2,3,6,7,8-	2788	2772	16
1,2,6,8-	2349	2281	68	1,2,3,7,8,9-	2802	2772	30
1,2,4,6-	2350	2281	69	1,2,3,4,6,7-	2806	2772	34
1,2,4,9-	2350	2281	69	1,2,3,4,6,7,9-	2961	3018	−57
1,4,7,8-	2353	2281	72	1,2,3,4,6,7,8-	2996	3018	−22
1,2,7,9-	2363	2281	82	1,2,3,4,6,7,8,9-	3197	3263	−66
1,2,3,6-	2378	2281	97				

[a] Numbers indicate the chlorinated positions.
[b] Calculated retention indices taken from ref. 33.

and V. As with other groups of chlorinated aromatics[31], *viz.*, benzenes, anisoles, veratroles, phenols, phenyl acetates and 2- and 4-hydroxybenzaldehydes, the I^* values of the compounds in the present investigation decrease with increasing degree of chlorination. This is due to the effect of the screened electrons of the halogen atoms,

TABLE V

RETENTION (I), DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES FOR CHLORINATED DIBENZOFURANS



Isomer[a]	I[b]	I_M	I^*	*Isomer*[a]	I[b]	I_M	I^*
1-	1728	1430	298	1,2,3,4-	2318	2167	151
3-	1745	1430	315	1,2,3,6-	2318	2167	151
2-	1751	1430	321	2,3,4,9-	2318	2167	151
4-	1761	1430	331	1,4,6,9-	2319	2167	152
1,3-	1875	1676	199	1,2,7,8-	2324	2167	157
1,4-	1901	1676	225	1,3,4,9-	2333	2167	166
1,7-	1908	1676	232	1,2,6,7-	2335	2167	168
1,8-	1913	1676	237	2,3,4,7-	2336	2167	169
2,4-	1914	1676	238	1,2,7,9-	2338	2167	171
1,6-	1923	1676	247	1,2,4,9-	2339	2167	172
3,7-	1925	1676	249	2,3,4,8-	2342	2167	175
2,7-	1931	1676	255	2,3,4,6-	2342	2167	175
1,2-	1936	1676	260	2,3,7,8-	2342	2167	175
2,8-	1936	1676	260	2,3,6,7-	2353	2167	186
3,6-	1941	1676	265	3,4,6,7-	2353	2167	186
2,6-	1946	1676	270	1,2,6,9-	2364	2167	197
4,6-	1947	1676	271	1,2,3,9-	2377	2167	210
2,3-	1954	1676	278	1,2,8,9-	2398	2167	231
3,4-	1964	1676	288	1,3,4,6,8-	2418	2413	5
1,9-	1982	1676	306	1,2,4,6,8-	2423	2413	10
1,3,7-	2055	1922	133	2,3,4,7,9-	2466	2413	53
1,3,8-	2060	1922	138	1,3,4,7,8-	2468	2413	55
1,3,6-	2071	1922	149	1,3,4,6,7-	2468	2413	55
2,4,9-	2076	1922	154	1,2,3,6,8-	2471	2413	58
1,3,4-	2079	1922	157	1,2,4,7,8-	2473	2413	60
1,4,7-	2081	1922	159	1,2,4,6,7-	2473	2413	60
1,2,4-	2084	1922	162	1,3,4,7,9-	2481	2413	68
1,4,8-	2087	1922	165	2,3,4,6,9-	2482	2413	69
1,4,6-	2087	1922	165	1,2,4,7,9-	2486	2413	73
2,4,7-	2094	1922	172	2,3,4,6,8-	2495	2413	82
2,4,8-	2099	1922	177	1,3,4,6,9-	2497	2413	84
2,4,6-	2100	1922	178	1,2,3,4,7-	2498	2413	85
2,3,9-	2116	1922	194	1,2,4,6,9-	2502	2413	89
1,2,7-	2116	1922	194	1,2,3,4,8-	2503	2413	90
1,2,8-	2121	1922	199	1,2,3,4,6-	2504	2413	91
1,2,3-	2123	1922	201	1,2,3,7,8-	2511	2413	98
3,4,9-	2126	1922	204	1,2,3,6,7-	2522	2413	109
1,3,9-	2129	1922	207	1,2,3,7,9-	2524	2413	111
1,2,6-	2132	1922	210	2,3,4,8,9-	2527	2413	114
2,3,7-	2134	1922	212	1,3,4,8,9-	2542	2413	129
2,3,8-	2139	1922	217	2,3,4,7,8-	2545	2413	132
3,4,7-	2144	1922	222	2,3,4,6,7-	2545	2413	132
3,4,8-	2149	1922	227	1,2,4,8,9-	2547	2413	134
2,3,6-	2149	1922	227	1,2,3,6,9-	2551	2413	138

(Continued on p. 334)

TABLE V *(continued)*

Isomer[a]	I^b	I_M	I^*	*Isomer*[a]	I^b	I_M	I^*
3,4,6-	2150	1922	228	1,2,3,4,9-	2572	2413	159
1,4,9-	2155	1922	233	1,2,3,8,9-	2585	2413	172
2,3,4-	2156	1922	234	1,2,3,4,6,8-	2656	2658	−2
1,2,9-	2190	1922	268	1,3,4,6,7,8-	2660	2658	2
1,3,6,8-	2224	2167	57	1,2,4,6,7,8-	2665	2658	7
1,4,6,8-	2240	2167	73	1,3,4,6,7,9-	2675	2658	17
2,4,6,8-	2252	2167	85	1,2,4,6,7,9-	2680	2658	22
1,3,4,7-	2259	2167	92	1,2,4,6,8,9-	2685	2658	27
1,3,7,8-	2264	2167	97	1,2,3,4,7,8-	2706	2658	48
1,2,4,7-	2264	2167	97	1,2,3,4,6,7-	2707	2658	49
1,3,4,8-	2264	2167	97	1,2,3,6,7,8-	2714	2658	56
1,3,4,6-	2265	2167	98	1,2,3,4,7,9-	2719	2658	61
1,2,4,8-	2270	2167	103	1,2,3,6,7,9-	2728	2658	70
1,2,4,6-	2270	2167	103	1,2,3,6,8,9-	2734	2658	76
1,3,6,7-	2274	2167	107	1,2,3,4,6,9-	2736	2658	78
1,3,7,9-	2277	2167	110	2,3,4,6,7,8-	2737	2658	79
1,2,6,8-	2284	2167	117	1,2,3,7,8,9-	2772	2658	114
1,4,7,8-	2290	2167	123	1,2,3,4,8,9-	2780	2658	122
1,4,6,7-	2290	2167	123	1,2,3,4,6,7,8-	2899	2904	−5
2,3,6,8-	2302	2167	135	1,2,3,4,6,7,9-	2914	2904	10
2,4,6,7-	2303	2167	136	1,2,3,4,6,8,9-	2919	2904	15
1,3,6,9-	2303	2167	136	1,2,3,4,7,8,9-	2967	2904	63
1,2,3,7-	2303	2167	136	1,2,3,4,6,7,8,9-	3152	3149	3
1,2,3,8-	2308	2167	141				

[a] Numbers indicate the chlorinated positions.
[b] Calculated retention indices taken from ref. 34.

as clearly shown in Fig. 3, which indicates a dependence of the selectivity indices of compounds on the degree of chlorine substitution. The values are considerably higher as with other aromatics, where negative I^* values obtained on a low-polarity stationary phase seem to be usual. Tables IV and V show that the negative values for I^* are very rare, particularly with dibenzofurans having lower molecular weights than the corresponding dibenzo-*p*-dioxins. This results in lower I_M values for dibenzofurans, although the retention indices of analogous isomers in both groups would be near to each other. As with chlorobenzenes, the greatest retention occurs with the isomers where the chlorine substituents are closest together and decreases as the position around the ring allows greater separation[2]. Thus, for example, the 1,3,6,8- is the first tetrachloro isomer to elute and 1,2,8,9- is the last in both groups of compounds.

The relationship between the retention characteristics on a gas chromatographic column and the molecular structure of the chlorinated dibenzo-*p*-dioxins[33] and dibenzofurans[34,35] have been thoroughly discussed and the trends are evident from the data in Tables I–V. It is apparent that the models developed can be applied to other similar systems.

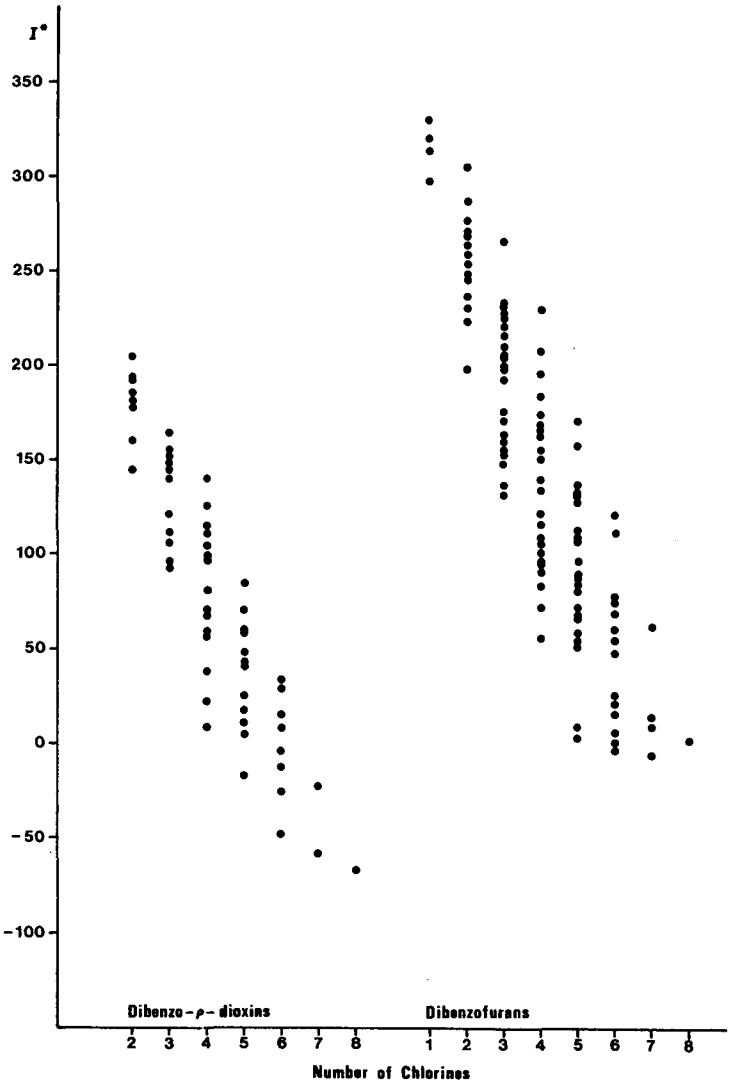


Fig. 3. Dependence of selectivity indices of compounds of interest on the degree of chlorine substitution (Tables IV and V).

ACKNOWLEDGEMENT

K. M. Mäntykoski gratefully thanks the Imatran Voima Foundation for financial support.

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Journal of Chromatography, 477 (1989) 337–344
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 572

COLUMN PERFORMANCE OF Q-SEPHAROSE HP IN ANALYTICAL- AND PREPARATIVE-SCALE CHROMATOGRAPHY

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SUMMARY

The chromatographic behaviour of two Q-Sepharose® HP HR 16/10 columns was tested under analytical- and preparative-scale conditions. Protein clogging of the top filter and the packing was the main reason for the observed decrease in retention volume, gel bed height and peak height during 300 analytical separations of a protein mixture (5 mg of protein per injection). Different washing procedures proved that adsorbed protein molecules decreased the availability of the sample molecules to the ion-exchange groups. By using a column where the top filter was fixed to the adaptor (XK 16/20 column), the stability of the column bed height was improved.

Purification of ovalbumin from egg white with large sample loads showed that washing with 0.1% pepsin solution maintained the optimum recovery. The column performance was evaluated in 50 purification cycles, corresponding to *ca.* 25 g of purified ovalbumin.

INTRODUCTION

With the development of bioindustries, chromatographic methods have become widely used in the purification of a variety of biological materials. To make the purification processes as inexpensive as possible, chromatographic media that provide a long lifetime are sought. In preparative-scale chromatography, the chemical and physical properties of the media are very important because they determine to a great extent the reusability of the support. It is essential that they can withstand rigorous cleaning and sanitation procedures. Further, it would be an advantage if the support is "user-friendly" so it could be used without a decrease in performance after practical mistakes such as pumping air into the column.

In a series of papers[1–4] it has been shown that media for analytical-scale separations can be used for at least 1000 repetitive injections of a protein or a serum sample. However, it was shown recently[5] that preparative-scale separations sometimes demand a washing procedure between every run in order to sustain optimum sample recovery.

Recently, a new cross-linked agarose-based anion exchanger, Q-Sepharose HP, has become commercially available. This has an average bead size of 34 μm and is

0021-9673/89/$03.50

intended especially for process development and the small-scale production of proteins and peptides. The objective of this work was to determine the ability of Q-Sepharose HP to withstand exposure to operations such as pumping air into the column, compression of the gel bed, different washing procedures, repetitive injection of an analytical protein sample and repetitive protein preparations on a large scale. Further, the importance of having the top filter fixed to the flow adaptor was elucidated.

EXPERIMENTAL

Chemicals and apparatus

Human transferrin was obtained from KabiVitrum (Stockholm, Sweden) and ovalbumin, β-lactoglobulin and pepsin from Sigma (St. Louis, MO, U.S.A.). Piperazine was of purum quality (Fluka, Buchs, Switzerland) and all inorganic compounds were of analytical-reagent grade. Decon 90 was obtained from Decon Labs. (Brighton, U.K.). Q-Sepharose HP, HR 16/10 and XK 16/20 columns and two FPLC® systems were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). An FPLC system consisted of an LCC-500 control unit, two P-500 high-precision pumps, a P-1 peristaltic pump, a UV-1 UV monitor (280 nm, HR 10 cell), MV 7 and MV 8 valves and a FRAC-100 fraction collector. A Shimadzu C-R3A integrator was used to collect and store data. The titrations of chloride were performed on a Mettler Memo Titrator DL 40 RC.

Column packing procedure

A 23-g amount of pre-washed settled gel was gently mixed with an equal amount of distilled water. The gel slurry was poured into an HR 16/10 or an X K16/20 column with packing equipment[6,7], and the flow-rate was set at 2.0 ml/min. When the gel had settled, the flow-rate was set at 8.0 ml/min and after 5 min the packing equipment was removed, the bed height adjusted to 10 cm and the top adaptor mounted. Finally, distilled water was allowed to pass through the gel bed at 8.0 ml/min for 90 min.

Sample preparation

Preparative sample. The egg white was diluted with ten volumes of 0.02 *M* piperazine (pH 6.0) (solvent A) and stirred on a magnetic stirrer for 10 min. It was then left overnight in a refrigerator for insolubles to settle before filtering through a 0.45-μm filter.

Analytical samples. The sample used for the lifetime test with 300 injections was a protein mixture consisting of transferrin (2.3 mg/ml), ovalbumin (3.3 mg/ml) and β-lactoglobulin (3.3 mg/ml). When the XK column was studied, a sample consisting of ovalbumin (10 mg/ml) was utilized. Both samples were solubilized in solvent A and were filtered through a 0.45-μm filter.

All samples were kept frozen until used.

Elution procedures for analytical-scale separations

Gradient elution. A 500-μl volume of the protein mixture was injected on to one of the HR 16/10 columns. The proteins were eluted at a flow-rate of 3.0 ml/min and a

linear gradient from solvent A to solvent B. The gradient time was 30 min. Solvent A consisted of 0.02 *M* piperazine (pH 6.0) and solvent B consisted of solvent A with the addition of 0.3 *M* sodium chloride. This elution procedure was also used when the XK 16/20 column was studied.

Isocratic elution. The two peaks from β-lactoglobulin were isocratically eluted with 55% solvent B at a flow-rate of 3.0 ml/min and acetone (1%, v/v) was eluted with distilled water at a flow-rate of 1.0 ml/min. The injection volume was 500 μl. These tests were performed on the occasions indicated in Table II.

Sample application and elution procedure for preparative-scale chromatography

A 50-ml volume of egg white diluted 10-fold with solvent A was applied to an HR 16/10 column at a flow-rate of 1.0 ml/min. The flow-rate was adjusted to 3.0 ml/min after the sample application. A 90-ml volume of solvent A was allowed to pass through the column before the gradient was started. The gradient and the solvents were the same as for the analytical column. The eluted ovalbumin was collected and a UV spectrum between 190 and 320 nm was recorded after a 10-fold dilution with solvent A. The amount of purified ovalbumin was evaluated from a calibration graph obtained from solutions of pure ovalbumin. The absorption of ovalbumin at 277 nm was used for quantification. Fifty preparations of ovalbumin from egg white were done. The column was rinsed from contaminating materials every six runs by filling it with 0.1% pepsin in 0.01 *M* hydrochloric acid. The enzymatic treatment was performed at room temperature (22°C) and lasted 24 h.

Determination of column ion-exchange capacity

Determinations of the total column ion-exchange capacity were performed by passing 40 ml of 1.0 *M* sodium chloride through the column at a flow-rate of 4.0 ml/min. The excess of ions was washed out with 40 ml of distilled water. The adsorbed ions were desorbed with 1.0 *M* sodium acetate, collected and then titrated potentiometrically with 0.1 *M* silver nitrate by using a silver electrode as the measuring electrode.

RESULTS AND DISCUSSION

In the first part of this investigation, two Q-Sepharose HP HR 16/10 columns were studied with respect to column performance. One column was used under analytical-scale and the other under preparative-scale conditions. In both instances samples containing ovalbumin were utilized. This protein was chosen because it has a strong tendency to denature and adsorb on filters and column packing materials.

Column performance in analytical-scale chromatography

Gradient elution of a protein mixture injected repetitively was used to test the column performance (Fig. 1). To stress the column further it was exposed to different washing procedures according to Table I. The column showed no significant change in performance during the first 50 injections even though air (20 ml) was pumped once into the column. However, after these injections the gel bed started to sink (the top filter was also displaced, see below) continuously during 200 injections (Table II). This behaviour was probably related to clogging of the top filter and the gel bed by

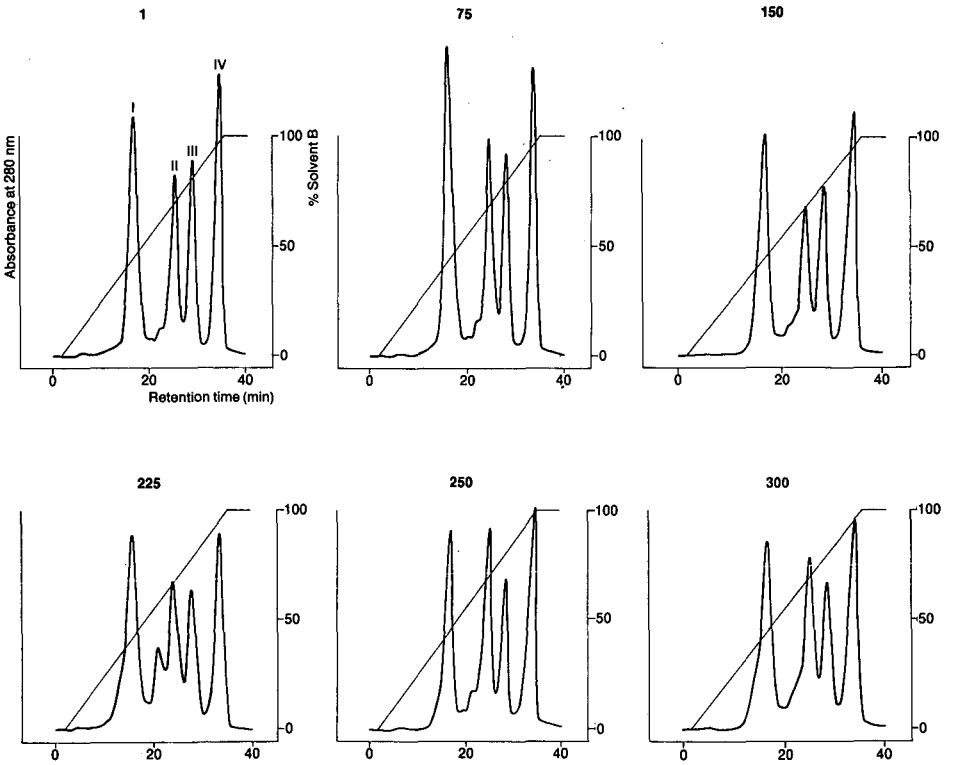

Fig. 1. Chromatograms from a Q-Sepharose HP column (HR 16/10 column) showing the separation of a test mixture containing transferrin (peak I), ovalbumin (peak II) and β-lactoglobulin (peaks III and IV). Events between the chromatograms are given in Table I. See Experimental for chromatographic conditions.

denatured proteins. From Tables I and II it can be seen that cleaning with 0.5 *M* of sodium hydroxide (20 ml) and 13 *M* acetic acid (40 ml) did not affect the continuous compression of the gel bed. However, the cleaning procedures after the 249th injection seemed to stop the reduction of the gel bed height. Moreover, the cleaning procedures with 0.1 *M* sodium hydroxide or 1.0 *M* acetic acid for 7 and 5 days, respectively, were also effective washing steps (Tabel II).

To study more carefully the change in retention characteristics of Q-Sepharose HP during the test, acetone and β-lactoglobulin were isocratically eluted on certain occasions. From Table II it can be seen that the retention volume of acetone decreased as much as the observed reduction in the total bed volume (2.6 ml). However, the bed compression cannot totally explain the decrease in the retention volume (V_R) of the two peaks emanating from β-lactoglobulin with isocratic elution (Table II). Moreover, the cleaning procedures after the 249th injection (Table I) resulted in a temporary increase in V_R for these peaks (Table II). A possible explanation of these results could be that the support matrix was gradually contaminated with proteins and, as a logical consequence, the amount of available ion-exchange groups became reduced. From this explanation the observed increase in V_R after a protein cleaning

TABLE I

WASHING PROCEDURES AND OTHER EVENTS IN ANALYTICAL-SCALE SEPARATIONS ON Q-SEPHAROSE HP

Injection No.[a]	*Washing solution or event*	*Washing conditions*
24	Gel run dry	
74	Adaptor adjusted	
95	Adaptor adjusted	
149	0.5 *M* NaOH	20 ml, 1.3 ml/min
174	Adaptor adjusted	
195	Adaptor adjusted	
199	0.5 *M* NaOH	20 ml, 1.3 ml/min
	13 *M* acetic acid	40 ml, 1.3 ml/min, reversed flow
	Adaptor adjusted	
219	Adaptor adjusted	
244	Adaptor adjusted	
249	2 *M* NaCl	20 ml, 1.3 ml/min
	1 *M* NaOH	80 ml, 1.3 ml/min, reversed flow
	13 *M* acetic acid	40 ml, 1.3 ml/min, reversed flow
	5% Decon	40 ml, 1.3 ml/min, reversed flow and 60 min without flow
	40% ethanol	200 ml, 1.3 ml/min, reversed flow
274	0.1 *M* NaOH	7 days without flow
299	1 *M* acetic acid	5 days without flow

[a] The injection number given is that just before the event indicated.

TABLE II

VARIATION OF BED HEIGHT, CHLORIDE ION CAPACITY AND RETENTION VOLUME IN ISOCRATIC ELUTIONS IN ANALYTICAL-SCALE SEPARATIONS ON Q-SEPHAROSE HP

Injection No.	*Bed height (cm)*	*Chloride ion capacity*[a] *(mmol)*	*Retention volume (ml)*		
			Acetone	*β-Lactoglobulin*[b]	
				Peak I	*Peak II*
1	[c]	[c]	19.0	36.0	92.5
50	9.9	3.56 ± 0.07	18.0	35.4	93.0
100	9.8	n.d.[d]	17.4	33.6	84.9
150	9.5	3.53 ± 0.08	17.2	32.7	79.5
200	9.1	n.d.	17.0	32.4	84.0
250	8.9	n.d.	16.4	33.0	88.8
275	8.9	n.d.	16.4	n.d.	n.d.
300	8.9	3.54 ± 0.07	16.2	32.4	87.0

[a] The capacity is given for the total gel volume. The values are given with a 95% confidence interval. The pooled standard deviation is 0.05 mmol (degrees of freedom = 6).

[b] The β-lactoglobulin peaks are eluted at 55% solvent B.

[c] Prior to the start the bed height was 10.2 cm and the chloride ion capacity was 3.53 ± 0.08 mmol, corresponding to 0.173 mmol/ml gel.

[d] n.d., not determined.

TABLE III

VARIATION OF THE RETENTION VOLUME OF THE FOUR MAIN PEAKS DURING 300 INJECTIONS OF A PROTEIN MIXTURE ON A Q-SEPHAROSE HP COLUMN

Injection No.	*Peak I (ml)*	*Peak II (ml)*	*Peak III (ml)*	*Peak IV (ml)*
1	48.87	74.04	84.69	100.74
25	48.24	73.77	84.75	100.53
50	47.97	73.65	84.58	100.26
75	47.85	73.41	83.85	100.05
100	46.77	72.15	82.74	98.73
125	46.53	72.12	82.08	98.64
150	47.19	72.81	82.89	99.48
175	46.47	71.85	82.41	99.21
200	46.59	71.46	82.17	98.52
225	46.83	71.28	82.05	98.13
250	46.98	71.70	82.62	99.27
275	48.00	73.11	84.00	99.57
300	47.91	73.14	84.00	99.12

step can also be rationalized. The fact that the chloride capacity remained constant during the lifetime test (Table II) indicates that the ion-exchange groups are chemically stable.

From Tables I and III it can be seen that the retention volumes under gradient elution also decreased with the number of sample injections until the column was cleaned. However, no effect on V_R from the cleaning process after the 199th injection was observed because the adaptor was adjusted at the same time (Table I). The column cleaning procedure with 0.1 *M* sodium hydroxide for 7 days gave rise to the largest changes in V_R of the four peaks (Table III), indicating that this was the most effective cleaning procedure investigated.

The peak heights had also decreased after 300 injections (Fig. 1). This reduction may reflect a decrease in the column plate count due to bed compression. In addition, peak II was split at the 225th injection. From separate experiments it was concluded that this extra peak resulted from denaturation of ovalbumin. The chromatographic pattern was restored after the injection of a fresh sample.

The results presented indicate that if the performance of a Q-Sepharose HP column is to remain unchanged over a long period of use, it must be cleaned regularly.

Column lifetime in preparative-scale chromatography

Repetitive injections of 50 ml of egg white, diluted 10-fold with solvent A, were used to investigate the fouling of Q-Sepharose HP under preparative conditions. A preparative and an analytical separation of egg white are depicted in Figs. 2 and 3, respectively. During each preparative run about 0.5 g of ovalbumin was purified but the recovery of ovalbumin had decreased by about 25% after six repetitive preparations (Fig. 4). Moreover, the gel bed height was reduced by 3 cm. After the first six preparations the column was treated with 1.0 *M* sodium hydroxide for 2 h. As this treatment did not restore either the recovery or the bed height, a 0.1% pepsin solution was used to clean the column. It was found that enzymatic cleaning of the column after every sixth preparation restored the recovery to 100% (Fig. 4) and the gel bed

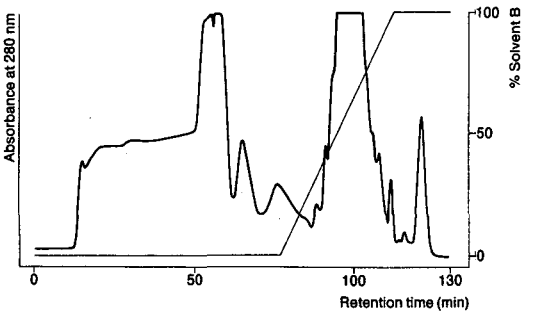

Fig. 2. Preparative-scale chromatogram of egg white on Q-Sepharose HP packed in an HR 16/10 column. A 50-ml sample of 10-fold diluted egg white was injected at a flow-rate of 1.0 ml/min. See Experimental for details of chromatographic conditions.

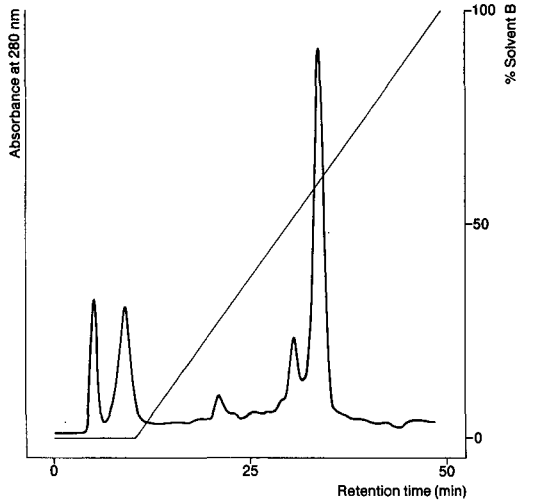

Fig. 3. Analytical-scale chromatogram of egg white on Q-Sepharose HP packed in an HR 16/10 column. A 500-μl sample of 10-fold diluted egg white was injected at a flow-rate of 3.0 ml/min. See Experimental for details of chromatographic conditions.

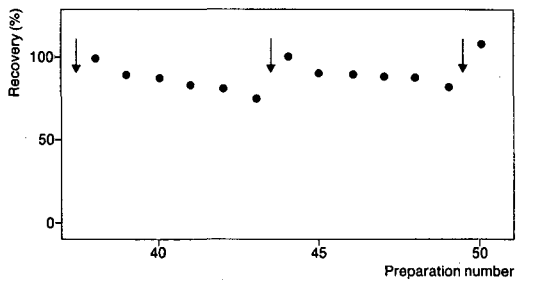

Fig. 4. Recovery of ovalbumin from egg white using a Q-Sepharose HP column (HR 16/10). The recovery of the original preparation was set at 100%. Arrows indicate that the gel has been rinsed with 0.1% pepsin in 0.01*M* hydrochloric acid between the runs.

height to a great extent. The only effect observed after 50 preparations was a 10% decrease in bed height.

Bed compression

The results presented above indicate that the movable top filter may be an important cause of bed compression. To investigate this, an XK 16/20 column, which has a top filter attached to the flow adaptor, was packed with Q-Sepharose HP. It was found that after 100 repetitive injections of 10 mg/ml ovalbumin (injection volume 500 μl), the bed height was the same as at the beginning of the test (10.0 cm). This result indicates that a column with the top filter attached to the adaptor is to be preferred for semi-rigid gels when clogging proteins are to be separated.

CONCLUSION

It can be conluded that the durability of column performance in the preparative and analytical chromatography of proteins such as ovalbumin depends to a great extent on the choice of cleaning solution and how often the column is cleaned. Therefore, it is important that the packing material can withstand different washing solutions. In this investigation it has been shown that Q-Sepharose HP withstands treatment for long periods of time at both high and low pH values. However, the most effective cleaning solution in this instance was a pepsin solution. This cleaning procedure is probably also applicable to common analytical LC columns.

Further, to reduce the bed compression caused by filter clogging, it is important to use a top filter adaptor to which the filter is attached or to change the top filter regularly.

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Journal of Chromatography, 477 (1989) 345–357
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IDENTIFICATION OF REACTION PRODUCTS FROM THE PYRIDINIUM DICHROMATE DERIVATIZATION OF PROSTAGLANDINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND DIRECT CHEMICAL IONIZATION MASS SPECTROMETRY

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(First received January 18th, 1989; revised manuscript received April 17th, 1989)

SUMMARY

The reaction products from the oxidation of prostaglandins with pyridinium dichromate have been identified by direct chemical ionization mass spectrometry of the underivatized compounds after separation by reversed-phase high-performance liquid chromatography and ultraviolet diode-array detection. The thermal influence on the reproducibility of the dehydration patterns of the mass spectra was studied. The main products from the prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ were the corresponding 15-oxo derivatives. Minor amounts of the 9,11,15-trioxoprostaglandin were formed from PGE, while the oxidation of PGF was less selective, yielding additional dioxo derivatives. Addition of water to the reagent reduced the reactivity, but increased the selectivity in favour of the formation of 15-oxo-PGF during the oxidation of PGF.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become a widespread technique for the separation of prostaglandins (PGs)[b]. After separation, the isolated double bonds of non-derivatized PGs have been detected by UV spectrophotometry at low wavelengths (190–215 nm)[1–8]. However, derivatization is usually preferred in order to increase the sensitivity and selectivity. This includes fluorescent labelling of PGs with 4-bromomethyl-7-methoxycoumarin[9,10], 7-acetoxy-4-bromomethylcoumarin[11,12], 7-[(chlorocarbonyl)methoxy]coumarin[13], aromatic isocyanates[10], anthroylnitrile[14], *p*-(9-anthroyloxy)phenacylbromide[15,16] and anthryldiazomethane[17–19]. Recently, detection limits of 10–30 fmol (PGE, PGF; signal-to-noise ratio, S/N = 5, on column) have been obtained with 3-bromomethyl-6,7-

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[b] In this text, prostaglandins are collectively referred to as PGs, ($\Delta^{13,14}$)-prostaglandins as PG_1 and ($\Delta^{13,14}$,$\Delta^{5,6}$)-prostaglandins as PG_2, while PG without a subscript denotes PG_1 *and* PG_2 (PGE = PGE_1 *and* PGE_2).

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dimethoxy-1-methyl-2(1H)-quinoxalinone[20] and 1-pyrenyldiazomethane[21]. The formation of UV-absorbing substituted phenacyl esters[22–26], benzyloximes[27], naphthacyl esters[28–30] and benzyl esters[31] as well as the base catalysed conversion of E and A prostaglandins (PGE and PGA) into PGB (λ_{max} = 278 nm)[32–36] have been described. Detection limits of 140 fmol (S/N = 5, on column) have been achieved with 2,4-dimethoxyanilides and electrochemical detection[37].

We have previously reported a rapid UV derivatization procedure in which E-type PGs (PGEs) are selectively oxidized to their 15-oxo derivatives by pyridinium dichromate (PDC) in acetonitrile with detection limits of 0.14 pmol (S/N = 2, on column)[38,39]. In this paper the derivatization is further characterized with respect to the reactivity, selectivity and identity of the reaction products.

EXPERIMENTAL

Chemicals

All PGs were obtained from Sigma (St. Louis, MO, U.S.A.), PDC (98%) from Aldrich (Milwaukee, WI, U.S.A.) and formic acid (p.a.) from Merck (Darmstadt, F.R.G.). Acetonitrile, ethyl acetate and dichloromethane were HPLC grade from Rathburn (Walkerburn, U.K.). Deionized water was distilled once. PG standards were dissolved in acetonitrile ($2.8 \cdot 10^{-4} M$) and stored at −20°C.

HPLC equipment

The HPLC instrumentation consisted of a solvent-delivery system (Waters Model 590), a valve loop injector (Waters U6K) connected to a C_{18} column [Brownlee MPLC™, 200 mm × 2.1 mm, 5-μm C_{18} silica (Spheri-5) or Perkin-Elmer, 33 mm × 4.6 mm, 3-μm C_{18} silica (Pecosphere)] and a diode-array UV detector (Hewlett-Packard 1040A). Water, acetonitrile (HPLC grade S) and formic acid were used as the mobile phase.

Mass spectrometry

Direct chemical ionization mass spectrometry (DCI-MS) was performed on a double focusing mass spectrometer (JEOL JMS-DX 303) with methane or isobutane (AGA Specialgas) as the reagent gas. Measurement conditions: accelerating voltage, 3 kV; ionizing voltage, 200 eV; ionizing current, 300 μA; electron multiplier voltage, 1.5 kV and chamber temperature 210°C.

Derivatization of PGs

Unless stated otherwise, PGs were oxidized by PDC in acetonitrile, diluted in water and extracted with ethyl acetate to remove the excess of reagent as previously described[39].

RESULTS AND DISCUSSION

Identification of reaction products

Oxidation of PGE under the conditions stated above resulted in one major product in high yields, whereas the oxidation of PGF resulted in a more complicated pattern. This is illustrated in Figs. 1–4, in which the three-dimensional isoabsorbance

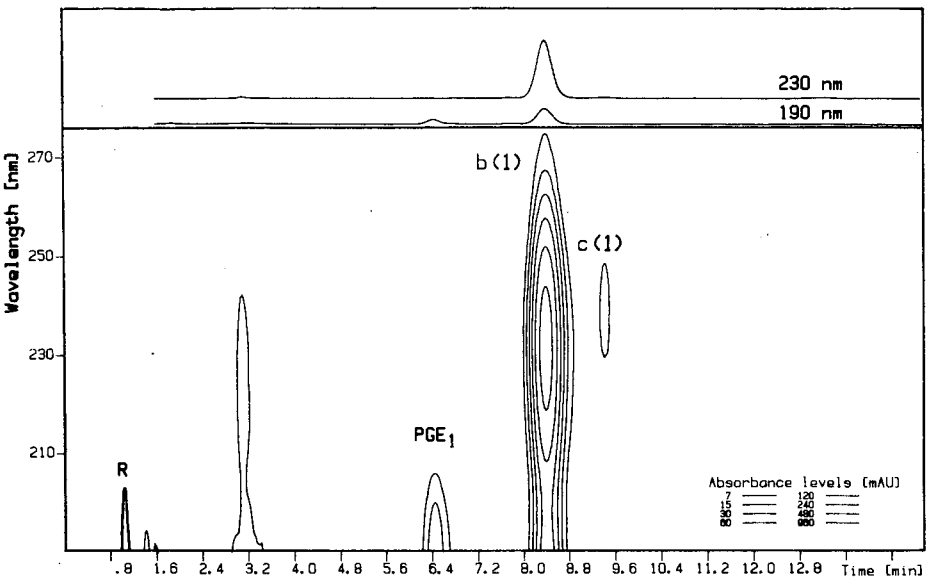

Fig. 1. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of PGE_1. Upper section: single-channel chromatograms; UV detection at 190 and 230 nm. Lower section: three-dimensional isoabsorbance diagram; UV diode-array detection. Column: C_{18} (Brownlee, 5-μm RP-18 silica, 200 mm × 2.1 mm I.D.). Mobile phase: acetonitrile–5 m*M* aqueous formic acid (36:64) at 0.4 ml/min. R = Reagent peak (PDC). For other structures see Fig. 7.

C_{18} reversed-phase chromatograms of the reaction mixtures of each PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are shown. The PGE chromatogram (Figs. 1 and 2) are dominated by compound b[a] and a minor contribution from the later-eluting compound c. The PGF chromatograms (Figs. 3 and 4) reveal three major components, a, b and d, under similar reaction conditions. Compound b from PGF will be coeluted with compound b from the corresponding PGE. The spectroscopic properties of all compounds a–d (Figs. 1–4, Table I) are clearly indicative of the presence of a conjugated enone chromophore.

To obtain further structural information, the reaction products of each PG were subjected to mass spectrometric analysis after isolation by fraction collection and extraction from the acidic aqueous mobile phase by dichloromethane (1:1, v/v). Besides being a convenient solvent for application on the solid sample probe, the concentrated dichloromethane solution (0.5 ml reduced to 10 μl) resulted in far less chemical noise than did unpurified HPLC grade ethyl acetate. Underivatized PGs can be analysed by electron-impact ionization (EI-MS), resulting in numerous fragment ions and complex spectra[40–42] which obscure the information on molecular weights[43]. By exposing non-volatile compounds on a heated probe to an appropriate ionized gas, molecular weight information can be obtained from the resulting CI spectra. This is frequently referred to as "direct exposure", "desorption CI", "direct CI (DCI)", "in-beam" or "surface ionization"[43–45]. Hydroxylated compounds like

[a] Abbreviated PG derivative names without an index denote PG_1 *and* PG_2 [b = b(1) *and* b(2)]. See Fig. 7.

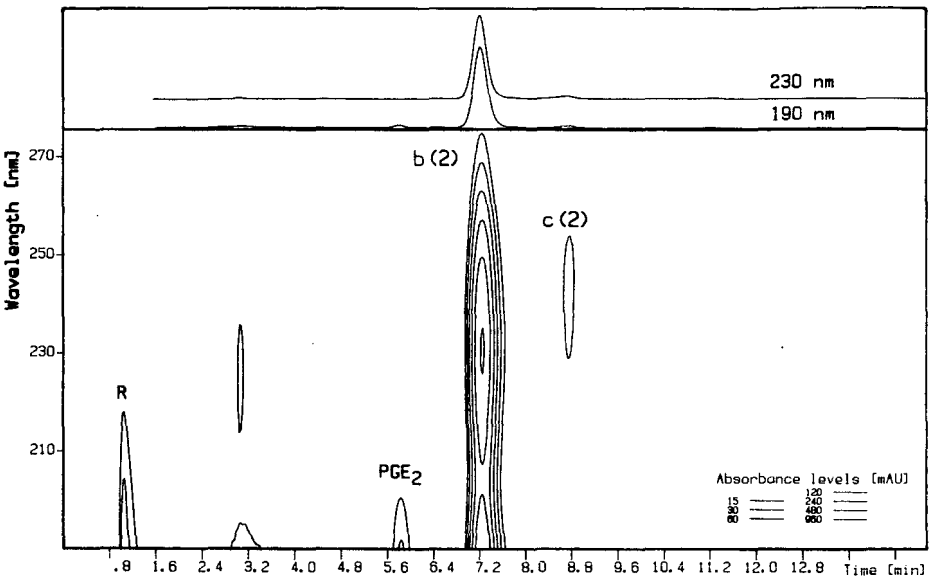

Fig. 2. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of PGE_2. Conditions as in Fig. 1.

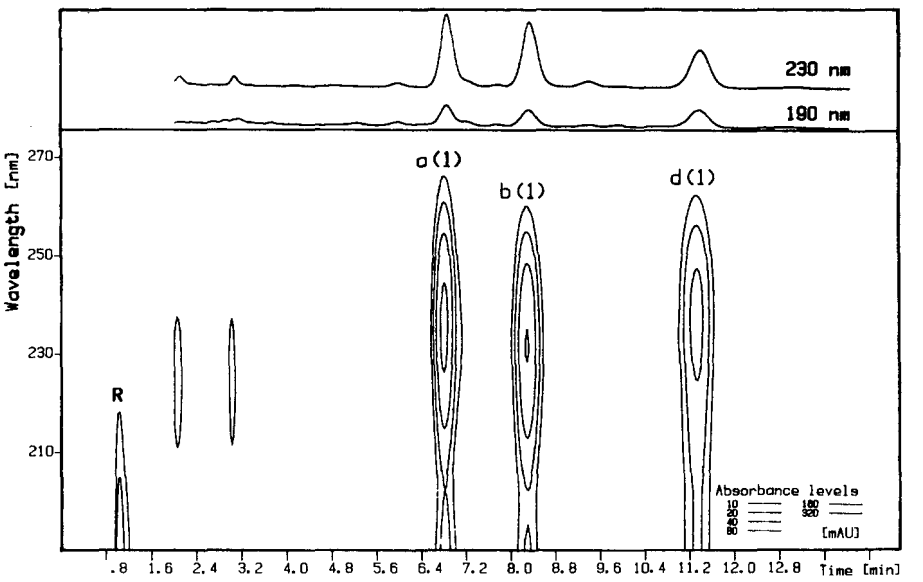

Fig. 3. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGF_{1\alpha}$. Conditions as in Fig. 1.

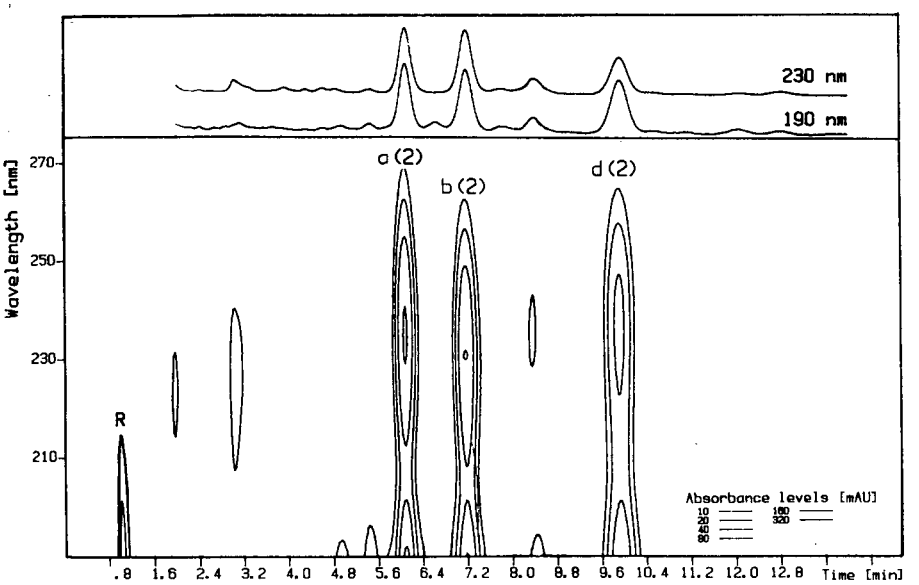

Fig. 4. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGF_{2\alpha}$. Conditions as in Fig. 1.

PGs will undergo dehydration under protonating CI conditions. The extent of dehydration can be reduced by increasing the proton affinity of the reagent gas relative to the analyte, *e.g.*, replacing methane with ammonia[45]. A protonating reagent gas can, however, be advantageous with respect to the identification of polar substituents, since the number of water losses will reflect the number of hydroxyl and/or ketone functions in the molecule, as observed in thermospray MS of PGs[46]. The dehydration process is clearly demonstrated by the isobutane CI spectra of $PGF_{2\alpha}$ and $PGF_{1\alpha}$ (Fig. 5) where three consecutive water losses were observed, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 339/337, 321/319 and 303/301, in addition to ions formed by elimination of CO_2. The possible combinations of these neutral losses are shown in Fig. 6. Ions MH^+ and $[M-H]^+$ of low abundances were also observed, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 357/355 and 355/353, as was a series of dehydrated ions from the $[M+C_4H_9]^+$ adduct, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 395/393, 377/375 and 359/357.

TABLE I

UV ABSORPTION MAXIMA IN ACETONITRILE–5 m*M* AQUEOUS FORMIC ACID OF PGs AFTER PDC OXIDATION

Abbreviated names refer to Figs. 1–4 and 7.

	λ_{max} (nm)
PGE_1/PGE_2	<190/<190
PGF_1/PGF_2	<190/<190
a(1)/a(2)	234/234
b(1)/b(2)	230/230
c(1)/c(2)	242/242
d(1)/d(2)	234/234

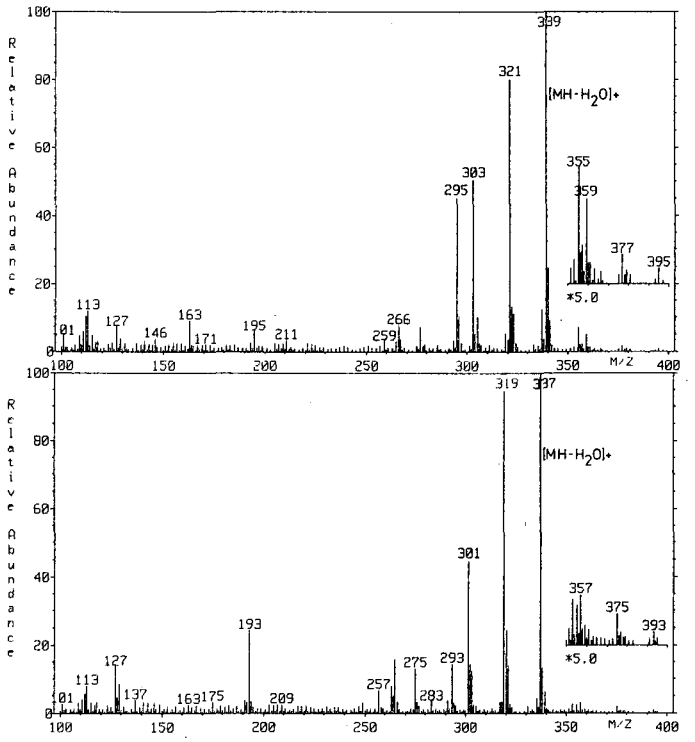

Fig. 5. Direct CI-MS (DCI-MS) of $PGF_{1\alpha}$ (MW 356) (upper spectrum) and $PGF_{2\alpha}$ (MW 354) (lower spectrum). Reagent gas: isobutane.

The partial isobutane DCI mass spectra of oxidized and non-oxidized PGs are compared in Table II. Spectra were obtained by heating a conventional quartz capillary solid sample probe from ambient temperature to 100°C in 6 s, keeping it isothermal for 10 s and finally raising the temperature to 200°C in 20–30 s. The ion source was kept at 210°C. As is seen from Table II, protonated molecules (MH^+) of the native PGs were low in abundance (1–4%) as compared to those for the oxidized PGs (7–100%). This is probably reflecting the presence of the allylic activated hydroxyl group at C-15 and/or a β-ketol (PGE) from which dehydration will easily occur.

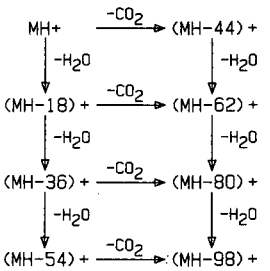

Fig. 6. Formation of fragment ions in DCI-MS of PGs by successive dehydration and decarboxylation.

The mass spectrum of each compound in the reaction sequence PGE->a->c (Table II) revealed a molecular weight reduction of 2 mass units and the disappearance of one hydroxyl group, *i.e.*, one hydroxyl group is oxidized, in each step. The same observation was made for the sequence PGF->a->b,d. Together with the UV spectroscopic and chromatographic data (Figs. 1–4, Table I), the structures in Fig. 7 have been assigned. The proposed structure of d(2) was confirmed by PDC oxidation of PGD_2 which yielded a compound with identical DCI mass spectrum, UV spectrum and HPLC retention time. The reversed-phase retention of compound c, eluted between the di oxo derivatives b and d, is not readily explained from its assigned tri oxo structure, suggesting a keto–enol conversion of the 9,13-dione to an hydroxyenone.

Although elimination of water was not observed exclusively from hydroxyl groups, but also from ketones, the latter is likely to happen less frequently, requiring a double proton transfer. With the exception of compound d, the data were in accordance with this assumption. After the assigned number of hydroxyl groups had been eliminated, there was a drastic reduction in the abundance of ions from continued dehydration (Table II). Assuming that the first dehydration step of compounds b and d is the formation of a β-enone in the cyclopentane ring, further elimination of water from d might be facilitated by the formation of an extended conjugated system fol-

TABLE II

PARTIAL ISOBUTANE DCI MASS SPECTRA OF PGs AND PRODUCTS FROM THE OXIDATION OF PGs WITH PDC

Abbreviated names (a–d) refer to corresponding components of the chromatograms in Figs. 1–4. The first and second entry in each column is the observed *m/z* value. Column indices 1 and 2 denote PG_1 and PG_2 respectively. Other entries are intensities relative to the base peak (= 100). An asterisk (*) denotes fragments resulting from dehydration of other than hydroxyl functional groups. Upper section: $[MH-nH_2O]^+$. Lower section: $[MH-CO_2-nH_2O]^+$.

		PG	*Products*		*PG*	*Products*		
		PGE ⟶	*a*	*c*	*PGF* ⟶	*a*	*b*	*d*
MH (*m/z*)	1:	355	353	351	357	355	353	353
	2:	353	351	349	355	353	351	351
MH^+	1:	1	9	100	3	100	10	11
	2:	1	8	100	4	100	8	7
$[MH-18]^+$	1:	36	100	7*	100	88	100	100
	2:	30	100	11*	100	57	100	100
$[MH-36]^+$	1:	100	5*	2*	80	16	7*	27*
	2:	100	6*	1*	95	19	6*	23*
$[MH-54]^+$	1:	7*	1*	1*	50	1*	1*	1*
	2:	5*	1*	3*	45	4*	0*	2*
$[MH-44]^+$	1:	2	3	11	1	6	9	2
	2:	1	4	10	2	5	1	3
$[MH-62]^+$	1:	10	11	2*	45	8	14	29
	2:	13	30	5*	17	5	22	30
$[MH-80]^+$	1:	25	3*	0*	25	3	2*	3*
	2:	27	2*	1*	10	3	0*	4*
$[MH-98]^+$	1:	2*	1*	2*	3	0*	1*	0*
	2:	2*	2*	3*	6	0*	1*	0*

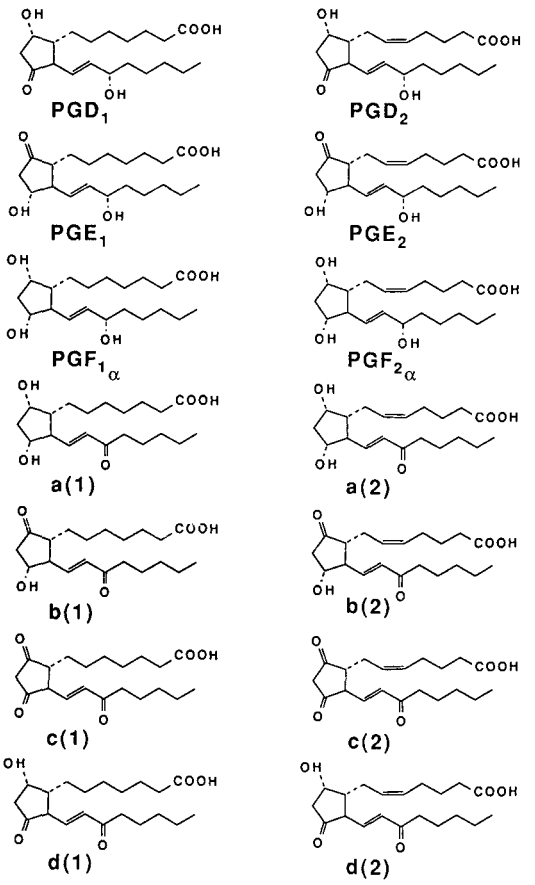

Fig. 7. PG structures and proposed structures of reaction products from the PDC oxidation of PGs.

lowing the 1,2-elimination of water from a protonated ketone at C-11, with possible contributions from the keto–enol tautomeric properties of the PGD C-11 keto group[47]. This may explain the difference in abundance between the $[MH-36]^+$ of b and d.

Relative ion abundances, in particular of the protonated molecules, varied both within and between experiments. In some cases, the variations between corresponding PG_1 and PG_2 were also larger than expected (Table II). In Fig. 8a is shown the variation in the relative ion abundances of the isobutane DCI spectrum through the total ion current (TIC) evaporation profile of $PGF_{1\alpha}$. The abundances of multi-dehydrated ions increased with time at the expense of MH^+ (not shown) and $[MH-H_2O]^+$, leading to a changeover in the base peak from $[MH-H_2O]^+$ to $(MH-2H_2O)^+$. Decarboxylation ions (*m/z* 295,277) were less influenced. The spectral variations of PGF were considerably reduced with methane as the reagent gas (Fig. 8b). Evidence for a possible thermal origin for these variations was provided by the work of Field[48], who found isobutane spectra of acetates to be strongly dependent on temperature, whereas methane spectra were less affected. Apparently, the

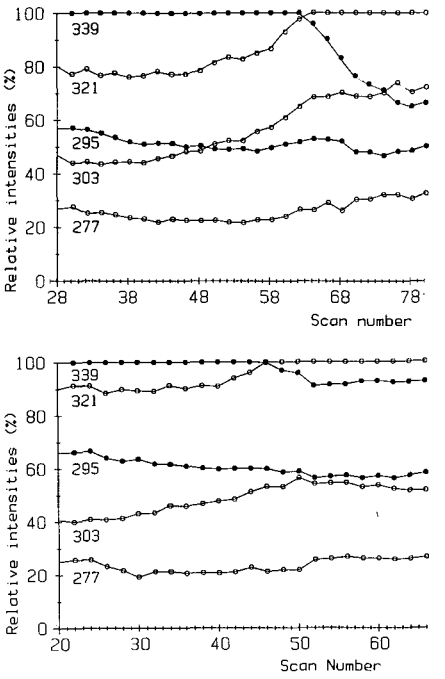

Fig. 8. Ion current profiles indicating the consistency of the DCI mass spectrum of $PGF_{1\alpha}$. Reagent gases: isobutane (upper spectrum) and methane (lower spectrum).

reduced exothermic nature of the isobutane protonation [proton affinity (PA) = 824 kJ/mol] compared to methane (PA = 546 kJ/mol) makes the fragmentation of the former more sensitive to variation in the internal energy prior to ionization. As previously questioned[45], an additional contribution to the spectral variations from thermal preionization dehydration cannot be excluded, since increased dehydration with time was observed even with methane CI. In particular, a time-dependent strong reduction of the ion $(MH-H_2O)^+$ was found in the CI spectra of PGE and PGD_2, independent of the choice of methane or isobutane as the reagent gas. Although the probe temperature was rapidly increased, the time needed for heat transfer to the quartz capillary and the sample may very well be responsible for the observed time-dependent abundance variations. Lowering the probe temperature somewhat reduced this effect, but resulted in reduced spectrum intensities due to broadening of the TIC profile, in particular of the more polar samples, *e.g.*, PGF. Below a probe temperature of 150°C the signal disappeared, as was the case below a source temperature of 150°C. Although the thermal effects will limit the reproducibility of ion abundances, the spectra can still be interpreted on a qualitative basis. In some cases, the DCI mass spectrum can even differentiate between positional isomers: the methane DCI spectrum of PGE_2 was readily distinguished from that of PGD_2 by the abundance of the ion $(MH-3H_2O)^+$ (*m/z* 299, Fig. 9), which was considerably higher with PGE_2 [relative abundance (R.A.) = 30%] than with PGD_2 (R.A. = 7%). This property did not change through the probe heating process. Isobutane as a reagent gas gave a much smaller difference.

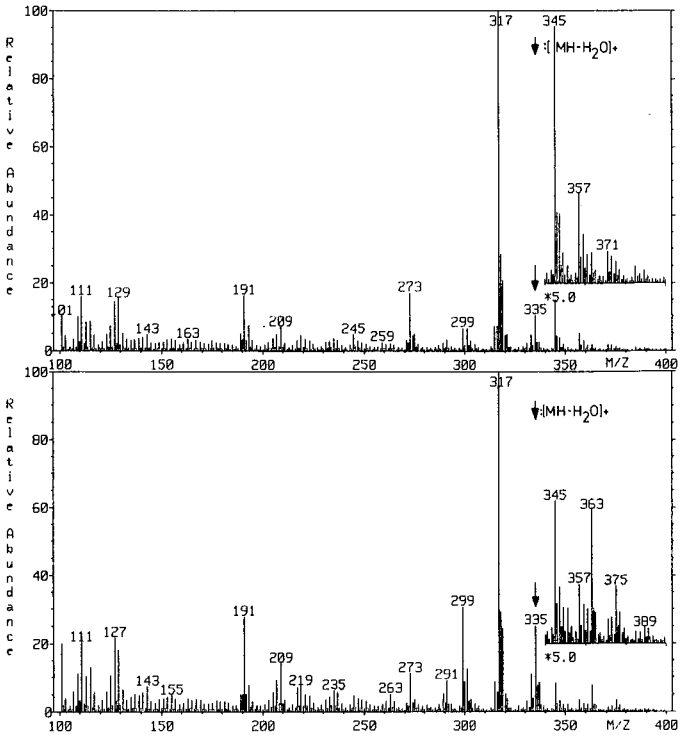

Fig. 9. DCI-MS of PGD_2 (MW 352) (upper spectrum) and PGE_2 (MW 352) (lower spectrum). Reagent gas: methane.

Increasing the solvent polarity will reduce the reactivity of PDC. To determine the influence of the solvent water content on the reactivity and selectivity, $PGF_{1\alpha}$ was oxidized in the presence of various concentrations of water. By direct injection of 1-μl aliquots of the reaction mixture on the short 3-μm particle column with a solvent flow-rate of 2.0 ml/min, the separation of all PGs was complete in less than 2 min, permitting a nearly real time monitoring of the oxidation, as shown in Figs. 10–12. According to the reaction kinetics, there is no need for extra drying of acetonitrile since a water content of 0.1% did not influence the reaction rate. On the other hand, a

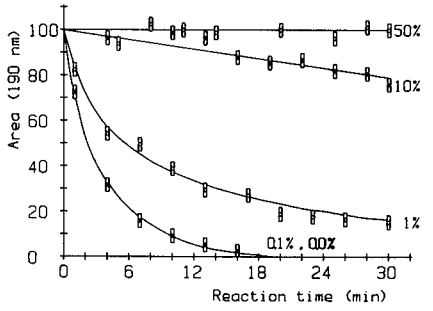

Fig. 10. Reaction of $PGF_{1\alpha}$ with PDC–acetonitrile in the presence of various concentrations of water.

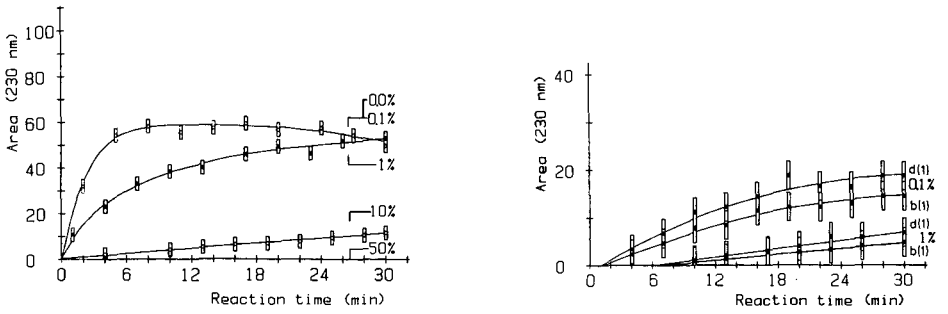

Fig. 11. Formation of the primary oxidation product a(1) from $PGF_{1\alpha}$ in the presence of various concentrations of water.

Fig. 12. Formation of the secondary oxidation products b(1) and d(1) from $PGF_{1\alpha}$ in the presence of various concentrations of water.

water content of 1% resulted in a selectivity change by reducing the formation of the by-products b and d (Fig. 12), although at the cost of reaction time (>30 min) (Figs. 10 and 11).

Peak areas of the derivatized relative to the underivatized PGs obtained from equal amounts of PGs are compared in Table III. PGE shows excellent response and selectivity, whereas the detection of PGF is impaired by less than half the response of

TABLE III

RELATIVE CHROMATOGRAPHIC RESPONSES (%) OF EQUAL AMOUNTS (100 ng) OF OXIDIZED AND NON-OXIDIZED PGs MEASURED AS: PEAK AREA (15-OXO-PG) (230 nm)/PEAK AREA (15-HYDROXY-PG) (190 nm)

PG	*15-oxo-PG*	*By-products*
	PG	*PG*
E_1	b(1): 140	c(1): < 1
E_2	b(2): 70	c(2): < 1
F_1	a(1): 58	b(1): 6
		d(1): 12
F_2	a(2): 30	b(2): 3
		d(2): 6

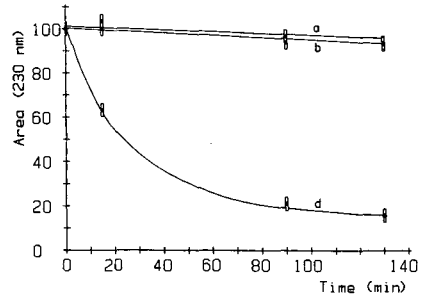

Fig. 13. Stability of oxidation products a, b and d from PGF in acetonitrile solution.

PGE and by-products at the 10–20% level. The response difference between oxidized PG_1 and PG_2 is not a property of the 15-oxo derivative, but reflects the larger absorptivity of underivatized PG_2 at 190 nm caused by the additional $\Delta^{5,6}$ double bond.

The low response of 15-oxo-PGF was, in addition to the effect of being distributed into several compounds, in part caused by the formation of compound d, which was not stable in solution, as shown in Fig. 13. Compounds a and b were stable for several hours in acetonitrile solution. This can be explained by the structural differences in the cyclopentane ring, since d possesses a reactive proton at C-12, being simultaneously allylic and α to a carbonyl group[47].

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Journal of Chromatography, 477 (1989) 359–365
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 549

COUPLED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY FOR THE DETERMINATION OF PESTICIDE RESIDUES IN BIOLOGICAL MATRICES

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(First received January 2nd, 1989; revised manuscript received April 4th, 1989)

SUMMARY

A fully automated high-performance liquid chromatography–gas chromatography (HPLC–GC) network is described. A ten-port valve set up as a loop type LC–GC interface allowed the transfer of large LC effluent fractions into the gas chromatograph by concurrent solvent evaporation. The system performed highly efficient sample enrichment and clean up by LC and on-line GC separation with sensitive electron-capture detection. The efficiency of the system was demonstrated by application to the trace analysis of N-(3-chloro-2,6-dimethylphenyl)-N-(2-oxotetrahydrofuranyl)-2-methoxyacetamide (CGA 80000) in various crops and soil samples. The residue level determined was 0.02 mg/kg for crop samples and 0.01 mg/kg for soil samples. The relative standard deviations of the calibration graphs were in the range 2–5%; the mean recovery was >85%.

INTRODUCTION

Recent trends towards automated analyses combine on-line sample preparation with the final determination[1]. High-performance liquid chromatography (HPLC) is a very efficient method to separate trace components from coextractives of the matrices. Coupled HPLC–gas chromatography (GC) systems allow the direct transfer of a selected LC fraction into a GC capillary column; they produce high analyte/matrix selectivities, lower the detection limits and improve quantitation[2]. The coupling of LC and GC has recently been made more feasible by the development of evaporation techniques for large solvent volumes in capillary GC retention gaps[3]. Using

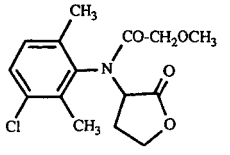

Fig. 1. Structure of CGA 80000, N-(3-chloro-2,6-dimethylphenyl)-N-(2-oxotetrahydrofuranyl)-2-methoxyacetamide.

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a loop-type LC–GC interface[4] in combination with automated column switching techniques, routine analyses are less time consuming and more reproducible.

CGA 80000, *i.e.*, N-(3-chloro-2,6-dimethylphenyl)-N-(2-oxotetrahydrofuranyl)-2-methoxyacetamide is a systemic experimental fungicide (Fig. 1). Residue methods developed so far use GC with electron-capture detection. The rather poor sensitivity of the component requires several labour intensive clean up and concentration steps.

EXPERIMENTAL

HPLC conditions and equipment

The LC system consisted of a 200 mm × 4.6 mm I.D. column packed with Nucleosil CN 5 μm (Macherey-Nagel, Düren, F.R.G.). Injections were made with a Valco injection valve (Model C6W; Valco, Houston, TX, U.S.A.); the injection volume was 500 μl. The solvent delivery system consisted of a piston pump (Model 420; Kontron Instruments, Zurich, Switzerland); the flow-rate was 1 ml/min. The mobile phase used was *n*-hexane–ethanol (8:2, v/v); the monitoring UV detector was a Pye Unicam LC-UV-detector (Pye Unicam, Cambridge, U.K.), operated at 220 nm.

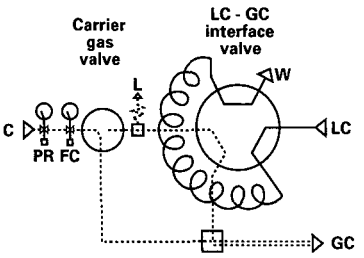
GC conditions and equipment

The GC system consisted of an Hewlett-Packard Model 5700 gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with an electron-capture detector. The analytical column was a 15 m × 0.53 mm I.D. fused-silica column (DB-5, film thickness 1.5 μm; J&W Scientific, Cordova, CA, U.S.A.). A retention gap of 3 m (530 μm I.D., fused-silica column phenyl-deactivated; Macherey-Nagel) coupled to the GC column with a press-fit connection (J&W Scientific) was used to reconcentrate the components of interest. The carrier gas was helium at a flow-rate of 8–10 ml/min during GC analysis and 1–2 ml/min during introduction of the LC fraction. Nitrogen at a flow-rate of 40 ml/min was used as a make-up gas for the electron-capture detector operated at 300°C. The GC oven temperature was held at 120°C during solvent evaporation, then programmed to 240°C at 16°C/min and held at 240°C for 8 min.

LC–GC interface

The HPLC–GC interface was assembled with commercially available components following suggestions by Grob[4] but modified for automated routine analysis. It consisted of a ten-port switching valve (Model C10W, Valco), a low dead volume T-piece (Model ZTl, Valco), a variable restrictor (Model 47220; Kuhnke, Malente, F.R.G.), a pressure regulator (Model 8286 ANVS-30; Porter Instruments, Hatfield, PA, U.S.A.), a flow controller (Model VDC-1000 AVF-10, Porter) and two pressure gauges (Model 111.10.40; WIKA, Klingenberg, F.R.G.).

Fig. 2 shows the loop-type LC–GC interface suggested by Grob[4] consisting of a sample valve and a carrier gas valve. The carrier gas is regulated by a pressure regulator followed by a flow controller. During GC analysis the carrier gas is flow controlled. However, while the LC fraction evaporates, the column inlet pressure increases. To accelerate the discharge of solvent vapours through the column and to maintain a constant solvent flow into the retention gap against the increased inlet pressure, a relatively high overall pressure setting is required[5]. As a consequence the



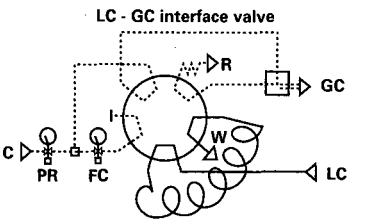


Fig. 2. Loop-type HPLC–GC interface according to suggestions by Grob[4]. The carrier gas switching valve and the interface valve are set to the mode for GC elution. C = Carrier gas supply; PR = pressure regulator; FC = flow controller; L = leak; GC = flow direction of carrier gas (to GC column); LC = flow direction of mobile phase (exit from LC column); W = waste.

Fig. 3. Loop-type HPLC–GC interface used in the present study. The ten-port interface valve is set to the mode for GC elution; the carrier gas flow is pressure regulated. R = Variable restrictor.

boiling point of the solvent increases. This must be compensated by higher initial transfer temperatures to maintain concurrent solvent evaporation conditions[6]. Hence, the minimum temperatures for eluting sharp peaks from the GC column are increased due to the reduced efficiency of the phase soaking effect[3]. The sample valve and its connection tubes are backflushed after solvent introduction to prevent contamination of the carrier gas with solvent residues.

Fig. 3 shows the modified LC–GC interface. A ten-port interface valve replaces the separate sample valve and carrier gas valve. Between the pressure regulator and the following flow controller a T-piece is installed which allows pressure regulation of the carrier gas during GC analysis. The connections between the interface valve and the T-piece above the GC oven are made of deactivated fused-silica capillaries or of inert polymeric capillaries. In contrast to the system set up by Grob using a 310-μm retention gap and analytical column, the interface valve described here is connected to 530-μm capillaries. The larger column diameter allows a low pressure setting of the carrier gas at 0.4 bar (with a resulting flow-rate of 8–10 ml/min at the initial transfer temperature of 120°C). The transfer flow-rate was set to 1–2 ml/min. During solvent evaporation the column inlet pressure is $\approx$0.3 bar permitting low initial transfer temperatures. The full power of the phase soaking process can be reached at lower GC oven temperatures and extends therefore the use of LC–GC loop transfer with concurrent solvent evaporation to lower boiling components.

LC–GC network

A sampler (Model 7671 or 7672, Hewlett-Packard) was adapted for HPLC loop sampling and for sample transfer into the gas chromatograph. A detailed description is given in ref. 7.

Fig. 4A–D show the LC–GC network used in this study with a single LC column. In Fig. 4A and B the sample loop (500 μl) is filled and, after rotating the sampling valve into the ON position, LC elution starts. The effluent of the LC column passes through the GC injection loop (size 1000 μl) of the ten-port interface valve. After rotating the interface valve to the injection position (Fig. 4C, position ON) the flow controlled carrier gas forces the solvent fraction containing the analyte of interest from the loop

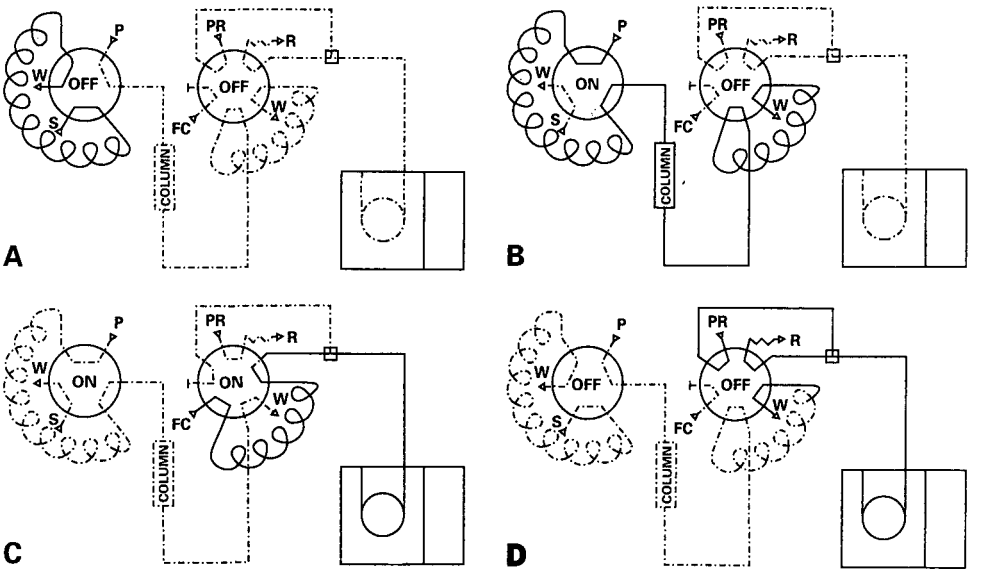


Fig. 4. HPLC–GC network. LC sampling valve and loop-type HPLC–GC interface valve. W (six-port sampling valve) = Waste exit of the LC sampling loop connected to the sucking pump; S (six-port sampling valve) = sample inlet port from the automated liquid sampler; P (six-port sampling valve) = eluent pump; PR = pressure regulated carrier gas inlet; FC = flow controlled transfer gas inlet; R = variable restrictor; W = waste exit of the LC–GC transfer loop; ON = injection position; OFF = standby position. Solid lines show the actual liquid sample and gas flow, dashed lines are standby positions. (A) Standby position, LC loop being filled; (B) injection and elution of the LC column; (C) transfer of the loop fraction into the gas chromatograph with the flow controlled carrier gas flow; (D) GC elution and detection with the pressure regulated carrier gas flow, purging of the liquid transfer line.

into the retention gap. As the column inlet pressure increases during solvent evaporation, the flow controller shuts the gas line and prevents a back flow of solvent vapours. Part of the solvent is pressed through the T-piece connector into the carrier gas line, but the compressed gas prevents the solvent reaching the interface valve. The increase of the pressure during the transfer of the LC fraction is monitored by the pressure gauge of the flow controller. At the end of the transfer period the pressure decreases, the flow controller opens the gas line and residual solvent is flushed into the retention gap. The interface valve is now rotated back (Fig. 4D, position OFF), the carrier gas supply returns to pressure regulation and the elution of the GC column is started. The solvent transfer line is backflushed through the variable restrictor (flow-rate $\approx$0.3 ml/min) to prevent residual solvent vapours from entering the GC column during GC analysis.

This LC–GC network with a single LC column can be extended to multiple column networks[8] for trace enrichment[9] and clean up of complex sample matrices[10]. The sampler with the ten-port interface valve can also be used as a large volume GC sampler suitable for direct sample enrichment on the GC column. This GC injection mode can easily be achieved by connecting the interface valve directly to the liquid sampler.

Sample preparation

The whole crop samples were homogenized with a cutter (horizontal cutter Model H4/4-2R; Schwabenland, Zurich, Switzerland). Subsamples of 10 g were extracted with 150 ml methanol–0.1% phosphoric acid (9:1, v/v) by shaking for 120 min. A 9-ml volume of the extract was transferred to a round-bottom flask and concentrated to 4 ml using a rotating evaporator. After dilution in 10 ml methanol–0.1% phosphoric acid (2:8, v/v) the solution was transferred to a Chem Elut® 1020 column (No. AI CE 1020; Analytichem, Harbor City, CA, U.S.A.). The round-bottom flask was rinsed twice with 2 ml methanol–0.1% phosphoric acid (2:8, v/v) and the rinsing solutions were transferred to the column. CGA 80000 was eluted with 80 ml toluene–*tert.*-butyl methyl ether (8:2, v/v); the eluate was evaporated to dryness and redissolved in 6 ml hexane–ethanol (8:2, v/v).

RESULTS AND DISCUSSION

Injection of large volumes in GC

To test the performance of the LC–GC interface, standard samples were injected directly into the retention gap by the loop interface and compared to manual injections made with a micro syringe into the standard GC injection port. The interface valve was connected to the liquid sampler and 500 μl of standard solutions containing 5 ng CGA 80000 were injected. The repeatability of this injection technique was determined from a set of ten injections. Mean and standard deviation were calculated to be 4.91 $\pm$ 0.16 ng ($N = 10$). Volumes of 2 μl of standard solutions containing 5 ng CGA 80000 were injected manually and mean and standard deviations were calculated to be 5.34 $\pm$ 0.38 ng ($N = 10$). The results demonstrate the possibility to enrich trace samples on the GC column from large solvent volumes without disturbing the performance of the electron-capture detector flushed with large volumes of solvent vapours.

The GC system was calibrated by injecting 500 μl of standard solutions (loop injection) in the range 1–25 ng CGA 80000. The resulting peak heights were used to calculate a linear regression. The standard deviations of the relative deviations of the measured peak heights from the calculated regression lines were in the range of 2–5%.

Direct GC injection with the loop type interface was also used to optimize the transfer time and the solvent evaporation temperature. The initial transfer temperature was set to 120°C for routine analysis. A reduction of the transfer temperature to 100°C did not increase the sensitivity significantly. Using a transfer flow-rate of 1–2 ml/min, good reproducibility was achieved with transfer times of about 5 min for 500-μl samples in hexane–ethanol (8:2, v/v) and of about 11 min for 1000-μl samples.

The solvent evaporation temperature and transfer times are not very critical for the routine analysis of CGA 80000 as the elution temperature of the compound is relatively high. In our experience the crucial factor is the purity of the solvents when using electron-capture detection (ECD) and large injection volumes. Similar problems were also reported in the application of ECD in LC[11]. The authors recommend a purification procedure to remove electron-absorbing contaminants from solvents. So far these clean up procedures have not been tested because changing to another batch or to another quality of the solvents solved the problem.

TABLE I

CALIBRATION OF THE HPLC–GC SYSTEM AND RECOVERIES OF SAMPLES FORTIFIED WITH CGA 80000

Mean and standard deviation of recoveries: 87 ± 8% (N=42).

Crop analyzed	*Range of standard deviations (%)*	*Range of recoveries (%)*
Pepper	2.6–5.2	70–96
Strawberry	1.4, 4.5	84–97
Citrus	1.2, 3.3	86–114
Soy bean	3.9	79, 85
Soil	2.7–4.8	80–91

Coupled HPLC–GC

For the determination of low pesticide concentrations in soil and crop samples, off-line LC techniques are commonly used for pretreatment and clean up purposes. Since CGA 80000 is separated into the isomeric components on silica columns, prepacked cartridges packed with C_{18} chemically modified silica gel were used. These columns however showed irreproducible performance due to batch to batch variablity caused by residual silanol groups. Reusable LC colunns for sample clean up are therefore an attractive alternative to prefilled cartridges.

The HPLC and GC conditions were optimized separately. The sensitivity of CGA 80000 was of the order of 10 ng by UV detection, while the sensitivity by GC with ECD was 0.5 ng.

The repeatability of the overall LC–GC system was determined from ten injections of standard solutions with 5 ng CGA 80000. Mean and standard deviation were calculated to be 5.17 ± 0.04 ng (N=10). The system was calibrated by injecting standard solutions of CGA 80000 ranging from 1 to 25 ng. The resulting GC peak heights were used to calculate the linear regression with the relative standard deviation. The data for the calibrations are summarized in Table I.

Analysis of residue samples

The described LC–GC network is being used for routine trace analysis of CGA 80000 and a wide variety of crops and soil samples has been analyzed. To check the performance of the system, samples were fortified with 0.04 and 0.2 mg/kg of CGA 80000. Soil samples were also fortified at levels of 0.02 and 0.1 mg/kg. Standard and sample injections were made alternately. The linear regression, the relative standard deviation of each calibration graph and the recoveries of the fortified samples were calculated. The results are given in Table I.

Fig. 5A and B show the chromatograms of orange peel samples. Compared with the standard injection in Fig. 5C, only few peaks from crop coextractives influence the GC separation with the sensitive electron-capture detector.

The residue level for quantitation used for routine analyses is 0.02 mg/kg of CGA 80000 in various crops and 0.01 mg/kg in soil samples. The limit of determination was not fully exploited as the LC sampling volume may be increased to several millilitres and the efficiency of the LC clean up can be increased by column switching depending on the crop to be analyzed.

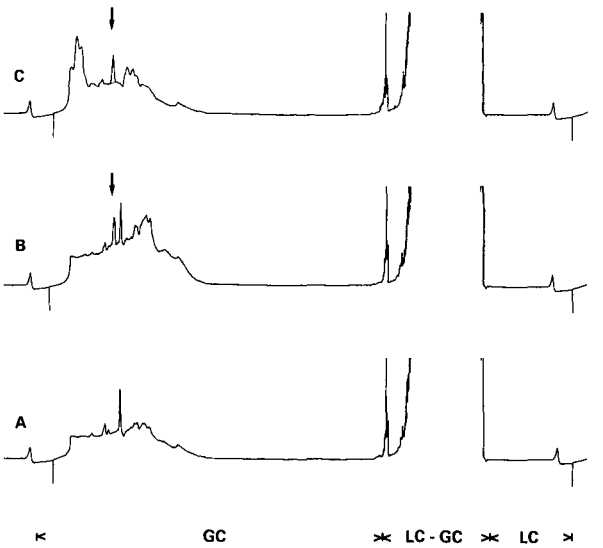


Fig. 5. (A) Gas chromatogram of an extract of orange peels after on-line HPLC clean up. The aliquot injected corresponds to 50 mg of the extract. (B) Gas chromatogram after on-line HPLC clean up of an extract of orange peels spiked before extraction with 0.04 mg/kg of CGA 80000. The aliquot injected corresponds to 50 mg of the extract. (C) Standard injection of 2 ng of CGA 80000 into the coupled HPLC–GC system.

CONCLUSIONS

Coupled HPLC–GC has been shown to be highly suitable for automation of the trace analysis of CGA 80000. On-line sample concentration and LC clean up prior to GC detection reduced the analysis times. Using a loop-type LC–GC interface with the possibility of injecting large sample volumes into capillary columns, the required limits of determination were obtained with good reproducibility.

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Journal of Chromatography, 477 (1989) 367–376
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 592

OPTICAL RESOLUTION OF AMINO ACID DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON TRIS(PHENYLCARBAMATE)S OF CELLULOSE AND AMYLOSE

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(Received February 13th, 1989)

SUMMARY

The optical resolution of ten N-protected alanine esters was examined by high-performance liquid chromatography using six cellulose and five amylose tris(phenylcarbamate) derivatives as chiral stationary phases. Tris(3,5-dimethylphenylcarbamate)s of both cellulose and amylose showed high resolving power for these racemates. The resolution of 23 N-benzyloxycarbonyl α-amino acid esters was also tested on tris(3,5-dimethylphenylcarbamate)s of cellulose and amylose. All amino acid derivatives except two were completely resolved at least by one of the colums. On cellulose tris(3,5-dimethylphenylcarbamate), the L-isomers of the amino acids except for threonine were eluted first.

INTRODUCTION

Recently, we reported that phenylcarbamate derivatives of polysaccharides, particularly cellulose tris(phenylcarbamate)s (CTPCs)[1–3] and amylose tris(phenylcarbamate)s (ATCPs)[4], showed characteristic optical resolving abilities for various enantiomers when used as a chiral stationary phase (CSP) for high-performance liquid chromatography (HPLC). The optical resolving abilities of the derivatives having various substituents on the phenyl groups were dependent greatly on the inductive effects of the substituents, and either 3,5-dimethyl- or 3,5-dichlorophenylcarbamates of cellulose and amylose often showed the best chiral recognition abilities for many racemic compounds.

In this work, we investigated the resolution of N-protected amino acid esters by using CTPC (**1–6**) and ATPC (**7–11**) derivatives. The amino groups of racemic amino acids were protected with acetyl (Ac), benzoyl (Bz), *tert.*-butoxycarbonyl (Boc), phenoxycarbonyl (PhOC) or benzyloxycarbonyl (CBZ) groups and the carboxy group with ethyl (Et) or benzyl (Bzl) groups.

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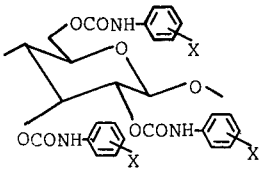
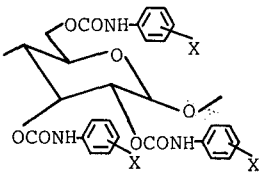


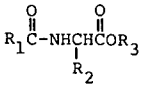
CTPC

	X =
1	3,5-Me_2
2	4-Me
3	4-Et
4	H
5	4-F
6	4-Br

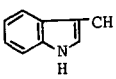


ATPC

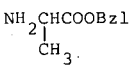
	X =
7	3,5-Me_2
8	4-Me
9	H
10	4-Cl
11	3,5-Cl_2



$R_1 = CH_3$, Ph, $t\text{-}C_4H_9O$, PhO, $PhCH_2O$

$R_2 = CH_3$, C_2H_5, $(CH_3)_2CH$, $CH_3(CH_2)_2$, $(CH_3)_2CHCH_2$, $CH_3(CH_2)_3$, $CH_3CH_2(CH_3)CH$, Ph, $PhCH_2$, $HOCH_2$, $HO(CH_3)CH$, Bzl-O-$C_6H_4CH_2$, R_3OOCCH_2, $R_3OOC(CH_2)_2$, H_2NCOCH_2, $H_2NCO(CH_2)_2$,  ,  , $CBZ\text{-}NH(CH_2)_4$, $CH_3CH_2SCH_2$, $Bzl\text{-}SCH_2$, $-(CH_2)_3-$, $-(CH_2)_4-$

$R_3 = C_2H_5$, $PhCH_2$



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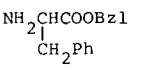

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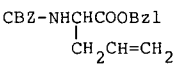

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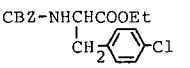

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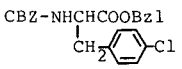

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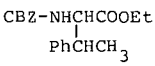

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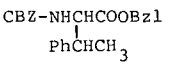

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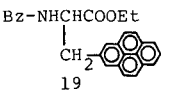

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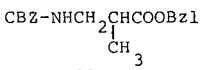

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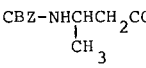

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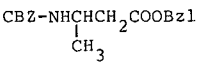

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EXPERIMENTAL

Details on the preparation of CSPs were described previously[2]. CTPC and ATPC derivatives (25%, w/w of silica gel) were adsorbed on macroporous silica gel (Nucleosil 4000-7), which had been treated with 3-aminopropyltriethoxysilane.

Each CSP was packed in a stainless-steel tube (25 cm × 0.46 cm I.D.) by a

slurry method. Chromatographic analyses were performed on a JASCO Trirotar-II chromatograph equipped with UV (JASCO UVIDEC-100-III) and polarimetric (JASCO DIP-181C) detectors. The optical resolution was monitored with a flow cell (50 mm × 2 mm (I.D.) at full lamp (mercury) intensity without a filter. Optical resolution was performed with an hexane–2-propanol mixture at a flow-rate of 0.5 ml min^{-1} at 25°C. The dead time, t_0, was estimated with 1,3,5-tri-*tert.*-butylbenzene as a non-retained compound[5]. ^1H NMR spectra were measured with a JEOL-MH-100 (100 MHz) spectrometer.

Racemic and optically active samples of amino acid derivatives were prepared by the conventional methods and were identified by IR, ^1H NMR and elemental analyses.

RESULTS AND DISCUSSION

Fig. 1 shows the complete resolution of alanine as its N-benzyloxycarbonyl ethyl ester on cellulose tris(3,5-dimethylphenylcarbamate) **1**. The capacity factors, $k'_1 = (t_1 - t_0)/t_0$ and $k'_2 = (t_2 - t_0)/t_0$, for the first and second isomers eluted were 1.56 and 2.82, respectively, and the separation factor, $\alpha = k'_2/k'_1$, which represents the chiral recognition ability of CSPs, was 1.82. The resolution factor, R_s, which can be estimated by $2(t_2 - t_1)/(W_1 + W_2)$, was 4.82.

Ten N-protected alanine esters were chromatographed on CTPC derivatives **1**–**6** (Table I). The chiral recognition abilities of CSPs depended greatly on the substituents of CTPCs. Among the six CTPC derivatives, the 4-bromo derivative **6** exhibited the best resolving power for Ac-Ala-OEt, Ac-Ala-OBzl, Bz-Ala-OBzl, PhOC-Ala-OEt and PhOC-Ala-OBzl. However, these alanine derivatives gave rather broad peaks on **6** as shown by the relatively small R_s values. On the other hand, compound **1** exhibited the highest α and R_s values for N-CBZ alanine derivatives.

Recently, we reported that compound **1** exhibits high optical resolving power for the phenylcarbamates (PhNHCOOR*) of racemic secondary alcohols (R*OH)

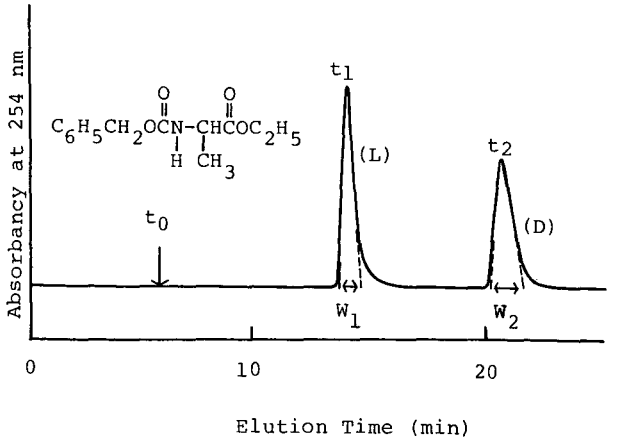

Fig. 1. Chromatographic resolution of N-benzyloxycarbonyl alanine ethyl ester on cellulose tris(3,5-dimethylphenylcarbamate) **1** (Column: 25 cm × 0.46 cm I.D. Eluent: hexane–2-propanol (90:10); flow-rate: 0.5 ml/min. Temperature: 25°C.

TABLE I

RESOLUTION OF N-PROTECTED ALANINE DERIVATIVES ON CTPC DERIVATIVES **1–6**

Eluents: hexane–2-propanol (80:20); for column **1**, hexane–2-propanol (90:10).

Column	*Ac-Ala-OEt*			*Ac-Ala-OBzl*			*Bz-Ala-OEt*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
1 3,5-Me_2	1.09(+)	1.12		5.52(−)	1.04	0.72	1.51(D)	1.34	2.30
2 4-Me	1.05(−)	1.25	0.78	1.65(−)	1.32	1.28	1.26(L)	1.12	
3 4-Et	0.68(−)	1.49	1.87	1.18(−)	1.58	2.06	0.66(L)	1.29	0.93
4 H	4.74(−)	1.14	0.88	7.69(−)	1.22	1.57	2.01	1.00	
5 4-F	2.08(−)	1.36	1.11	3.11(−)	1.51	1.58	2.62(L)	1.49	1.29
6 4-Br	1.61(−)	1.63	1.20	2.58(−)	1.65	1.61	2.74(L)	1.22	0.91
	Bz-Ala-OBzl			*Boc-Ala-OEt*[a]			*Boc-Ala-OBzl*[a]		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
1	3.52(D)	1.15	1.26	0.88(D)	*ca.* 1		1.89(D)	1.31	2.13
2	2.10(D)	1.25	1.06	0.48(D)	*ca.* 1		0.87(L)	*ca.* 1	
3	1.20(D)	1.23	1.07	0.19(D)	*ca.* 1		0.61(L)	1.07	
4	3.22(D)	1.15		1.13(D)	*ca.* 1		1.21(L)	1.25	0.68
5	3.87(D)	1.32	1.50	0.76(D)	1.21		0.82(L)	1.28	0.92
6	4.98(D)	1.37	1.71	0.69(D)	*ca.* 1		0.58(L)	1.27	
	PhOC-Ala-OEt			*PhOC-Ala-OBzl*			*CBZ-Ala-OEt*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
1	1.89(D)	1.30	1.98	4.02(L)	1.03		1.56(L)	1.82	4.82
2	1.53(D)	1.20	0.99	2.79(L)	1.33	2.04	1.28(L)	1.32	1.48
3	0.98(D)	1.09		1.64(L)	1.23	2.04	0.95(L)	1.42	1.86
4	1.81(D)	1.12		2.98(L)	1.31	1.09	3.63(L)	1.20	1.02
5	2.10(D)	1.35	1.32	3.36(L)	1.52	1.86	2.68(L)	1.50	0.79
6	2.37(D)	1.46	1.33	3.39(L)	1.62	2.18	4.37(L)	1.05	
	CBZ-Ala-OBzl								
	k'_1	α	R_s						
1	3.08(L)	2.20	7.26						
2	2.11(L)	1.30	1.53						
3	1.57(L)	1.47	2.59						
4	6.13(L)	1.23	1.41						
5	4.66(L)	1.74	1.93						
6	4.36(L)	1.04							

[a] Eluent: hexane–2-propanol (90:10).

but low resolving power for the benzoyl esters (PhCOOR*)[6]. This suggests that the interaction between the urethane groups of (±)-PhNHCOOR* and the urethane groups of **1** is much more important for the resolution than the interaction between the ester group of (±)-PhCOOR* and **1**. Therefore, in the present optical resolution,

the interaction between the N-protecting groups and the urethane groups of CSPs may be more important than that between the ester groups and CSPs. However, the elution order of enantiomers depended greatly on the protecting groups for both the NH_2 and COOH groups. D-Isomers of Bz-Ala-OBzl, Boc-Ala-OEt and PhOC-Ala-OEt were always eluted first regardless of the nature of the CSPs; in the resolution of other derivatives, L-isomers were less strongly retained except in the separations of Bz-Ala-OEt and Boc-Ala-OBzl on **1**. The absolute configurations of the N-acetyl derivates are not clear. The reversed elution order of enantiomers on **1** compared to that on other CTPC derivates has been occasionally recognized[2]. When the protecting group of the amino group was Bz, Boc or PhOC, the elution order of the enantiomers of ethyl esters was mostly opposite to that of benzyl esters. In these separations, the ester groups seem to play an important rôle for chiral recognition. On the other hand, for Ac and CBZ derivates, the difference in the ester groups appears to be less important. In this case, the interaction between CSPs and N-protected groups may be more influential for chiral discrimination than that between CSPs with ester groups. For the ethyl esters, the difference between PhOC and CBZ was important, whereas for the benzyl ester this difference did not affect the elution order of the enantiomers. Boc-Ala esters were weakly retained on the colums compared with other derivatives, and therefore a less polar eluent, hexane–2-propanol (90:10), was used. The Boc group apparently prevents adsorption of the derivatives on the CSPs, which resulted in a low degree of separation.

Table II showed the results of the optical resolution of the ten alanine derivatives on five ATPC derivatives (**7**–**11**). Amylose tris(3,5-dimethylphenylcarbamate) **7** exhibited good resolving power for most alanine derivatives, except for the N-CBZ derivatives. The elution order of the enantiomers of Bz-Ala-OBzl and PhOC-Ala-OEt was mostly reversed compared with that on the CTPC derivates.

The resolution of the ethyl and benzyl esters of 23 N-CBZ α-amino acids was examined on 3,5-dimethylphenylcarbamates **1** and **7** (Table III). Most amino acids, except Phegly and Lys were resolved completely on CTPC derivative **1**. The optical resolving power of compound **7** was low compared with that of **1**. However, Phegly which was not resolved on **1** was completely resolved as its benzyl ester. Lys derivatives were not completely resolved on these columns. Since an additional CBZ group protecting the ε-amino group of Lys exists far from a chiral carbon, the adsorption of this group on CSP may result in low chiral discrimination. On compound **1**, the amino acids where R_2 = alkyl group were better resolved than those where R_2 contained an heteroatom. The adsorption of R_2 on the CSP may disturb the chiral discrimination as in the case of the Lys derivatives. The cyclic amino acids (Pro and pipecolin) were separated with high separation factors. On compound **1**, the L-isomers of all the amino acid derivatives except for CBZ-Thr-OBzl were eluted first. The polar hydroxy group of Thr may be responsible for this exceptional elution order. Many CBZ amino acids with a free carboxy group have been directly resolved on **1** by using hexane–2-propanol containing a small amount of a strong acid like trifluoroacetic acid[7]. In this case, the L-isomers of most amino acids were also eluted first. Therefore, in the resolution of CBZ amino acids and their esters, L-isomers are less strongly retained regardless of the structure of the COOR group of the amino acids. The interaction between the CBZ group and the urethane group of compound **1** appears to govern the chiral discrimination.

TABLE II

RESOLUTION OF N-PROTECTED ALANINE DERIVATIVES ON ATPC DERIVATIVES 7–11

Eluent: hexane–2-propanol (90:10).

Column	*Ac-Ala-OEt*			*Ac-Ala-OBzl*			*Bz-Ala-OEt*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
7 3,5-Me_2	0.73(+)	1.53	1.76	2.33(+)	1.37	2.59	1.64(D)	1.43	2.33
8 4-Me	2.93(−)	1.15		3.73(−)	1.24	0.95	3.26	1.00	
9 H	3.19(+)	*ca.* 1		4.12(−)	*ca.* 1		3.58(L)	*ca.* 1	
10 4-Cl	4.11(−)	*ca.* 1		5.53(−)	*ca.* 1		4.18(L)	*ca.* 1	
11 3,5-Cl_2	2.27(−)	1.09	1.01	4.57(+)	1.14	1.06	3.56(D)	*ca.* 1	
	Bz-Ala-OBzl			*Boc-Ala-OEt*			*Boc-Ala-OBzl*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
7	3.33(D)	1.41	3.27	0.51(L)	*ca.* 1		0.89(L)	1.27	1.17
8	4.69	1.00		0.41(L)	1.09		0.67(L)	1.08	
9	4.16(L)	1.04		0.33(D)	*ca.* 1		0.67(L)	*ca.* 1	
10	3.32(L)	1.10		0.75(L)	*ca.* 1		0.93	1.00	
11	5.25(L)	1.12	1.29	1.04(L)	1.15	1.10	1.77(D)	1.05	
	PhOC-Ala-OEt			*PhOC-Ala-OBzl*			*CBZ-Ala-OEt*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
7	1.25(L)	1.25	1.41	2.18(L)	1.25	1.95	1.37(L)	*ca.* 1	
8	2.20(L)	1.44	2.29	2.86(L)	1.37	1.70	1.42(L)	*ca.* 1	
9	2.71(D)	1.23	0.74	3.92(L)	1.33	1.02	5.09(L)	*ca.* 1	
10	3.06(L)	*ca.* 1		3.71(L)	1.05		1.80(L)	*ca.* 1	
11	1.81(L)	1.33		3.26(L)	1.05		2.05(L)	1.19	
	CBZ-Ala-OBzl								
	k'_1	α	R_s						
7	2.18(L)	*ca.* 1							
8	2.15(L)	1.11							
9	3.41(L)	1.12							
10	2.19(L)	1.06							
11	4.57(D)	1.14	1.06						

N-Unprotected benzyl esters of Ala (**12**) and Phe (**13**) were not effectively resolved on **1** as compared with the corresponding N-CBZ derivatives (Table IV). This also suggests that the CBZ group may be very important in attaining efficient resolution.

The resolution of other amino acid derivatives (**14–22**) on compound **1** and **7** was also examined (Table IV). Most compounds were completely resolved on either **1** or **7** or on both. The separation factors for β-amino acid derivatives (**20–22**) were smaller than those for structurally similar alanine derivatives. These results indicate

TABLE III

RESOLUTION OF N-CBZ AMINO ACID ESTERS ON COLUMNS 1 AND 7

Eluent: hexane–2-propanol (90:10).

Amino acid	***1***						***7***					
	Et			*Bzl*			*Et*			*Bzl*		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
Ala	1.56(L)	1.82	4.82	3.08(L)	2.20	7.26	1.37(L)	*ca.* 1		2.18(L)	1.03	
Butyline[b]	0.89(L)	2.91	3.07	2.40(L)	3.28	4.68	2.53	1.00		3.86(L)	1.13	
Val	0.89(L)	2.76	6.65	1.51(L)	4.12	9.88	1.89(L)	1.17	0.80	3.19(L)	1.33	1.69
Nva	1.14(L)	1.77	2.70	2.22(L)	2.32	6.73	2.51(L)	1.06		3.65(L)	1.12	0.97
Leu	1.35(L)	1.57	3.34	2.23(L)	1.83	4.46	2.51(D)	1.29	1.19	3.68(L)	1.10	
Nle	1.07(L)	1.40	1.78	1.93(L)	1.64	3.48	2.39(D)	1.09		3.13(L)	1.13	0.79
Ile	0.77(L)	3.10	3.42	1.36(L)	4.03	6.40				3.60(L)	1.48	3.10
Phegly	4.03(D)	*ca.* 1		7.85(L)	1.04		3.55(L)	1.10		6.05(L)	1.25	1.12
Phe	1.41(L)	1.45	1.59	3.61(L)	1.21	1.45	3.97(L)	*ca.* 1		6.01	1.00	
Ser	4.26(L)	1.17	1.35	7.77(D)	*ca.* 1		4.90(D)	1.27	1.47	6.78(D)	1.34	2.81
Thr	3.40(D)	*ca.* 1		5.53(D)	1.37		5.81(L)	1.08		6.92(L)	1.10	
Tyr(OBzl)	2.45(L)	1.33	1.00	4.91	1.00		4.19(L)	1.36	2.16	6.35(L)	*ca.* 1	
Asp	2.35(L)	1.31	1.98	3.12(L)	1.11	0.87	4.15(L)	1.08		5.53(D)	1.10	0.94
Glu[a]	1.38(L)	1.42	1.26	3.35(L)	1.58	1.12	2.07(L)	1.07		5.03(D)	*ca.* 1	
Asn[a]	2.15(L)	1.33	1.69	3.82(L)	1.34	2.77	2.90(D)	1.06		4.46(L)	1.11	0.59
Gln[a]	2.26(L)	1.31	1.39	3.67(L)	1.35	1.63	2.91(L)	*ca.* 1		3.75(D)	1.10	
His[a]	1.35(−)	1.41	2.33				2.87(−)	1.21	0.98			
Trp[a]	2.96(L)	1.50	3.61	4.53(L)	1.55	1.84	2.89(L)	1.14	1.06	4.11(L)	1.19	1.57
Lys(CBZ)[a]	3.19(+)	1.13		5.20(+)	*ca.* 1		6.22(+)	*ca.* 1		8.61(+)	1.14	0.95
Cys(SBzl)	1.60(L)	1.51	1.53	2.82(L)	1.62	2.31	2.69(L)	*ca.* 1		4.21(D)	1.05	
Met	1.07(L)	1.36	1.41	1.38(L)	1.66	4.35	4.40(D)	1.04		8.40(L)	1.11	1.10
Pro	0.91(L)	2.19	1.91	1.81(L)	2.51	4.09	1.89(D)	1.17	0.80	3.33(D)	1.06	
Pip	0.82(L)	1.95	2.54	1.36(L)	2.10	3.18	1.44(D)	1.27		2.25(D)	*ca.* 1	

[a] Eluent: hexane–2-propanol–diethylamine (80:20:0.1).
[b] 2-Aminobutyric acid.

TABLE IV

RESOLUTION OF OTHER AMINO ACID DERIVATIVES **12–22** ON **1** AND **7**

Eluent: hexane–2-propanol (90:10).

Racemate	*1*			*7*		
	k'_1	α	R_s	k'_1	α	R_s
12	1.98(L)	1.20	1.10	1.33(D)	*ca.* 1	
13	2.18(L)	1.17	0.78	2.14(L)	1.11	
14	2.57(−)	2.22	6.40	4.04(−)	1.13	1.31
15	2.45(+)	1.41	2.56	5.03(+)	1.11	1.02
16	4.23(−)	*ca.* 1		7.30(−)	*ca.* 1	
17	1.00(+)	1.70	3.89	2.04(−)	1.08	
18	1.69(−)	1.79	4.09	4.33(+)	1.02	
19	4.31(+)	1.39	3.04	9.01(+)	1.25	
20	1.47(+)	1.08		2.23(−)	1.03	
21	3.20(+)	1.20	0.98	3.13(−)	1.06	
22	3.49(−)	1.06		3.00(−)	1.11	

that the existence of CBZNH and ester groups on the same asymmetric carbon is important to attain effective chiral discrimination.

In order to estimate the electronic effect of N-protecting groups, seven 4-substituted benzoyl alanine benzyl esters were synthesized. Fig. 2 shows the relationship between the separation factors and Hammett's σ constants of the substituents. Good resolutions were attained with the benzoyl derivatives having either electron-donating

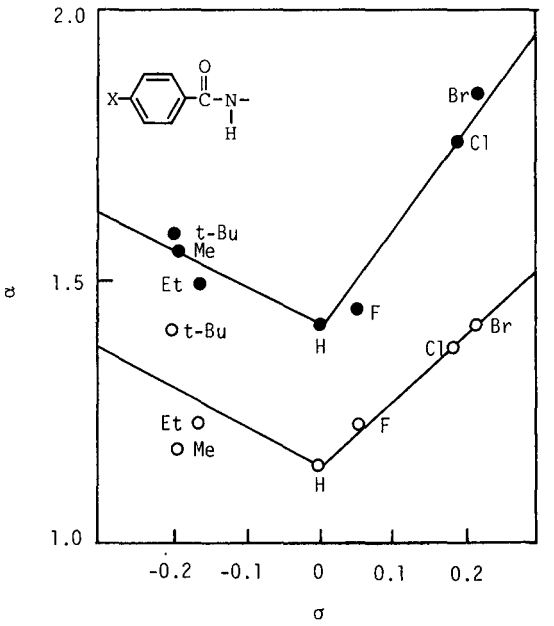

Fig. 2. Plots of the separation factor, α, of N-(4-substituted benzoyl)alanine benzyl esters on columns **1** (○) and **7** (●) against Hammett's σ values.

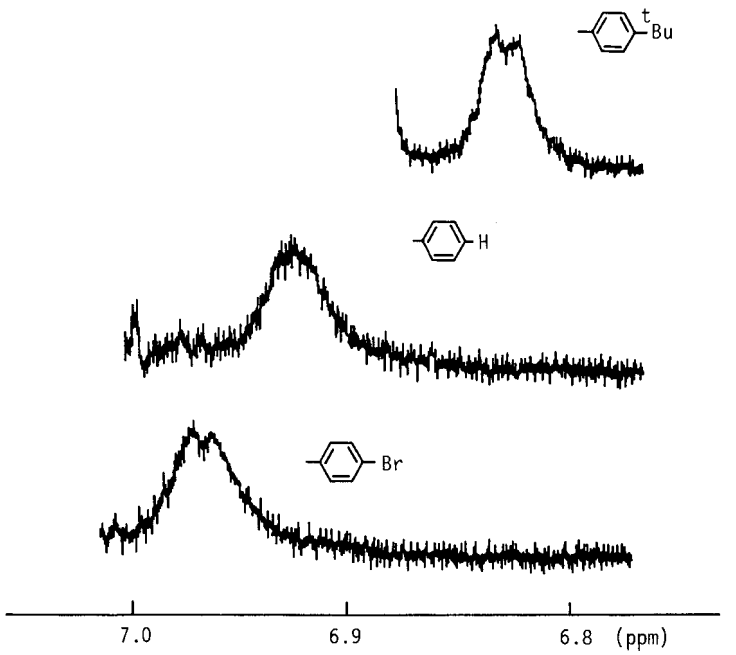

Fig. 3. ^{1}H NMR spectra of the NH protons of N-(4-substituted benzoyl)alanine benzyl esters (C^2HCl_3, 35°C, relative to tetramethylsilane).

or electron-withdrawing substituents. The unsubstituted N-benzoyl derivative was resolved with the lowest separation factor on these CSPs. Since the substituents themselves on the phenyl group do not seem to interact with the CSPs[2], the changes in the separation factors may be attributed mainly to the change in polarity of the amide bond. The NMR spectra of the –NH proton of 4-substituted benzoyl alanine benzyl esters in C^2HCl_3 are shown in Fig. 3. The NH resonances shift downfield as the electron-withdrawing power of the substituents on the phenyl group increases. This indicates that the acidity of the NH proton increases with increasing electron-withdrawing power of the substituents. The NH proton probably interacts with the CSP through an hydrogen bond formed with the carbonyl oxygen of the CSP (Fig. 4), and

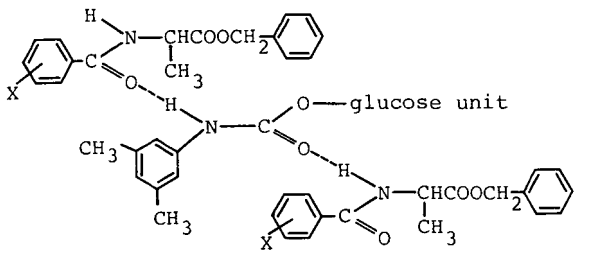

Fig. 4. Adsorption of N-(4-substituted benzoyl)alanine benzyl esters on cellulose tris(3,5-dimethylphenylcarbamate).

therefore this interaction may be more important for the amino acid derivatives having electron-withdrawing substituents. On the other hand, the electron density of the carbonyl oxygen of the alanine derivative, which is also considered to be an important adsorbing site and may interact with the NH proton of the CSP, probably increases with increasing electron-donating power of the substituents. The combination of these factors appears to lead to the results shown in Fig. 2.

ACKNOWLEDGEMENTS

The authors thank Dr. Masahiko Sisido of Tokyo Institute of Technology for providing amino acid derivative **19** and Dr. Kunisuke Izawa of Ajinomoto Co. for derivatives **14**, **20**, **21** and **22**.

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Journal of Chromatography, 477 (1989) 377–385
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MONITORING OF TRANSPEPTIDATION REACTIONS IN ANALOGUES OF GONADOTROPIN RELEASING HORMONE CONTAINING ASPARTIC ACID DERIVATIVES IN POSITION SIX

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(First received January 9th, 1989; revised manuscript received April 24th, 1989)

SUMMARY

High-performance liquid chromatographic systems were used for monitoring the hydrolysis of five potent agonistic gonadotropin releasing hormone analogues containing aspartic acid derivatives in position six. To separate the closely related nonapeptides formed during the hydrolysis, columns with reversed-phase packings were used under isocratic conditions. The mobile phases were methanol containing ammonium acetate or triethylammonium phosphate buffers. Good separations of the hydrolysis products from the investigated peptides allowed the reaction rate constants for the transformations examined to be calculated.

INTRODUCTION

Recently we have reported the synthesis and *in vivo* biological activity of some potent agonistic analogues of gonadotropin releasing hormone (GnRH) containing L-aspartyl or L-β-aspartyl benzyl or methyl ester or an aminosuccinyl residue in position six[1,2].

In order to interpret the *in vivo* biological activities of these analogues, account must be taken of the fact that after the injection a rapid chemical transformation of these peptides can occur in the animal at physiological pH (*ca.* 7.3) and temperature (*ca.* 37°C).

The α→β transpeptidation which may occur in peptides containing aspartic acid derivatives results in β-aspartyl peptide formation. This rearrangement involves an aminosuccinyl intermediate and is especially rapid at alkaline pH[3,4]. However, the same aminosuccinyl-peptide intermediate can be formed from β-aspartyl peptides as well (Scheme 1). A significant transpeptidation rate of a tetrapeptide with an -Asp(OCH_3)-Phe- sequence at pH 7.3 and 37°C was reported by McFadden and Clarke[5]. The formation of aminosuccinyl-peptides and transpeptidation is strongly sequence dependent[6].

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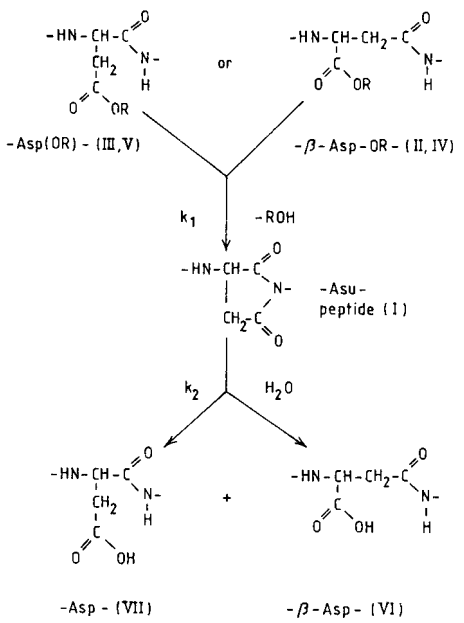


Scheme 1. The transformation of peptides containing an aspartyl- or β-aspartyl-ester moiety in aqueous media. The reactions follow the general scheme of consecutive reactions $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ where (in this case) C represents two different end products. Roman numerals refer to peptides listed in Table I.

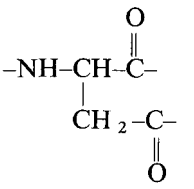
Numerous recent studies have documented the advantage of high-performance liquid chromatography (HPLC) in peptide analyses[7,8]. Its superior performance with reversed-phase (RP), chemically bonded packings has been utilized in the separation of several normal and isopeptides[9–11].

In this paper the usefulness of the RP-HPLC method for monitoring the transformations of our potent [aspartyl^6(ester)]-, [β-aspartyl^6-ester]- and [aminosuccinyl^6]-GnRH(1–9)-EA analogues is demonstrated. Several baseline separations achieved in isocratic systems enabled us to determine the kinetic parameters of the transformations and in one case a separation of epimer nonapeptides was achieved too.

EXPERIMENTAL

Materials

Peptides were synthesized by classical solution phase methods as described previously[1,2] and are listed in Table I. The abbreviations used follow the rules of the IUPAC-IUB commission on Biochemical Nomenclature[12]. Other abbreviations are:

Asu aminosuccinyl 

EA ethylamide $-NH-C_2H_5$

TABLE I
STRUCTURES OF PEPTIDES INVESTIGATED

	Peptide	*Structure*
	GnRH	Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
I	[Asu^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asu-Leu-Arg-Pro-EA
II	[β-Asp^6-OCH_3]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OCH_3 | Leu-Arg-Pro-EA
III	[Asp^6(OCH_3)]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp(OCH_3)-Leu-Arg-Pro-EA
IV	[β-Asp^6-OBzl]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OBzl | Leu-Arg-Pro-EA
V	[Asp^6(OBzl)]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp(OBzl)-Leu-Arg-Pro-EA
VI	[β-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OH | Leu-Arg-Pro-EA
VII	[Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-Leu-Arg-Pro-EA
VIII	[D-Asu^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asu-Leu-Arg-Pro-EA
IX	[D-β-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asp-OH | Leu-Arg-Pro-EA
X	[D-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asp-Leu-Arg-Pro-EA

All amino acids are in the L-configuration and in normal (α) linkage if not stated otherwise.

HPLC

The HPLC analysis of samples was performed on a Waters chromatograph consisting of an M 6000A pump, an U6K injector, an M450 variable wavelength UV detector and a BBC Goerz 220 recorder. Samples were analysed on a Shandon ODS Hypersil 5-μm, 25 cm × 0.5 cm column or on a Shandon 25 cm × 0.46 cm column with the same packing material. The dimensions of the quard column were 2.3 cm × 0.4 cm.

The mobile phases used are listed in Table II. The chromatograph was operated isocratically at ambient temperature and the mobile phase flow-rates were between 0.6 and 1.2 ml/min. The absorbance of the column effluents was monitored at 280 nm.

Hydrolysis of peptides

A 1-mg amount of GnRH analogue (peptides I–V or VIII in Table I) was dissolved in 0.5 ml of water; then 0.5 ml of 0.1 *M* buffer solution (pH 7.3, sodium phosphate; pH 6.0, ammonium acetate; pH 4.5, sodium acetate) were added to achieve the required pH. The incubation mixtures were kept in sealed glass tubes at 2, 22 and 37°C respectively. At preselected times, 100-μl aliquots were withdrawn from each incubation mixture and the reactions stopped by the addition of 20 μl of acetic acid. The samples were stored at −20°C before being analysed by HPLC.

TABLE II

RETENTION DATA FOR THE GnRH ANALOGUES INVESTIGATED

Column: Shandon ODS-Hypersil 5 μm (25 cm × 0.5 cm) with guard column in experiments A, B and D; without guard column in experiment G and 25 cm × 0.46 cm in experiments C, E and F. Flow-rates: 0.8 (A, B, D); 0.6 (C, E, F) and 1.2 ml/min (G).

Experiment	*Peptides*	t_R	k'	*Eluent*
A	[β-Asp^6]-GnRH(1–9)-EA (VI)	11.6	1.58	Methanol–0.1 *M* ammonium
	[Asp^6]-GnRH(1–9)-EA (VII)	13.0	1.89	acetate (pH 4.0) (48:52, v/v)
	[Asu^6]-GnRH(1–9)-EA (I)	16.2	2.60	
B	VI	14.2	1.96	Methanol–0.1 *M* ammonium
	VII	16.4	2.42	acetate (pH 4.0) (47:53, v/v)
	[β-Asp^6-OCH$_3$]-GnRH(1–9)-EA (II)	20.4	3.25	
	I	21.0	3.38	
C	VI	14.4	1.77	Methanol–0.25 *M* TEAP
	VII	16.9	2.25	(pH 3.0) (47:53, v/v)
	II	19.4	2.73	
	I	20.6	2.96	
D	VI	12.0	1.61	Methanol–0.1 *M* ammonium
	VII	13.6	1.96	acetate (pH 4.0) (48:52, v/v)
	I	17.2	2.74	
	[Asp^6(OCH$_3$)]-GnRH(1–9)-EA (III)	18.0	2.91	
E	VI	7.6	0.46	Methanol–0.25 *M* TEAP
	VII	8.1	0.56	(pH 3.0) (62:38, v/v)
	I	8.1	0.56	
	[β-Asp^6-OBzl]-GnRH(1–9)-EA (IV)	14.5	1.79	
F	VI	7.4	0.42	Methanol–0.25 *M* TEAP
	VII	8.0	0.53	(pH 3.0) (62:38, v/v)
	I	8.0	0.53	
	[Asp^6(OBzl)]-GnRH(1–9)-EA (V)	13.4	1.58	
G	[D-β-Asp^6]-GnRH(1–9)-EA (IX)	27.4	11.2	Methanol–0.25 *M* TEAP
	[D-Asp^6]-GnRH(1–9)-EA (X)	30.7	12.5	(pH 3.0) (35:65, v/v)
	VI	32.0	13.2	
	VII	45.5	18.8	

Reaction kinetic calculations

The percentage of the peptides I–V (Table I) after hydrolysis for time t was determined by cutting and weighing the peak areas in the HPLC chromatograms. Supposing a pseudo-first order reaction for each transformation summarized in Scheme 1, the reaction rate constants were calculated according to

$$k = \frac{1}{t} \ln \frac{A_0}{A} \tag{1}$$

where A_0 represents the initial concentration of the investigated peptide chosen to be 100%, and A is equal to the percentage of the peptide after hydrolysis for time t (ref. 13). The extinction coefficients at 280 nm ε_{280}, of the peptides I–VII were calculated from data in the literature[14,15]. Though peptides with a benzyl ester group have

TABLE III

REACTION RATE CONSTANTS (h^{-1}) FOR TRANSFORMATIONS OF PEPTIDES I–V AT VARIOUS pH VALUES AND TEMPERATURES

Peptide	*37°C*	*22°C*			*2°C*		
	pH 7.3	*pH 7.3*	*6.0*	*4.5*	*pH 7.3*	*6.0*	*4.5*
I	0.41 ± 0.028	0.10 ± $6.1 \cdot 10^{-3}$	$9.3 \cdot 10^{-3}$ ± $3.4 \cdot 10^{-4}$	$4.6 \cdot 10^{-4}$ ± $2.4 \cdot 10^{-5}$	$6.8 \cdot 10^{-3}$ ± $5.3 \cdot 10^{-4}$	$5.9 \cdot 10^{-4}$ ± $4.2 \cdot 10^{-5}$	–
II	0.49 ± 0.046	–	–	–	–	–	–
III	0.25 ± 0.016	–	–	–	–	–	–
IV	0.94 ± 0.079	0.19 ± 0.012	$2.0 \cdot 10^{-2}$ ± $1.0 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$ ± $4.9 \cdot 10^{-5}$	$1.2 \cdot 10^{-2}$ ± $6.6 \cdot 10^{-4}$	$1.6 \cdot 10^{-3}$ ± $1.2 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$ ± $6.1 \cdot 10^{-6}$
V	0.39 ± 0.015	–	–	–	–	–	–

a slightly (by 1.7%) greater ε_{280} value than those without it, this effect seems to be negligible for our purposes. Each k value was determined from at least three different measurements at different times and is presented as mean ± standard deviation in Table III.

Knowing the experimentally determined k_2 and the k_1 values for the transformations of peptides II–V to peptide I (see Schema A and Table I), the percentage composition of any incubation mixture can be calculated at any time applying the well known equations[13] for consecutive reactions

$$B = \frac{k_1}{k_2 - k_1} \cdot A_0(e^{-k_1 t} - e^{-k_2 t}) \tag{2}$$

$$C = \frac{1}{k_2 - k_1} \cdot A_0 \left[k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})\right] \tag{3}$$

where B represents the percentage amount of peptide I, C represents the sum of the percentage amounts of peptides VI and VII. To calculate the individual concentrations of VI and VII, it had to be taken into account that the ratio of VI to VII was 3.5:1 in our experiments.

RESULTS AND DISCUSSION

For the RP-HPLC chromatography of GnRH and its analogues the use of ammonium acetate buffers in combination with methanol, ethanol or acetonitrile as organic components has been reported several times[16,17]. The application of triethylammonium phosphate (TEAP) buffer for the RP-HPLC separation of peptides gave good results in the case of GnRH, too[18]. For the resolution of aspartyl- and β-aspartyl-peptides, methanol–water as the mobile phase containing acetic acid (2%), sodium acetate (pH 4.0) or ammonium acetate (pH 3.5) as a modifier was reported[9–11].

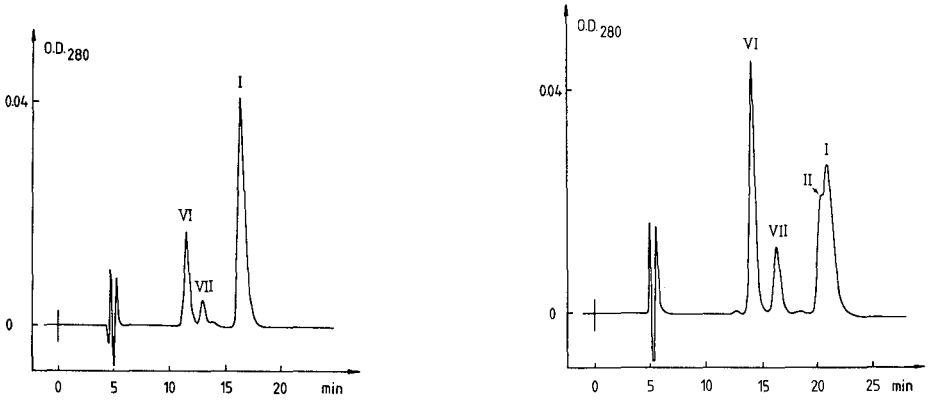


Fig. 1. HPLC analysis of the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) at pH 7.3 and 37°C for 40 min. VI = [β-Asp^6]-GnRH(1–9)-EA; VII = [Asp^6]-GnRH(1–9)-EA. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.5 cm). Eluent: methanol–0.1 *M* ammonium acetate buffer (pH 4.0) (48:52, v/v); flow-rate 0.8 ml/min. Detection: 280 nm.

Fig. 2. HPLC chromatogram of the hydrolysis of [β-Asp^6–OCH$_3$]-GnRH(1–9)-EA (II) at pH 7.3 and 37°C for 180 min. Other peptides as in Fig. 1. Eluent: methanol–0.1 *M* ammonium acetate buffer (pH 4.0) (47:53, v/v). Other conditions as in Fig. 1.

In our experiments on the separation of GnRH analogues I–VII satisfactory results were obtained with mobile phase combinations of methanol–0.1 *M* ammonium acetate buffer (pH 4.0) or methanol–0.25 *M* TEAP buffer (pH 3.0). During the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) two products, [Asp^6]-GnRH(1–9)-EA (VII) and [β-Asp^6]-GnRH(1–9)-EA (VI), were formed (Fig. 1). Peptides I, VI, and VII can easily be separated presumably because they are differently ionized at pH 4.0.

After a short time of hydrolysis of [β-Asp^6-OCH$_3$]-GnRH(1–9)-EA (II), four peaks were detected by HPLC (Fig. 2). Peptides I and II were not well separated in this system, presumably because they have the same isoelectric points and can only have a slight difference in their hydrophobic characters. Changing the mobile phase to methanol–0.25 *M* TEAP (pH 3.0), almost baseline separations were achieved (Fig. 3).

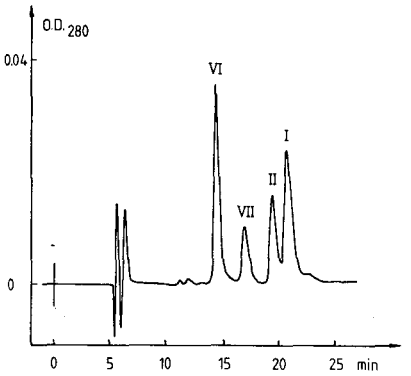


Fig. 3. HPLC analysis of the hydrolysis of [β-Asp^6-OCH$_3$]-GnRH(1–9)-EA (II) at pH 7.3 and 37°C for 180 min. Peptides as in Fig. 1. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.46 cm). Eluent: methanol–0.25 *M* TEAP buffer (pH 3.0) (47:53, v/v); flow-rate 0.6 ml/min. Detection: 280 nm.

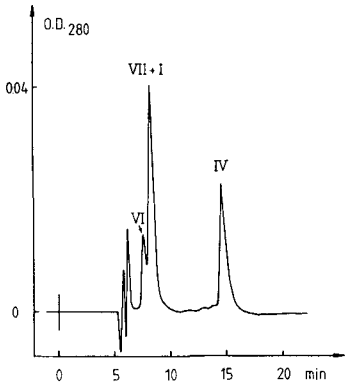


Fig. 4. HPLC diagram of the hydrolysis of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) at pH 7.3 and 37°C for 60 min. Peptides as in Fig. 1. Conditions as in Fig. 3, except methanol–0.25 *M* TEAP buffer (pH 3.0) (62:38, v/v).

The best HPLC separation of [Asp^6 (OCH_3)]-GnRH(1–9)-EA (III) from its hydrolysis producs was obtained in methanol–0.1 *M* ammonium actate (pH 4.0) as the mobile phase. Retention times and k' value for these and all the other peptides investigated are listed in Table II.

Monitoring the hydrolysis of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV), a good separation of IV from I was observed which can be explained by the strong hydrophobic nature of the benzyl ester group in IV (Fig. 4). A coelution of peptides I and VII was observed in this HPLC system.

Satisfactory resolutions were also achieved by analysing the hydrolysis samples of [Asp^6(OBzl)]-GnRH(1–9)-EA (V.) For k' values and chromatographic conditions see Table II.

In each case, the separation of the starting peptides from the hydrolysis products allowed us to determine the percentages of these peptides after hydrolysis for time t, and thus to calculate the reaction rate constants of these transformations (Table III).

The values of the hydrolysis (pH 7.3, 37°C) reaction rate constants of peptides I (0.41 h^{-1}) and III (0.25 h^{-1}) are similar to those observed for two hexapeptides with -Asu-Phe- (0.37 h^{-1}) and -Asp(OCH_3)-Phe- (0.34 h^{-1}) sequences[5].

Knowing the reaction rate constants of all transformations examined, we can calculate the percentages of the peptides in any incubation mixture at any time (for detail see Experimental), *e.g.*, the incubation mixture (37°C, pH 7.3) of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) (the most labile GnRH analogue examined in this work) contains 33.5% [Asu^6]-GnRH(1–9)-EA (I), 3.0% [β-Asp^6]-GnRH(1–9)-EA (VI), 0.9%[Asp^6]-GnRH(1–9)-EA (VII) and only 62.6% IV after hydrolysis for 30 min (Fig. 5).

We have suggested in an earlier paper that the striking biological activity of peptides II and IV may be attributed to their ability to form [Asu^6]-GnRH(1–9)-EA (I)[1]. Our recent findings indicate that there is no direct correlation between the order of the observed biological activities of I–V (I ≈ II > III > V ≈ IV) and their ability to form I at pH 7.3 and 37°C: IV > II > V > III (Table III). The impact of the rapid chemical transformation of peptides I–V on the observed biological activities will be discussed in detail elsewhere[19].

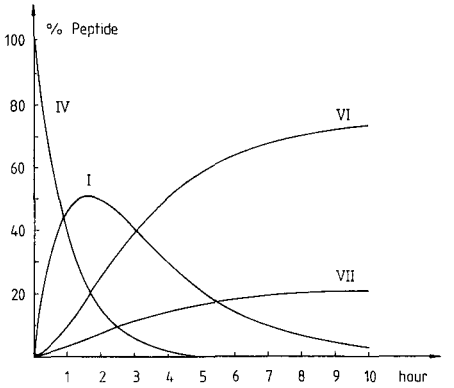


Fig. 5. The hydrolysis pattern of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) at pH 7.3 and 37°C calculated from the experimentally determined reaction rate constants. Peptides as in Fig. 1.

In order to determine the pH and temperature range at which the purification of peptides I–V can be carried out without significant transformation, the reaction rate constants of the transformations examined were determined at different pH values and temperatures. From the k values listed in Table III the time required for minimum (1%) transformation of these peptides can be calculated. In the case of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV), the less stabile GnRH analogue examined in this work, 7.7 h are necessary for 1% transformation at pH 4.5 and 22°C which means that lower pH values and/or temperatures are required during the purification of these analogues.

As a result of the hydrolysis of an hexapeptide containing the -Asu-Gly- sequence, beside the L-aspartyl and L-β-aspartyl peptides the formation of the corresponding D-aspartyl and D-β-aspartyl peptides was also reported, but the amount

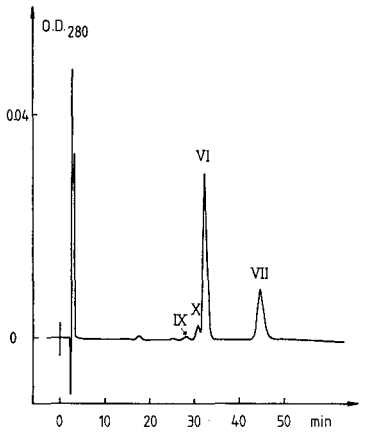


Fig. 6. HPLC analysis of the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) at pH 7.3 and 37°C for 19 h. Peptides as in Fig. 1, except IX = [D-β-Asp^6]-GnRH(1–9)-EA and X = [D-Asp^6]-GnRH(1–9)-EA. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.5 cm). Eluent: methanol–0.25 *M* TEAP buffer (pH 3.0) (35:65, v/v); flow-rate 1.2 ml/min. Detection: 280 nm.

of D-epimers was less than 10%[20]. This epimerization side reaction can be attributed to the tendency of the amino succinimide part to racemization[21]. We were curious to know whether or not the unidentified small impurities in Figs. 1–4 are the result of a similar epimerization process during the hydrolysis of our peptides.

Recently we have synthesized [D-Asu^6]-GnRH(1–9)-EA (VIII)[19]. Its hydrolysis resulted in two materials as detected by HPLC (conditions were the same as in experiment C in Table II). We arbitrarily assigned the peak which eluted first from the HPLC column as [D-β-Asp^6]-GnRH(1–9)-EA (IX) and the other product as [D-Asp^6]-GnRH(1–9)-EA (X). After this, we mixed the hydrolysis samples of I and VIII in 1:1 ratio and tried to separate the epimer nonapeptides but failed under these conditions: VI and X coeluted and IX appeared as a shoulder of VI+X. Changing the mobile phase composition to 35% methanol–65% TEAP (0.25 *M*, pH 3.0), good separation was achieved on the same column. Then, on analysing the hydrolysis products of peptide I (pH 7.3, 37°C, 19 h), 0.7% IX and 4.4% X were detected beside VI and VII (Fig. 6).

ACKNOWLEDGEMENT

This work was supported by the Hungarian Academy of Sciences: the Research Fund for Basic Research in Biology No. 2.1.5.4.

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Journal of Chromatography, 477 (1989) 387–395
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 593

DETERMINATION OF TOLUENEDIAMINES IN POLYURETHANE FOAMS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received January 3rd, 1989; revised manuscript received April 17th, 1989)

SUMMARY

A method is presented for the determination of 2,4- and 2,6-toluenediamine (TDA) in aqueous extracts of polyurethane foams. Foam samples are extracted with methyl-*tert.*-butyl ether followed by back extraction into dilute hydrochloric acid. The method utilizes reversed-phase high-performance liquid chromatography with electrochemical detection. The lower limit of detection is 1.0 ppb (ng/g) for a 10.0-g sample. The response of TDA is linear over three orders of magnitude and recovery data averaged 50% for samples spiked in the 25–50 ppb range. Confirmation of peaks attributed to TDA in the chromatographic analyses was carried out using liquid chromatography–mass spectrometry.

INTRODUCTION

Flexible polyurethane foams have had wide application in a multitude of commercial products including furniture and automotive seating, mattresses and carpet padding. It is estimated that the U.S.A. alone will have consumed about $6.0 \cdot 10^8$ kg of flexible foam in 1988 for such consumer products [1]. Toluene diisocyanate (TDI) is used in the production of a significant portion of such foams. TDI-based products would be expected to contain residual amounts of toluenediamine (TDA) as a result of hydrolysis of the free unreacted isocyanate.

Aromatic amines such as TDA are of significant health concern because several studies have indicated toxic properties of TDA isomers. The 2,4-TDA isomer has been shown to be carcinogenic in animals[2–4], giving a positive Ames test[5] and to be responsible for reproductive toxicity in rats[6,7]. Several other toxic effects of 2,4-TDA have been reported[8–10]. The 2,6-TDA isomer has been identified as a suspected carcinogen in at least one animal study[11], but other reports indicate that 2,6-TDA causes no statistically significant differences in tumor occurrence from controls[12]. The 2,5-TDA isomer, a rarely occurring isomer in commercial TDA production, has also been indicated as a toxic agent [13,14].

Though the toxic effects of 2,4- and 2,6-TDA have not been demonstrated in

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humans, they appear potentially harmful. The analysis of amines such as TDA in polyurethane foams should be of increasing interest because there are many consumer goods containing polyurethanes which involve human contact and exposure. Thus, the availability of a sensitive analytical technique for monitoring TDA in polyurethane foams and other products used in consumer products is of substantial concern.

Very little has been published on the detection of aromatic amines in foams. We previously have published a fluorimetric thin-layer chromatographic (TLC) method for TDA analysis in foams but the sensitivity was poor[15]. Several reports have focused on TDA analysis in aqueous extracts from polyurethane food bags[16–18] and analysis of airborne diamines[19]. To obtain the detection limits reported in most of these studies, laborious concentration steps and large injection volumes were required. These methods were reported to have on column detection limits of 1 ng of TDA.

Toluenediamine analysis has been done using gas chromatography[20,16], TLC[15,21], and liquid chromatography with UV detection[18,19]. These methods require significant time in sample preparation or generally are not sensitive enough to detect aromatic amines at the concentration at which they exist in actual samples, *e.g.* at ppb[a] levels. Several workers report using high-performance liquid chromatography (HPLC) with electrochemical detection (ED)[18,19], but none for TDA analysis in foams. Because solid polyurethane foams present a significant challenge in preparation for analysis, a method for such analysis is desirable.

This report presents an HPLC method which can detect 1 ppb of 2,4- or 2,6-TDA in polyurethane foams (50 pg on column). This method utilizes ED of the aromatic amines to achieve this detection limit.

EXPERIMENTAL

Reagents and materials

Methanol, methyl-*tert.*-butyl ether (MTBE), hydrochloric acid, acetic acid and ammonium acetate were purchased from Baker Chemicals (Phillipsburg, NJ, U.S.A.). The 2,4- and 2,6-toluenediamine isomers were from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Purity of the diamines was assessed by proton NMR. Acetonitrile was from Burdick & Jackson (Muskegon, MI, U.S.A.). The infusers were either purchased locally or constructed by a machine shop. The infuser consists of a heavy duty 600-ml beaker and a stainless-steel plunger that fits tightly into the beaker. The circular bottom of the plunger has a fine mesh wire which allows passage of solvent but retention of foam. Buffers were prepared with water purified by a Milli-Q water purification system (Millipore) and filtered through a nylon-66 filter (0.45 μm). Foams were prepared from prepolymer in our laboratory or purchased locally and represent a variety of industrial foams containing various additives. Polyurethane foams are typically composed of diols and triols derived from ethylene or propylene oxides and isocyanates (aromatic or aliphatic) such as TDI. To these structural components are added catalysts such as tin salts or amines. Other additives include dyes, pigments, antioxidants, and surfactants.

[a] Throughout this article, the American billion (10^9) is meant.

Equipment

The chromatographic system consisted of: an SSI 222B pump (Scientific Systems, State College, PA, U.S.A.), a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.) and electrochemical detector (Model 400 EG&G PAR, Lawrenceville, NJ U.S.A.) equipped with a glassy carbon electrode, an LCI-100 integrator (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Supelcosil C_{18} column (15 cm × 4.6 mm, 5 μm particle size) Supelco, Bellefonte, PA, U.S.A.). The detector was set at +875 mV *versus* Ag/AgCl and the output at 20 nA full scale. The eluent was 3% acetonitrile, 25 m*M* ammonium acetate, 1 m*M* propylamine, pH 5.5. The flow-rate was 1.0 ml/min and the injection volume was 50 μl. The liquid chromatography–mass spectrometry (LC–MS) experiments were conducted under the same chromatographic conditions and with a Finnigan TSQ 70 mass spectrometer equipped with a thermospray interface. LC–MS data were obtained by employing buffer ionization and scanning the third quadrupole from *m/z* 121 to *m/z* 500 in 1 s. The vaporizer temperature of the interface and the source block temperature were maintained at 118°C and 220°C, respectively during the experiments.

Extraction procedure

A 10.0-g foam sample is cut into 1-cm^3 pieces or smaller, or shredded in a bean grinder. The foam is placed into the infuser beaker and 60 ml of MTBE is added. The plunger is inserted and compressed such that the entire foam is submerged. The foam is compressed and released repeatedly for 5 min. The MTBE is expressed from the foam and decanted. This extraction is repeated two more times. The extracts are combined and blown down to *ca.* 80 ml. After transferring to a 125 ml separatory funnel, 4 ml of 20 m*M* hydrochloric acid (pH 2) is added and the mixture is extracted for 10 minutes on a mechanical shaker. After the phases have separated, the lower aqueous layer is removed and an additional 4 ml of 20 m*M* hydrochloric acid is added and the extraction is performed for an additional 10 min. The aqueous extracts are combined in a 10.0-ml volumetric flask (the organic phase is discarded) and the samples brought to volume with water. This extract should be protected from light and analyzed within 48 h.

Standard preparation

Standard stock solutions of 2,4- and 2,6-TDA are prepared in methanol at a concentration of 1 μg/μl and are stable for at least 4 weeks when kept refrigerated and protected from light. From this preparation, a 2 ng/μl intermediate standard was made in methanol. Appropriate dilutions from this stock are made in water to cover the concentration range of 0.5 to 50 pg/μl. The intermediate stock solution is stable for 1 week. Working standards are prepared fresh daily.

RESULTS AND DISCUSSION

The two major difficulties encountered in the analysis of toluenediamine in polyurethane foams were obtaining adequate sensitivity and developing a quantitative extraction protocol for the solid foam samples.

TABLE I

CONCENTRATION OF TDA ISOMERS IN FOAMS (ng/g)

Foam samples (10 g) were extracted with MTBE and analyzed by HPLC–ED as described in the text. Corrected values are based on 54% recovery of 2,6-TDA and 48% recovery of 2,4-TDA.

Foam	*2,6-TDA*	*2,6-TDA (corrected)*	*2,4-TDA*	*2,4-TDA (corrected)*	*Total (corrected)*
1	1.0	1.9	0.60	1.3	3.0
2	0.89	1.7	0.22	0.46	2.2
3	0.66	1.2	0.40	0.83	2.0
4	19	35	1.2	2.5	38
5	2.4	4.4	4.0	8.3	13
6	5.1	9.4	6.0	13	22
7	12	22	4.2	8.8	31
8	7.3	14	43	90	104

Electrochemical detection

The levels of TDA found in many foams were so low that conventional UV detection cited elsewhere [17] lacked the sensitivity for accurate quantitation at the ppb level. Our own experiments with UV detectors indicated a detection limit of around 100 ppb, well above the amounts that exist in many foams (Table I). Electrochemical detectors, in contrast, are extremely sensitive and are effective in measuring aromatic amines such as TDA by electrochemical oxidation. Several other laboratories[18,19] have succesfully used this mode of detection, though the applications were different. ED was found capable of detecting 50 pg of TDA on column (signal-to-noise ratio, S/N = 4), representing 1.0 ppb of TDA in a typical 10.0-g foam sample (Fig. 1).

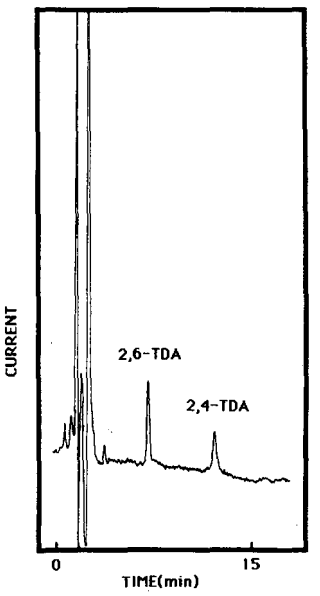

Fig. 1. Chromatogram of TDA standards. The chromatographic trace represents 1.0 ppb (50 pg on column) of each of the TDA isomers. Chromatographic conditions are given in the text.

Foam extraction study

Accurate foam analysis requires reproducible recovery and effective extraction of TDA from the solid foam matrix. Various solvents were examined for their effectiveness in extraction of TDA from spiked foams. The general protocol consisted of extraction of the foam with an organic solvent and back extraction into dilute hydrochloric acid. Organic solvents tested included MTBE, diethyl ether, ethyl acetate, chloroform methylene chloride and methanol. Also, 10–200 m*M* hydrochloric acid was tested, followed by back extraction into an organic solvent and then a final back extraction into dilute hydrocloric acid. Extractions with water and sterile saline solutions were also tested.

Recovery of TDA with methylene chloride and chloroform was poor (5–40%) and these solvents swelled the foam considerably, causing great difficulty in recovering the solvent from the foam. Ethyl acetate was judged as a poor solvent because its relatively high solubility in water (*ca.* 9%) did not allow a phase separation after the back extraction step. Diethyl ether gave relatively good results but MTBE was chosen because of its higher boiling point and the significantly cleaner chromatograms generated in its use (note Fig. 2). Overall, major considerations on the choice of solvent were immiscibility with water, volatility and efficacy in recovery of spiked TDA from the foam.

Further extraction studies indicated that three extractions of the foam with MTBE followed by two back extractions with dilute hydrochloric acid were sufficient in obtaining the maximum recovery. An additional important factor is the surface area of foam exposed. Cutting foam into small pieces or shredding of foam allowed greater recovery than that with large foam pieces.

High-performance liquid chromatography

LC analysis of TDA has been reported previously[18,19]. Both acetate and phosphate buffers have been employed. Acetate buffer at pH 5.5 with low concentrations of acetonitrile was well suited for good retention of TDA isomers and compatible with the electrochemical detector. The addition of 1 m*M* propylamine served to sharpen the TDA peaks thereby improving sensitivity slightly. Fig. 2 shows a typical chromatogram of a polyurethane foam.

Liquid chromatography–mass spectrometry

Many compounds may be extracted into the final aqueous fraction during the foam sample workup. Several peaks in addition to the TDA isomers are present, as noted in the chromatogram (Fig. 2). Co-elution of chromatographic peaks with standard materials does not constitute positive peak identification. Positive artifacts are possible, particularly in complex matrices. To confirm TDA isomers in foam extract, LC–MS was performed.

LC–MS was conducted under the same chromatographic conditions as in the HPLC analysis. The thermospray LC–MS data were obtained with a Finnigan MAT TSQ-70 mass spectrometer equipped with a Finnigan thermospray interface utilizing buffer ionization. Fig. 3 shows a total-ion chromatogram and a single-ion trace of m/z 123 (MH^+ ion of TDA) of a foam extract. Only the protonated molecular ion of TDA was observed in the LC–MS mass spectrum with no fragmentation.

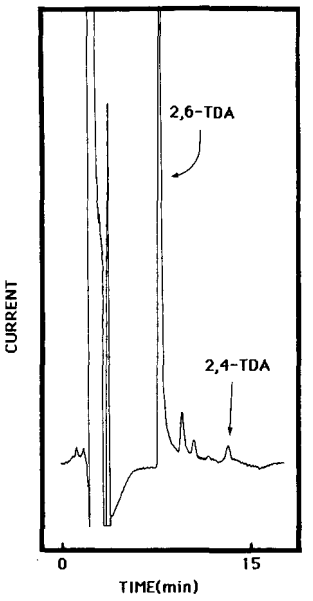

Fig. 2. Chromatogram of foam extract. The extract contained 20 ppb of 2,6-TDA and 1.5 ppb of 2,4-TDA.

Standard calibration

Response of TDA was linear in the range 0.5–500 ppb. Typically, a standard curve encompassed the range 0.5–50 ppb for a 20-nA full scale range on the electrochemical detector. Notably, even low concentrations of TDA (< 10 ppb) were stable for 2–3 days at room temperature. TDA was not irreversibly adsorbed to the glassware, as evidenced by comparison with silanized and untreated glass.

Sample preparation

Polyurethane foams typically contain significant moisture content. Freshly prepared foams contained approximately 40% water. Within 3 days at room temperature only about 10% moisture remained. Moisture content varied with relative humidity. No correction for this moisture was made for foams.

The foams were extracted as outlined in the methods section. After expression of MTBE, the foams were weighed and found to retain about 4 g solvent/10 g foam, or about 2% of the total solvent volume used in the three successive extractions. The results of LC–ED analysis from several foams are given in Table I. Corrected values of TDA below 1.0 ppb (ng/g), though less than the stated detection limit, are derived from peak areas where S/N is less than 4.

Precision and recovery

Precision was determined by analysis of six preparations of a single foam sample. Duplicate injections of each preparation were made. The precision of the method was 13% R.S.D.

Recovery was determined by spiking foams directly with TDA and then ex-

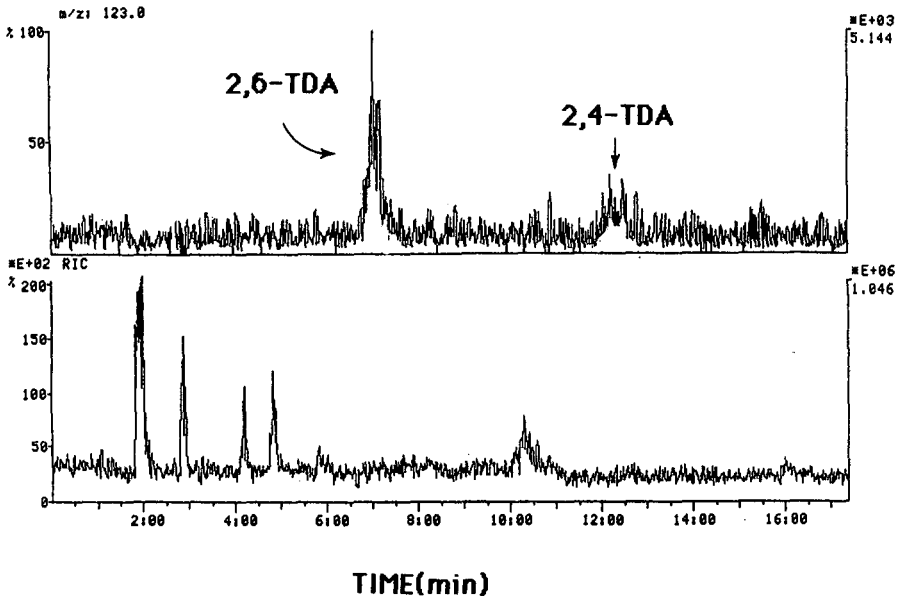

Fig. 3. Ion chromatograms from LC–MS analysis. Total (bottom) and selected (top) ion chromatograms from LC–MS foam extract. Conditions are given in the text.

tracting and analyzing by HPLC. Foams were spiked at 25 and 50 ppb. Results are summarized in Table II. Corrections were based on a typical TDA foam concentration.

It is unclear why the recovery of TDA from foams is so low, nevertheless it is

TABLE II

SPIKE AND RECOVERY DATA FOR 2,6-TDA AND 2,4-TDA

Foam samples (10 g) were spiked with 250 and 500 ng of TDA in methanol, extracted with MTBE and analyzed by HPLC–ED as described in the text.

Sample	*Recovery (%)*	
	2,6-TDA	*2,4-TDA*
1 at 25 ng/g	53	49
2	64	50
3	64	50
4	48	45
5	50	48
1 at 50 ng/g	55	48
2	51	49
3	59	55
4	60	57
5	40	32
	$\bar{x}$ = 54	$\bar{x}$ = 48

reproducible. Part of the difficulty may be in factors associated with the spiking of solid samples and spiking at such low levels. Extensive studies of the extraction steps showed very good recovery of TDA during the back extraction. The difficulty lies in the initial ether extraction step. It appears that the aromatic amines strongly adhere to the foam matrix in spite of the repeated extraction of shredded foam.

We reported in 1977[15] that several polyurethane foams contained up to 400 ppm of 2,4-TDA and 80 ppm of 2,6-TDA. The method, now obsolete, was based on methanol extraction and TLC with fluorimetric detection. Reinvestigation of this TLC procedure indicated that a urea derivative often overlapped the TDA spot causing gross overestimation of the TDA content. Later analysis of the same foams by an early version of the present HPLC method gave TDA values one to three orders of magnitude lower (0.1–5 ppm). We concluded that the TLC method had given artificially high values. This information, together with the present data indicate an apparent decline in free TDA over the last decade, but we are unable to conclude whether this reflects changes in foam-making technology or is simply an artifact of sample selection.

CONCLUSIONS

This is the first HPLC–ED method applied to the determination of TDA in polyurethane foams and provides the best sensitivity reported to date, allowing quantitative analysis of levels of TDA found in real samples. In addition, the presence of TDA isomers in the foam extract has been confirmed by LC–MS. Fig. 2 indicates that several other components are present in the foam extract. Attempts to identify any of these compounds were unsuccessful. It is assumed that they may be other types of aromatic amines or basic compounds.

Though this work was initially designed for analysis of relatively simple foams it appears to be applicable to many commercial foams based on our results with several industrial foams chosen at random. Our major interest was in foams that would be compatible with human tissues. This puts severe limitations on ingredients that may be used. In contrast, industrial foams may contain many components and additives such as dyes, antioxidants and surfactants. Such additives can contribute to problems with the extraction or interferences in chromatography though we experienced no significant problem with such. Thus, this HPLC–ED method provides excellent sensitivity for TDA analysis in a variety of commercial polyurethane foams.

ACKNOWLEDGEMENTS

The authors wish to thank Cliff Kehr for helpful discussions, S. Arquette for technical assistance, and K. Glover for typing of the manuscript.

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Journal of Chromatography, 477 (1989) 397–406
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 565

IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

XVI[a]. SIMPLE AND RAPID DETERMINATION OF RESIDUAL PYRIDONECARBOXYLIC ACID ANTIBACTERIALS IN FISH USING A PREPACKED AMINO CARTRIDGE

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(First received October 25th, 1988; revised manuscript received April 12th, 1989)

SUMMARY

A simple and rapid method for the determination of residual pyridonecarboxylic acid antibacterials (PCAs) (oxolinic acid, nalidixic acid and piromidic acid) in fish was developed using a combination of high-performance liquid chromatography (HPLC) and clean-up with an amino-type prepacked cartridge. PCAs were extracted with *n*-hexane–ethyl acetate (1:3) and the extract was applied to a Baker 10 amino cartridge. PCAs were eluted from the cartridge with acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) and were determined by HPLC. The separations were performed on Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) using a mobile phase containing oxalic acid. The recoveries of PCAs from various fishes fortified at the level of 1.0 ppm were 77.1–95.5%, and the detection limits were 0.05 ppm. The analytical time per sample was less than 30 min.

INTRODUCTION

Pyridonecarboxylic acid antibacterials (PCAs) are widely applied to cultured fishes to cure and prevent diseases, and oxolinic acid, nalidixic acid and piromidic acid are permitted in Japan. However, their residues in fish constitute one of the most serious problems for public health agencies. Although some high-performance liquid chromatographic (HPLC) methods[1–8] had been applied to the determination of residual PCAs in fish, we considered them to be unsuitable for routine analysis for the following reasons. For clean-up of PCAs, these methods require a long time because

[a] For Part XV, see *J. Chromatogr.*, 462 (1989) 315.

0021-9673/89/$03.50

they require some time-consuming treatments such as column chromatography[6], concentration in an evaporator[1–8], filtration[1–3,6,8] and partition in a separating funnel[1–7]. For determination, because PCAs appear as tailing peaks in reversed-phase HPLC, ion-pair chromatography[2,5,7,9,10], application of citrate buffer in the mobile phase[11,12] and derivatization of PCAs to methyl esters[1,13] have been attempted. However, ion-pair chromatography and the use of a citrate buffer did not give effective results, and the derivatization is very complicated. Although ion-exchange columns[3,4,14,15] and a wide-pore ODS column[8] were successfully applied to the determination of PCAs, a conventional ODS column is more desirable from considerations of cost and durability of the HPLC column.

In order to establish a suitable method for the routine determination of residual PCAs in fish, we tried to simplify the clean-up of PCAs by using a disposable prepacked cartridge and to control the tailing of PCAs on a conventional ODS column using the techniques we reported previously[16,17]. This paper describes techniques for the sensitive and rapid determination of PCAs using a conventional HPLC column with Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.), and for the simple and rapid clean-up of residual PCAs in fish using a Baker 10 amino cartridge.

EXPERIMENTAL

Materials

Acetic acid, acetonic acid (2-hydroxyisobutyric acid), acetonitrile, anhydrous sodium sulphate, citric acid, ethyl acetate, *n*-hexane, lactic acid, malonic acid, methanol, oxalic acid, phosphoric acid, sodium hydroxide (NaOH) and tartaric acid were analytical-reagent grade materials. Oxolinic acid (OA), nalidixic acid (NA) and piromidic acid (PA) were purchased from Sigma (St. Louis, MO, U.S.A.).

Baker 10 amino (catalogue No. 7088-3), Baker 10 cyano (7021-3), Baker 10 diol (7094-3), Baker 10 primary-secondary amino (7089-3), Baker 10 quaternary amine (7091-3), Baker 10 carboxylic acid (7211-3) and Baker 10 aromatic sulfonic acid (7090-3) cartridges were purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

Preparation of standard solution

Each standard (10 mg) was weighed accurately into a 100-ml volumetric flask and diluted to volume with acetonitrile. Subsequent dilutions were made with the eluent.

Clean-up procedure

A 5-g amount of sample and 10 g of anhydrous sodium sulphate were weighed into a 50-ml centrifuge tube, blended with 20 ml of extraction solvent for 30 s using a high-speed blender (Ultra-Turrax TP 18/2N, IKA WERK, Staufen, F.R.G.), centrifuged (1500 rpm, 300 *g*, for 2 min) and the supernatant was decanted. The above extraction procedure with the solvent was repeated once more and the combined extracts were applied to a Baker 10 amino cartridge pre-washed with 10 ml of methanol. The cartridge was washed with 5 ml of the extraction solvent, *n*-hexane–ethyl acetate (1:3), and air-dried by aspiration for 1 min. PCAs were eluted from the cartridge with 10 ml of acetonitrile–methanol–0.01 *M* aqueous oxalic acid (adjusted to pH 3.0 with NaOH) (3:1:6) and 1–20 μl of the eluate were injected into the HPLC system for the determination of PCAs.

High-performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant-flow pump (LC-5A, Shimadzu, Kyoto, Japan) was used, with a UV detector (Shimadzu SPD-2AM) operated at 295 nm. The separation was performed on Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) (Macherey, Nagel & Co., Düren, F.R.G.) with acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (3:1:6) as the mobile phase at a flow-rate of 1.0 ml/min at room temperature.

RESULTS AND DISCUSSION

Establishment of HPLC system

In a previous study[16,17], organic acids such as oxalic acid and acetonic acid were effective in control the tailing of tetracycline antibiotics and dehydroacetic acid, respectively, in reversed-phase (RP) HPLC. We considered that such reagents may also be effective in controlling the tailing of PCAs. In order to find a suitable reagent for the determination of PCAs by RP-HPLC, peak asymmetry factors (A_s) of PCAs were measured using mobile phases containing various acids (acetic, phosphoric, citric, tartaric, malonic, lactic, acetonic and oxalic acid). As shown in Table I, a significant effect was given with oxalic acid, in spite of the low concentration. Therefore, we tried to optimize the HPLC conditions for the determination of PCAs using oxalic acid in the mobile phase, and a satisfactory chromatogram of PCAs was obtained as shown in Fig. 1.

With regard to the stationary phase to be used for the separation of PCAs, Nucleosil C_{18} gave satisfactory separations and A_s values of PCAs using oxalic acid in the mobile phase. In contrast, satisfactory A_s values could not be obtained on LiChrosorb RP-18 (E. Merck, Darmstadt, F.R.G.) using any of the reagents described above in the mobile phase. The difference can be successfully explained by the assumptions that the tailing of PCAs is caused by the influence of residual silanol

TABLE I

COMPARISON OF ASYMMETRY FACTORS OF OA, NA AND PA USING VARIOUS AQUEOUS SOLUTIONS IN THE MOBILE PHASE

Peak asymmetry factors of OA, NA and PA were measured under the following HPLC conditions: column, Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–aqueous solution (3:1:6); flow-rate, 1.0 ml/min; detection, 295 nm.

Aqueous solution	*Asymmetry factor (A_s)*		
	OA	*NA*	*PA*
0.05 *M* acetic acid	9.5	>10.0	>10.0
0.05 *M* phosphoric acid	2.6	7.4	9.0
0.05 *M* citric acid	2.3	3.7	4.3
0.05 *M* tartaric acid	2.1	4.4	5.4
0.05 *M* malonic acid	1.9	4.0	5.5
0.05 *M* lactic acid	1.9	2.0	1.9
0.05 *M* acetonic acid	1.9	1.9	1.9
0.01 *M* oxalic acid	1.8	1.7	1.6

Fig. 1. Typical high-performance liquid chromatogram of OA, NA and PA (100 ng each) under the optimum conditions. Column, Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (3:1:6); flow-rate, 1.0 ml/min; detection, 295 nm.

groups on the surface of the stationary phase and that oxalic acid has the ability to mask such silanol groups. Nucleosil C_{18} is end-capped and LiChrosorb RP-18 is not. Therefore, Nucleosil C_{18} successfully undergoes such an effect with oxalic acid because of its lower content of silanol groups, but LiChrosorb RP-18 has too many silanol groups to do so. In addition to the satisfactory A_s values of PCAs, a Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) was advantageous for the sensitive and rapid determination of PCAs because of its small particle size and the short column length, and therefore we used it as an analytical HPLC column.

Concerning the monitoring wavelength, OA and NA were most sensitive at 255 nm and PA at 280 nm; however, simultaneous determination was difficult in the range 230–290 nm because of the difference in peak heights among PCAs. As shown in Fig. 1, PCAs were monitored with similar sensitivity at 295 nm, so this was chosen as a monitoring wavelength.

In order to separate PCAs successfully, the following parameters of the mobile phase were examined; concentration of aqueous oxalic acid solution, pH of aqueous oxalic acid solution and proportions of aqueous solution, acetonitrile and methanol.

The concentration of oxalic acid in the mobile phase hardly influenced the capacity factors (k') of PCAs, whereas the A_s values were improved with increasing concentration. However, they were almost constant above 0.01 *M* and therefore we used 0.01 *M* aqueous oxalic acid solution in the mobile phase.

Whereas, for the pH of aqueous oxalic acid solution in the mobile phase, the k' values of PCAs were almost constant between pH 2.2 and 5.0, the A_s values increased with increasing pH and the best values were given with a pH-unadjusted solution (pH 2.2). We therefore used 0.01 *M* aqueous oxalic acid solution without adjustment of pH in the mobile phase.

In relation to the proportions of acetonitrile and methanol in the mobile phase, a satisfactory separation of PCAs was obtained with any proportion of acetonitrile and methanol, as shown in Fig. 2. However, mobile phases containing acetonitrile

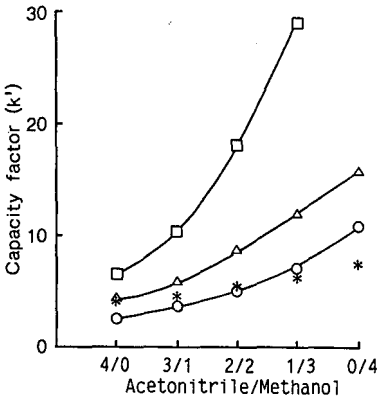

Fig. 2. Influence of ratio of methanol and acetonitrile on k' of PCAs and interfering substance. ○, OA; △, NA; □, PA; ✳, interfering substance from the amino cartridge. Mobile phase: (acetonitrile–methanol)–0.01 *M* aqueous oxalic acid solution (4:6).

and methanol in the ratios 4:0, 2:2 and 1:3 were not applicable in our clean-up system because of interfering peaks originating from the amino cartridge. On the other hand, for a constant ratio of organic solvent and aqueous solution, the k' values increased with increasing proportion of aqueous solution. Considering the retention times of PCAs and the influence of interfering peaks, we used acetonitrile, methanol and 0.01 *M* aqueous oxalic acid solution in the proportions 3:1:6 in the mobile phase.

As a result of these studies, we obtained the optimum HPLC conditions given under Experimental. According to these conditions, PCAs were successfully separated within 7 min. The detection limits of PCAs on the chromatogram were 0.5 ng (signal-to-noise ratio >5) and the calibration graphs were linear between 0.5 and 500 ng.

Establishment of clean-up system

In order to carry out the clean-up of PCAs simply and rapidly, we considered utilizing a disposable prepacked cartridge, and the following summarized clean-up procedure was applied. PCAs are extracted from the sample by blending with an organic solvent, the resulting extract is centrifuged and the supernatant is decanted and applied to a prepacked cartridge. PCAs are eluted from the cartridge with a suitable eluent and determined by HPLC. After the following studies of various aspects of this procedure, the simple and rapid clean-up system described under Experimental was established.

Comparison of prepacked cartridges. In order to select a suitable prepacked cartridge, commercially available normal-phase and ion-exchange type (cyano, diol, primary-secondary amino, amino, quaternary amine, carboxylic acid and aromatic sulphonic acid) cartridges were compared for their ability to retain PCAs. As the solvent to be applied to the cartridges, ethyl acetate was used in this examination, because it has often been used as an extraction solvent for PCAs. PCAs (5 μg each) were dissolved in 40 ml of ethyl acetate and were applied to each cartridge. The ethyl acetate solutions that had passed through the cartridge were collected and evaporated to dryness. PCAs in each residue were dissolved in the mobile phase and were deter-

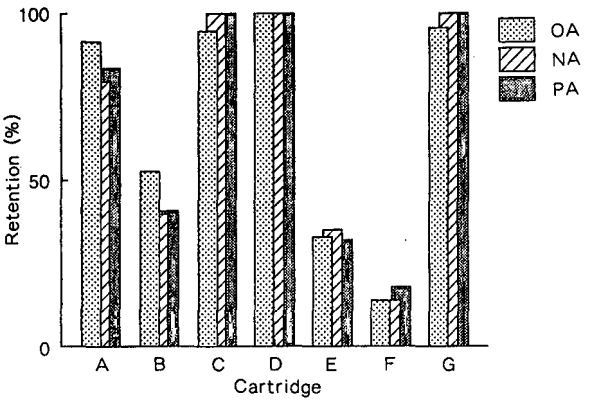

Fig. 3. Comparison of the abilities of prepacked cartridges to retain PCAs. Cartridges: A, Baker 10 cyano; B, Baker 10 diol; C, Baker 10 primary-secondary amino; D, Baker 10 amino; E, Baker 10 quaternary amine; F, Baker 10 carboxylic acid; G, Baker 10 aromatic sulfonic acid. HPLC conditions as in Fig. 1.

mined by HPLC. From the results, the percentages of PCAs retained on each cartridge were calculated. As shown in Fig. 3, PCAs were completely retained on the amino cartridge, so this was used in subsequent work.

Elution from the cartridge. Because the mobile phase is generally suitable for preparing sample and standard solutions to be injected into an HPLC system, we wished to elute PCAs from the cartridge with the mobile phase. However, satisfactory recoveries of PCAs from the cartridge could not be obtained in an attempt to use the mobile phase solution (acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution, 3:1:6) as the eluent. It was considered that the pH of the eluent is one of the most important factors for the elution of PCAs from the cartridge and that the elution of PCAs is expected to be improved by increasing the pH. In order to examine the influence of the pH of the eluent on the elution of PCAs from the cartridge, acetonitrile, methanol and 0.01 *M* aqueous oxalic acid solution (adjusted to various pH values with NaOH) were mixed in the same proportions as in the mobile phase and the recoveries of PCAs retained on the cartridge were investigated. The sample (eel, 5 g) and anhydrous sodium sulphate (10 g) were blended with 40 ml of *n*-hexane–ethyl acetate (1:3) and an extract of the sample was obtained. After addition of PCAs (5 μg each) to the extract, they were applied to the cartridge. PCAs were eluted with 10 ml of each eluent and were determined by HPLC. Satisfactory recoveries were obtained when the pH of 0.01 *M* aqueous oxalic acid solution was adjusted above 2.5. The elution pattern of PCAs from the cartridge was investigated using acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) as the eluent. After application of PCAs to the cartridge in the same manner as described above, the PCAs were eluted with the eluent. The eluate was fractionated into 2-ml fractions and PCAs in each fraction were determined by HPLC. As shown in Fig. 4, PCAs were satisfactorily eluted in 8 ml of eluate. Therefore, we used 10 ml of acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) as the eluent for the amino cartridge in subsequent work.

Comparison of extraction solvents. PCAs are slightly soluble in polar solvents such as water and insoluble in non-polar solvents such as benzene and hexane, so

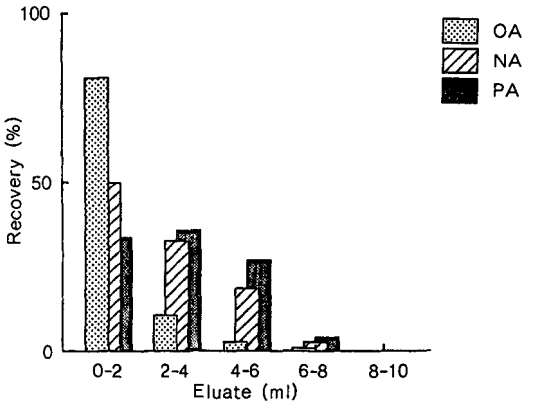

Fig. 4. Influence of elution volume on recoveries of PCAs from the amino cartridge. HPLC conditions as in Fig. 1.

methanol, acetonitrile, dichloromethane, chloroform and ethyl acetate have generally been used as extraction solvents from various samples[1–5,7–12]. However, chloroform and dichloromethane were unsuitable as extraction solvents for our clean-up system, because their high specific gravity prevented the decantation of the extracts. The applicability of methanol, acetonitrile and ethyl acetate was investigated by means of the following examination. Extracts of sample (eel, 5 g) with 40 ml of each extraction solvent were obtained in the same manner as described above. After addition of PCAs (5 μg each) to each extract, they were applied to the amino cartridge. PCAs were eluted from the cartridge with the eluent and were determined by HPLC. When methanol and acetonitrile were used as extraction solvents, the recoveries were very poor. Ethyl acetate gave satisfactory recoveries of NA and PA, but the recovery of OA was slightly lower than those of NA and PA. These results suggested that the retention power of the cartridge increases with decreasing polarity of the solvent and that the polarities of methanol and acetonitrile are too high to give sufficient retention of PCAs on the cartridge. As described above under *Comparison of prepacked cartridges*, because PCAs were completely retained on the cartridge using ethyl acetate alone as the solvent, the different retention behaviour was attributed to the influence of the sample matrix and OA was the most susceptible to such an influence. We considered that the use of a less polar extraction solvent than ethyl acetate would be effective for improving the retention of OA on the cartridge.

On the basis of the above considerations, several solvent systems were prepared by combination of *n*-hexane and ethyl acetate (1:3, 2:2, 3:1 and 4:0), and the recoveries of PCAs were investigated in the same manner as described above. Although satisfactory results were obtained in every instance with these mixtures, the efficiency of extraction of PCAs from the sample decreased with increasing proportion of *n*-hexane in ethyl acetate. In order to select a suitable mixing ratio of *n*-hexane and ethyl acetate, the overall recoveries of PCAs were investigated using various mixtures of *n*-hexane and ethyl acetate. After addition of PCAs (5 μg each) and anhydrous sodium sulphate (10 g) to the sample (eel, 5 g), they were extracted with 40 ml (2 × 20 ml) of *n*-hexane–ethyl acetate (0:4, 1:3, 2:2, 3:1 and 4:0) and applied to the cartridge. As

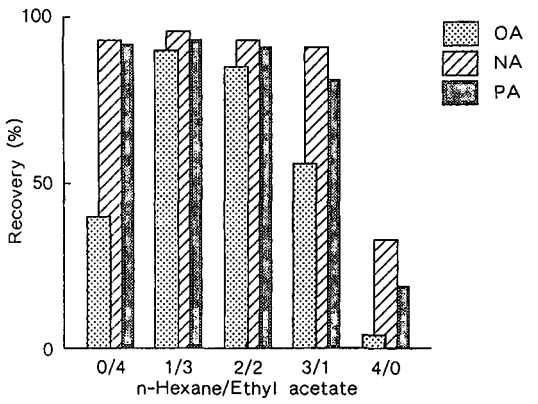

Fig. 5. Effect of ratio of *n*-hexane and ethyl acetate in the extraction solvent on recoveries of PCAs from fortified eel. HPLC conditions as in Fig. 1.

shown in Fig. 5, the best recoveries were obtained when *n*-hexane–ethyl acetate (1:3) was used as the extraction solvent. Next, the effect of extraction volume was examined using *n*-hexane–ethyl acetate (1:3). After addition of PCAs (5 μg each) and anhydrous sodium sulphate (10 g) to the sample (eel, 5 g), they were extracted with 20 ml, 40 ml (2 × 20 ml), 60 ml (3 × 20 ml) and 80 ml (4 × 20 ml) of the extraction solvent and each extract was applied to the cartridge. The best recoveries were obtained when 40 ml of extraction solvent were used. When more than 40 ml of extraction solvent was used, the recoveries of OA and NA became poorer because of weak retention on the cartridge. Therefore, we used 40 ml (2 × 20 ml) of *n*-hexane–ethyl acetate (1:3) as the extraction solvent in our clean-up system.

Effect of anhydrous sodium sulphate. In order to improve the efficiency of extraction of PCAs from the sample, anhydrous sodium sulphate was used, otherwise the sample formed a gummy mass at the bottom of the centrifuge tube during blending with the extraction solvent and satisfactory recoveries of PCAs could not be obtained. In addition, the use of anhydrous sodium sulphate was advantageous for the retention of PCAs on the cartridge, because the retention power of the cartridge was weakened by the influence of water in the extract. It was sufficient to use 10 g of anhydrous sodium sulphate for extraction of PCAs from 5 g of sample.

Application to various fish

The recoveries of PCAs from fortified eel, rainbow trout, sweetfish, red sea bream and yellowtail were investigated at the levels of 1.0 and 0.1 ppm. PCAs (5 and 0.5 μg) were spiked with 50 μl of acetonitrile to 5 g of each sample and determined in accordance with the present method.

As shown in Table II, satisfactory recoveries and coefficients of variations were obtained at the low concentration level of PCAs. The detection limits were 0.05 ppm and the time required for the analysis of one sample was less than 30 min. Typical high-performance liquid chromatograms of these fish are shown in Fig. 6. No interfering peaks appeared on the chromatograms.

In conclusion, a method for the determination of residual PCAs in fish was established using a combination of HPLC and clean-up with a Baker 10 amino car-

TABLE II

RECOVERIES OF OA, NA AND PA FROM FORTIFIED FISH

Recoveries of OA, NA and PA from 5 g of various fish fortified at the levels of 1.0 and 0.1 ppm according to the present method. Results for four replicates (1.0 ppm) and three replicates (0.1 ppm).

Sample	*Addition level (ppm)*	*Recovery (%)*[a]		
		OA	*NA*	*PA*
Eel	1.0	90.3 (2.4)	95.5 (1.3)	92.6 (1.7)
	0.1	92.4 (4.0)	95.2 (1.0)	89.8 (2.0)
Rainbow trout	1.0	87.5 (3.9)	93.2 (3.8)	87.3 (3.9)
	0.1	76.1 (9.8)	81.4 (4.8)	79.9 (8.3)
Sweetfish	1.0	88.3 (3.4)	93.2 (1.1)	90.0 (0.6)
	0.1	84.9 (2.2)	87.3 (4.5)	87.4 (1.5)
Red sea bream	1.0	85.8 (0.9)	88.4 (3.5)	82.0 (3.3)
	0.1	80.7 (2.8)	87.5 (3.7)	73.9 (3.5)
Yellowtail	1.0	77.1 (1.2)	90.4 (1.5)	83.9 (3.2)
	0.1	75.5 (6.8)	88.9 (6.8)	90.9 (7.8)

[a] Coefficients of variation (%) in parentheses.

tridge. For the determination of PCAs by HPLC, oxalic acid was effectively used to control the tailing of PCAs on a conventional RP-HPLC column of Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.). In the clean-up procedure, PCAs in fish were successfully extracted with *n*-hexane–ethyl acetate (1:3) and a Baker 10 amino cartridge was effective for the simple and rapid clean-up of PCAs. Using this method, PCAs spiked in various fishes were rapidly determined with good recoveries, coefficients of variation and sensitivity. Further, this method does not require any special HPLC columns and any time-consuming treatments. Therefore, we recommend it for the routine determination of residual PCAs in fish.

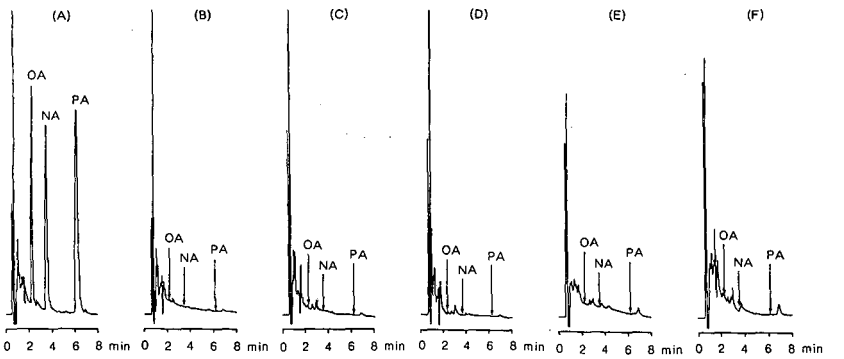

Fig. 6. Typical high-performance liquid chromatograms of different fish. (A) Fortified eel (1.0 ppm); (B) eel; (C) rainbow trout; (D) sweetfish; (E) red sea bream; (F) yellowtail. HPLC conditions as in Fig. 1.

ACKNOWLEDGEMENT

We greatly appreciate the encouragement given by Dr. S. Isomura, Director of the Aichi Prefectural Institute of Public Health.

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Journal of Chromatography, 477 (1989) 407–412
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 583

Note

Increments of the arithmetic retention index for polyoxyethylene glycol monoalkyl ethers and their degradation products

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In previous work[1], arithmetic retention indices were determined for succesive homologues of non-ionic surfactants having polyoxyethylene chains. OV-17 silicone resin was used as the liquid phase. The increments of the arithmetic index were calculated for the characteristic groups present in the surfactant molecules and can be used to predict the I_R values for different homologues having various numbers of oxyethylene groups and to identify these homologues on chromatograms of commercial polydisperse mixtures.

The degradation of polyoxyethylene glycol monoalkyl ethers was recently studied and various degradation products were identified[2,3]. The aim of this work was to determine the arithmetic retention indices for polyoxyethylene glycol monoalkyl ethers and their degradation products on liquid stationary phases used for the analysis of commercial non-ionic surfactants.

EXPERIMENTAL

Polydisperse polyoxyethylene glycol monoalkyl ethers, $RO(CH_2CH_2O)_nH$, having different lengths of the alkyl group and of the polyoxyethylene chain, were used. They were analysed directly as acetates and after their degradation at 150°C in the presence of acetyl chloride[2,3].

A Perkin-Elmer Model 900 gas chromatograph with a flame ionization detector was used. The separation was carried out in stainless-steel columns filled with (I) silicone resin OV-101 (3%) on Chromosorb G AW DMCS (60–80 mesh) (0.9 m × 2.7 mm I.D.), (II) Dexsil 400 (1%), on Chromosorb G AW DMCS (80–100 mesh) (0.4 m × 2.7 mm I.D.) and (III) Carbowax 20M-TPA (12%) on Chromosorb W AW DMCS, (80-100 mesh) (1.6 m × 2.7 mm I.D.) Columns I and II were used for the direct analysis of polyoxyethylene glycol monoalkyl ethers and column III only for the analysis of their degradation products. The temperatures of columns I, II and III were 100, 130 and 100°C for 1 min and were then raised to 300, 360 and 220°C at 8, 6 and 5°C/min, respectively. Argon was used as the carrier gas at a flow-rate of 30 cm^3/min.

0021-9673/89/$03.50

The arithmetic retention index[4] was determined for all the separated components of the analysed polydisperse mixtures.

RESULTS AND DISCUSSION

The values of the arithmetic retention indices determined are given in Tables I–III. The succesive homologues of polyoxyethylene glycol monoalkyl ethers elute in order of increasing number of oxyethylene groups and increasing length of the alkyl group up to the homologues having 17 oxyethylene groups[2,3]. However, the values of

TABLE I

ARITHMETIC RETENTION INDICES FOR POLYOXYETHYLENE GLYCOL MONOALKYL ETHERS, C_mH_{2m+1}–O–$(CH_2CH_2O)_nH$, AS ACETATES, ON OV-101

m	*n*	I_R *(exp.)*	I_R *(calc.)*	*Error*	
				Absolute	*Relative (%)*
12	0	1640	1650	10	0.6
	1	1900	1912	12	0.6
	2	2173	2173	0	0.0
	3	2448	2435	−13	0.5
	4	2715	2696	−19	0.7
	5	2962	2958	−4	0.1
	6	3225	3219	−6	0.2
	7	3471	3481	10	0.3
	8	3735	3742	7	0.2
14	0	1835	1850	15	0.8
	1	2109	2112	3	0.1
	2	2364	2373	9	0.4
	3	2642	2635	−7	0.3
	4	2908	2896	−12	0.4
	5	3172	3158	−14	0.4
	6	3423	3419	−5	0.1
	7	3683	3681	−2	0.1
16	0	2045	2050	5	0.2
	1	2309	2312	3	0.1
	2	2577	2573	−4	0.2
	3	2854	2835	−19	0.7
	4	3103	3096	−7	0.2
	5	3362	3358	−4	0.1
	6	3627	3619	−8	0.2
	7	3893	3881	−12	0.3
18	0	2245	2250	5	0.2
	1	2509	2512	3	0.1
	2	2788	2773	−15	0.5
	3	3034	3035	1	0.0
	4	3294	3296	29	0.1
	5	3529	3558	29	0.8
	6	3789	3819	30	0.8
Average error				9	0.3

TABLE II

ARITHMETIC RETENTION INDICES FOR POLYOXYETHYLENE GLYCOL MONODODECYL ETHERS, $C_{12}H_{25}O(CH_1CH_2O)_nH$, AS ACETATES ON DEXSIL 400

n	I_R *(exp.)*	I_R *(calc.)*	*Error*	
			Absolute	*Relative (%)*
0	1686	1653	−33	2.0
1	1929	1930	1	0.1
2	2196	2208	12	0.5
3	2475	2485	10	0.4
4	2750	2762	12	0.4
5	3020	3040	20	0.7
6	3312	3317	5	0.2
7	3600	3594	−6	0.2
8	3876	3871	−5	0.1
9	4167	4149	−18	0.4
Average error			12	0.5

I_R for only the first seven to nine homologues were determined because of the lack of standard alkanes with higher molecular masses.

The values of the arithmetic retention indices follow linear relationships correlating I_R with the number of oxyethylene groups. Hence the slopes determine the I_R increments for one oxyethylene group. Similar values were obtained for various homologous series of polyoxyethylene glycol monoalkyl ethers containing different

TABLE III

ARITHMETIC RETENTION INDICES FOR THE DEGRADATION PRODUCTS OF POLYOXYETHYLENE GLYCOL MONOALKYL ETHERS ON CARBOWAX 20M-TPA

Compound	I_R *(exp.)*	I_R *(calc.)*	*Error*	
			Absolute	*Relative (%)*
$CH_3COOCH_2CH_2Cl$	1318	1352	34	2.6
$CH_3COOCH_2CH_2OCH_2CH_2Cl$	1757	1747	−10	0.6
$CH_3COOCH_2CH_2OCH_2CH_2OCH_2CH_2Cl$	2157	2142	−15	0.7
$C_{10}H_{21}Cl$	1470	1467	−3	0.1
$C_{10}H_{21}OCH_2CH_2Cl$	1838	1862	24	1.3
$C_{12}H_{25}Cl$	1684	1667	−17	1.0
$C_{12}H_{25}OCH_2CH_2Cl$	2064	2062	−2	0.1
$ClCH_2CH_2OCH_2CH_2OCH_2CH_2Cl$	1935	1924	−11	0.6
$ClCH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2Cl$	2330	2319	−11	0.5
$CH_3COOC_{10}H_{21}$	1684	1685	1	0.1
$CH_3COOCH_2CH_2OC_{10}H_{21}$	2068	2080	12	0.6
$CH_3COOC_{12}H_{25}$	1893	1885	8	0.4
$CH_3COOCH_2CH_2OC_{12}H_{25}$	2278	2280	2	0.1
$CH_3COOCH_2CH_2OCH_2CH_2OOCCH_3$	1970	1965	−5	0.3
$CH_3COOCH_2CH_2OCH_2CH_2OCH_2CH_2OOCCH_3$	2375	2360	−15	0.6
Average error			10	0.7

TABLE IV

REGRESSION AND CORRELATION COEFFICIENTS FOR THE LINEAR RELATIONSHIP $I_R = A + Bn$, WHERE n DENOTES THE NUMBER OF OXYETHYLENE GROUPS

Liquid stationary phase	*Alkyl group*	*A*	*B*	*Correlation coefficient*
OV-101	$C_{12}H_{25}$	1649	261.9	0.99988
	$C_{14}H_{29}$	1842	264.2	0.99993
	$C_{16}H_{33}$	2049	263.5	0.99995
	$C_{18}H_{37}$	2258	256.4	0.99977
Dexsil 400	$C_{12}H_{25}$	1653	277.3	0.99984

numbers of carbon atoms in their alkyl groups (Table IV). The correlation coefficients are high (*ca.* 1.0) and demonstrate the statistical validity of the equations derived.

The chemical formulae of the separated components in the group of surfactants considered can be expressed as

$$A_i = (G_1)_{a_{1i}}, (G_2)_{a_{2i}}, \ldots, (G_l)_{a_{li}}$$

where $G_1, G_2, \ldots, G_l$ are the characteristic groups present in the system considered, a_{1a}, $a_{2i}, \ldots, a_{li}$ are the numbers of groups $G_1, G_2, \ldots, G_l$ in compound A_i, $i = 1, 2, \ldots, k$ are the numbers of compounds considered in the system and $j = 1, 2, \ldots, l$ are the numbers of the characteristic groups considered.

In our systems k is much greater than l; $k = 46$, 10 and 15 for OV-101, Dexsil 400 and Carbowax 20M-TPA, respectively, whereas $l = 3$ or 4. The following groups are considered: the methylene group equivalent to the methyl group ($-CH_2-$, $-CH_3$), the etheric oxygen –O–, the $-OOCCH_3$ group and the chlorine atom (–Cl). The oxyethylene group is considered as the sum of two methylene groups and the etheric oxygen.

The set of subscripts $\{a_{ji}\}$, $j = 1, 2, \ldots, l$, forms the formula vector, a_i, of the compound A_i:

$$a_i = [a_{1i}, a_{2i}, \ldots, a_{li}]^T$$

where T denotes transposition.

The formula matrix **A** of the system considered is then defined as $\mathbf{A} = \mathbf{a}_1, \mathbf{a}_2, .., \mathbf{a}_n$. Hence, if the products of degradation of polyoxyethylene glycol monoalkyl ethers are considered (Table III), this formula matrix is

$$\mathbf{A} = \begin{bmatrix} 2 & 4 & 6 & 10 & 12 & 12 & 14 & 5 & 7 & 10 & 12 & 12 & 14 \\ 0 & 1 & 2 & 0 & 1 & 0 & 1 & 2 & 3 & 0 & 1 & 0 & 1 \\ 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 \end{bmatrix}$$

where the order of compounds is the same as that in Table III. The groups considered, $-CH_2-$, $-CH_3$, –O–, $-OOCCH_3$ and –Cl, are numbered 1, 2, 3 and 4, respectively.

TABLE V
INCREMENTS OF THE ARITHMETIC RETENTION INDEX

Group	*OV-101*	*Dexil 400*	*Carbowax 20M-TPA*
$-CH_2-$, $-CH_3$	100	100	100
$-OCH_2CH_2-$	261.5	277	395
$-O-$	61.5	77	195
$-OOCCH_3$	450	453	685
$-Cl$	–	–	467

However, if the above formula matrix were considered, the increments of the arithmetic index would not be equal to 100. Therefore, the standard *n*-alkanes were also considered. Their formula can be expressed as $A_i = (CH_2)_{a_{CH_2 i}}$ (the methyl group is considered equivalent to the methylene group) and their formula vector is $\mathbf{a}_i = [a_{CH_2 i}$, 0,0,0]. The whole matrix of the system considers the formula vectors of all compounds present in the system, including standard alkanes.

Assuming additivity, the arithmetic retention index of a compound A_i can be expressed as:

$$I_{RA_i} = \sum_{j=1}^{k} a_{ji} \Delta I_{RG_j}$$

where it is assumed that the increment of I_R for a group G_j is constant for all compounds considered. Thus, for all compounds present in the system, the following set of linear equations is obtained:

$$\mathbf{I}_R = \mathbf{A} \Delta \mathbf{I}_R$$

where

$$I_R = [I_{RA_1}, I_{RA_2}, \ldots, I_{RA_k}]^T$$

$$\Delta I_R = [\Delta I_{RG_1}, \Delta I_{RG_2}, \ldots, \Delta I_{RG_k}]^T$$

The increments of the arithmetic retention index obtained by solving the above set of linear equations are given in Table V. These increments can be used to calculate the retention indices of the compounds considered (Table I–III). The errors in such predictions are relatively low and equal to about 10 units of the arithmetic retention index for polyoxyethylene glycol monoalkyl ethers and their degradation products. Hence the increments obtained can be used to predict the arithmetic retention index for different homologues of the groups of compounds considered.

CONCLUSIONS

The arithmetic retention index calculated from the increments determined for characteristic groups present in polyoxyethylene glycol monoalkyl ethers and their

degradation products can be used to identify peaks on chromatograms. The average absolute and relative errors of the arithmetic retention index calculation are 10 and 0.5%, respectively.

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Journal of Chromatography, 477 (1989) 413–419
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 581

Note

Extraction and high-performance liquid chromatographic determination of gypsogenin 3,O-glucuronide

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(First received January 11th, 1989; revised manuscript received April 12th, 1989)

The genus *Gypsophila* (Caryophyllaceae) is well known to contain saponins of industrial interest with various applications, *e.g.*, in the compositions of photosensitive surfaces in photography and of shampoos[1]. More recently, these saponins have been described as apparently being responsible for hypocholesterolemic effects[2]. Gypsogenin is the main pentacyclic triterpenoidal aglycone of these saponins[3]. Substituted on the 3-OH and on the 28-COOH by two chains of different glycosides, these saponins, then called bidesmosidic, are among the most glycosylated. Gypsoside, for example, extracted from *Gypsophila pacifica*, contains nine glycosides[4]. A commercial product well known as Saponin pure white (Merck), used in the past as a standard for haemolytic tests in most saponin determinations, was previously reported to be extracted from roots and rhizomes of *Gypsophila paniculata*[5]. These large saponins easily break during their extraction and purification, giving shorter saponins. Higuchi *et al.*[6] have demonstrated in this commercial product the presence of a gypsogenin 3-O-glycoside, [28-hydroxy-23,28-dioxoolean-12-en-3β-yl O-β-D-galactopyranosyl (1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranosid]uronic acid, and the roots and rhizomes of *G. paniculata* have been reported[7] to contain a main saponin, the gypsoside, the same as described in *G. pacifica*. However, high-performance liquid chromatography (HPLC) of Saponin pure white shows about nine saponins, none of them more abundant than the others (unpublished work; identification of some of them is in progress) and the routine quantitative determination of these saponins is difficult and time consuming. Tagiev and Ismailov[8] described a thin-layer chromatographic method for the determination of a *Gypsophila* saponin in the roots of *G. bicolor* after extraction, purification and measurement of the spot surface revealed by phosphotungstic acid. Although this method is interesting for a rapid evaluation, it seems very imprecise. We report here an efficient method for the determination of *Gypsophila* sp. saponins by the means of their prosapogenin, gypsogenin 3,O-glucuronide (I) (Fig. 1), which is much more stable than gypsogenin. This greater stability may be explained by the reversible formation of a hemiacetal group by the glucuronic acid carboxyl group and the genin CHO group[4]. I is present in most of the saponins isolated and identified from the genus *Gypsophila*[9,10]. We chose to test this method of quantification on Saponin pure white by hydrolysing this mixture of saponins to their prosapogenin, I, purifying it by liquid–liquid extraction with a polar solvent nonmiscible with water, in one step, and measuring it by HPLC.

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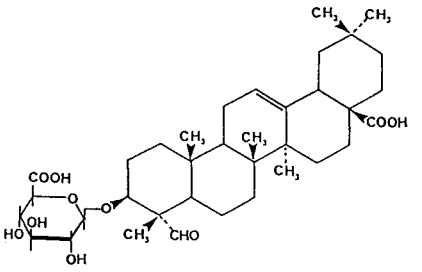

Fig. 1. Structure of gypsogenin 3,O-glucuronide.

EXPERIMENTAL

Commercial Saponin pure white (No. 7695) was purchased from Merck (Darmstadt, F.R.G.). Sep-Pak C_{18} reversed-phase cartridges were purchased from Millipore–Waters (Milford, MA, U.S.A.).

The standard hydrolysis conditions for quantitative experiments involved boiling with refluxing at 95–100°C of a maximum of 20 mg/ml of the Merck saponin mixture in 0.57 *M* sulphuric acid for at least 1 h. Under these conditions, aglycone (gypsogenin) or by-products (gypsogenin lactone) were not detected.

The extraction conditions were as follows. The carboxylic groups of the glucuronic acid-containing saponins undergo two types of dissociation. Glucuronic acid dissociates near pK_a 3.6 and the carboxylic group of triterpenoidal aglycones dissociates near pK_a 4.9[11]. Hence at pH 1 all acid moieties of I are in the proton-associated form and can be easily extracted with a solvent immiscible with water such as *n*-butanol or ethyl acetate with a very good yield (95%) in one step with 2 ml of solvent and 7 ml of hydrolysing mixture. The second step of the extraction gives only 5% of remaining product. *n*-Butanol and ethyl acetate show a different behaviour in neutral media owing to the difference in their polarities. I precipitates at pH 7 in ethyl acetate whereas it remains soluble in *n*-butanol and therefore it can even be purified in ethyl acetate and water by changing the pH near 7 and liquid–liquid extraction gives a yield of nearly 100% with I in the aqueous phase. The pH must be below 9 in order to avoid any degradation, even below 30°C. Further purification of the aqueous extract is effected with a single and rapid purification step on a Sep-Pak C_{18} reversed-phase cartridge. The aqueous extract, adjusted to pH 2–3 with acetic acid, was passed through the cartridge, which was then rinsed with methanol–water (60:40, v/v) containing 0.1% of acetic acid. I was then eluted with 2 ml of methanol and was then ready for injection into the HPLC system.

The HPLC equipment consisted of a Waters Assoc. Model 510 pump with a Lambda Max 481 spectrophotometer and three stainless-steel columns, the first (250 × 4 mm I.D.) packed with LiChrospher 100 RP-18e (end-capped) (Merck, 5 μm), the second (300 × 3.9 mm I.D.) with LiChrospher RP-8 (Merck, 10 μm) and the third (300 × 3.9 mm I.D.) with μBondapak Phenyl (end-capped) (Waters, 10 μm). The analysis was carried out at 25°C at a flow-rate of 1.0 ml/min. The mobile phase consisted of a mixture of methanol and water in different proportions containing 5

m*M* TBA and the pH was adjusted with 0.5 *M* phosphoric acid. I (2 mg/ml, volume injected 20 μl) was detected at 206 nm.

I had m.p. 270–272°C; $[\alpha]_D = +44$ (c = 10 mg/ml in methanol); IR (KBr, 1%) 3400 cm^{-1} (OH), 1700 cm^{-1} (CO acid); FAB-MS, with NaI, m/z 669 $[M + Na]^+$, 453 [gypsogenin $- H_2O - H^+]^+$ and with KI, m/z 723 $[M + K]^+$, 453 [gypsogenin $- H_2O - H^+]^+$ [M = $C_{36}H_{53}O_{10}$ (646)]; ^{13}C NMR, see Table I (chemical shifts consistent with those of Higuchi *et al.*[6] for gypsogenin methyl ester).

RESULTS

Ion-pair HPLC for the analysis of gypsogenin 3,O-glucuronide

Few HPLC studies of triterponoidal saponins have been reported. Most have involved ginsenosides[12,13], and diene-transformed saikosaponins[13]. Generally, HPLC is employed to separate and identify mixtures of saponins or to purify them by a preparative method (*e.g.*, ref. 14). Saponins are studied in the underivatized form[15–18] although they have no capacity to absorb UV light (except a few of them, such as glycyrrhizic acid). One of these studies[19] was carried out on I obtained from Sarsynthex (Merignac, France), who purchased it from our laboratory. Hence the purity of this product was confirmed. Saponins may show large differences in polarity. In the olean-12-ene group, a mixture of eight saponins of *Lonicera nigra* L. (Caprifoliceae) was separated on an RP-8 column using gradient elution with acetonitrile–water[17]. Among these saponins, two 3,O-glucuronides of hederagenin and oleanolic acid eluted with relatively sharp peaks, between more polar bidesmosidic saponins (with three or four sugars attached to the 3-OH and the 28-COOH of the aglycones, hederagenin and oleanolic acid) and monodesmosidic saponins (with one or two sugars attached to the 3-OH 3 of the same aglycones). Hence the efficiency of the RP-8 column was good enough to perform the HPLC separation of these glucuronides under the correct conditions. In spite of this, I shows a different behaviour, as previously observed[19] and as we also found; under the above classical conditions, I showed extensive band broadening with loss of product remaining attached to the

TABLE I

^{13}C NMR CHEMICAL SHIFTS (δ VALUES) OF GYPSOGENIN 3,O-GLUCURONIDE IN $C^2H_3O^2H$

Carbon	δ *(ppm)*	*Carbon*	δ *(ppm)*	*Carbon*	δ *(ppm)*	*Carbon*	δ *(ppm)*
C_1	39.4	C_{11}	24.3	C_{21}	35.2	C_1'	105.4
C_2	26.0	C_{12}	123.6	C_{22}	33.6	C_2'	76.7
C_3	73.3	C_{13}	145.5	C_{23}	209.3	C_3'	83.9
C_4	56.4	C_{14}	43.2	C_{24}	10.8	C_4'	75.1
C_5	49.7	C_{15}	29.1	C_{25}	16.4	C_5'	77.6
C_6	21.6	C_{16}	24.3	C_{26}	18.0	C_6'	172.7
C_7	34.0	C_{17}	48.6	C_{27}	26.8		
C_8	41.1	C_{18}	43.0	C_{28}	182.0		
C_9	49.1	C_{19}	47.9	C_{29}	33.9		
C_{10}	37.3	C_{20}	31.9	C_{30}	24.8		

reversed stationary phase in the column. The sole chemical difference between I and the two glucuronides of hederagenin and oleanolic acid is the presence of a CHO group in the 23-position instead of the CH_2OH and CH_3 groups in the latter two compounds. Hence a detailed study of the efficiency of HPLC under various conditions of the mobile and stationary phases combined with changes in the polarity of the molecule of I was necessary.

Recently, reversed-phase ion-pair chromatography has been increasingly used for the analysis of ionic compounds[20]. A rapid and precise method for the determination of glycyrrhizin in *Glycyrrhizae radix* utilizing an ion-pair technique was described by Sagara *et al.*[20]. Following their recommendations for glycyrrhizin, also a saponin with glucuronic acid (two glucuronic acid groups by molecule of saponin), we selected tetra-*n*-butylammonium (TBA) as the counter ion. The mixing ratio of methanol to water and the pH of the mobile phase were varied to find the optimum conditions for determination of I with different HPLC columns (Table II). With methanol-water (75:25) containing 5 m*M* TBA and adjusted to pH 5.0 with 0.5 *M* phosphoric acid, I (2 mg/ml) was eluted at an appropriate time (13 min with a capacity factor of 5.6) and with a relatively sharp peak and good effiency (22 400 theoretical

TABLE II

EFFICIENCY OF DIFFERENT COLUMNS IN THE HPLC DETERMINATION OF I (1 mg/ml) WITH DIFFERENT CONDITIONS OF THE MOBILE PHASE

Mobile phase, methanol–water in various proportions containing 5 m*M* TBA, pH adjusted with 0.5 *M* phosphoric acid; flow-rate, 1 ml/min; injection volume, 20 µl; detection, 206 nm. All assays were performed at least in triplicate. *N*/m = theoretical plates per metre; *k'* = capacity factor.

Methanol–water	*pH*	*RP-18 (end-capped)*		*RP-8*		*µBondapak Phenyl (end-capped)*	
		N/m	*k'*	*N/m*	*k'*	*N/m*	*k'*
78:22	7.3–7.6[a]	5000	3.0				
	6	7440	3.0				
	5	7200	3.9				
75:25	7.3–7.6[a]	13 600	4.0				
	6	15 200	5.0				
	5	22 400	5.6				
72:28	7.3–7.6[a]	12 400	6.5	1280	2.3		
	6	13 200	7.7	2370	3.2		
	5	20 600	10.0	ND[b]	ND		
70:30	7.3–7.6[a]			1280	2.8	1220	1.3
	6			1750	3.8	1330	1.4
	5			3236	5.5	2230	2.0
68:32	6			833	4.2		
65:35	7.3–7.6[a]					1530	2.8
						1570	3.2
						1770	3.5

[a] pH not adjusted.
[b] ND, not determined.

plates per meter) with an RP-18 end-capped column (5 μm). The HPLC trace for a typical determination is illustrated in Fig. 2.

Generally, the influence of the alkyl chain on retention and efficiency is secondary to the much greater influence of the mobile phase composition[21]. Although this assertion is in part verified here, the influence of the alkyl chain is greater than for other solutes. Under the optimum conditions of mobile phase composition, RP-8 and Phenyl reversed phases give similar efficiencies as measured by the theoretical plate number. However, the use of RP-18 increases the efficiency by a factor of about 7, especially when the remaining silanol groups are well end-capped. Tests with another C_{18} column, probably with the silanol groups less protected, (results not shown) showed an intermediate efficiency. However, end-capping was not sufficient to give the best efficiency because the μBondapak Phenyl column was end-capped. The polarity of the solute is emphasized by the use of TBA with adjustment of the pH with phosphoric acid. With all other conditions identical, the efficiency could be doubled when the pH was adjusted to 5, I giving the pairing reagent. An ion-suppression method has been described[19] for the analysis of I to overcome this difficulty, apparently giving a good efficiency, but no value for the theoretical plate number was given. In that study[19] a C_{18} column was used with a 25-min water–acetonitrile gradient in

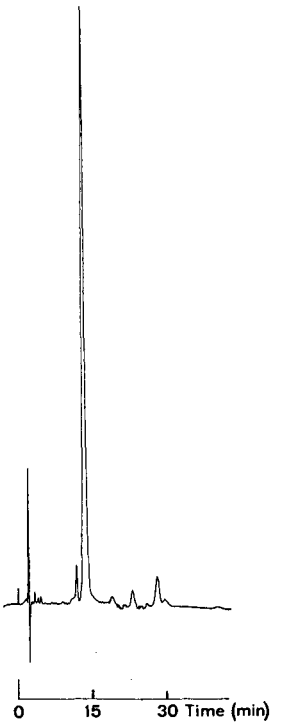

Fig. 2. Reversed-phase HPLC trace for a typical determination of I (2 mg/ml) with UV detection at 206 nm. Sample size, 20 μl; column, C_{18} (Merck, 5-μm LiChrospher 100 RP-18e, 250 mm × 4 mm I.D.). Isocratic elution with methanol–water (75:25) containing 5 m*M* TBA, pH adjusted with 0.5 *M* phosphoric acid, at a flow-rate of 1 ml/min.

TABLE III

HPLC DETERMINATIONS OF I IN INCREASING CONCENTRATIONS OF SAPONIN PURE WHITE (MERCK) AFTER HYDROLYSIS AND EXTRACTION AS DESCRIBED UNDER EXPERIMENTAL AND CONTROL OF THE METHOD BY THE YIELD OF I CONTAINED IN EACH CONCENTRATION OF SAPONIN PURE WHITE TESTED

Reproducibility of yields (1.0–15 mg/ml): X_s = 14.3%, S.D. = 2.15%.

	Saponin pure white (mg/ml)										
	0.5	*1.0*	*1.5*	*3.0*	*4.5*	*6.0*	*7.5*	*11.25*	*15.0*	*22.5*	*30.0*
I (mg/ml)	0.04	0.14	0.27	0.37	0.55	0.89	0.90	1.88	2.10	2.02	1.74
Yield of I as a % of Saponin pure white	8.7	14.0	18.0	12.5	12.3	14.8	12.0	16.7	14.0	9.0	5.8

the presence of trifluoroacetic acid (TFA). As TFA has a low UV absorbance, detection of I could be monitored at 210 nm. This ion-suppression method completes the results presented here with the ion-pair method. Comparison between these two methods is in progress, and the results will be reported in the near future.

Our results clearly show that, in contrast to other glucuronic acid-containing saponins, I requires a very apolar reversed-phase support with good end-capping and with an apolar protection of the molecule by a pairing reagent or transformation by acidification of the molecule to be eluted under HPLC conditions without band broadening and with a symmetrical peak.

Determination of gypsogenin 3,O-glucuronide in Saponin pure white (Merck)

The concentration of I in Saponin pure white and the reproducibility of the method were measured using the above HPLC conditions, with increasing concentrations of the analyte from 0.5 to 30 mg/ml submitted to the hydrolysis and extraction conditions described under Experimental. The results, given in Table III, show good correspondence between I concentration and Saponin pure white concentration in the range 1.5–15 mg/ml, with an average of 14.3% of I and a coefficient variation of 2.15%. In comparison, roots and rhizomes of *G. paniculata* contain 0.74 to 3.92% dry weight of I (unpublished results).

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Journal of Chromatography, 477 (1989) 420–426
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 575

Note

Reversed-phase high-performance liquid chromatography of very long oligodeoxyribonucleotides

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(First received November 11th, 1988; revised manuscript received March 21st, 1989)

The rapid development of automated DNA synthesis has facilitated the production of oligodeoxyribonucleotides (oligonucleotides) of up to 180 bases in length, that are predominantly used for gene synthesis[1,2]. While it is possible for some applications to use the crude product of synthesis directly, *e.g.*, as hybridization probes or sequencing primers, gene synthesis requires oligonucleotides conforming precisely to the desired sequence, since otherwise expensive and time-consuming site-specific mutagenesis procedures become necessary. During synthesis the individual coupling and deprotection steps are not completely quantitative, the crude product of synthesis containing, among other by-products, varying amounts of shorter oligonucleotides. Similarly, depurination under acidic conditions and strand scission in ammonia also lead to smaller sequences which need to be separated from the desired sequence by means of an high resolution technique.

Various high-performance liquid chromatography (HPLC) procedures have been reported for the purification and separation of oligonucleotides. However, most of these techniques are restricted to oligonucleotides of 45–60 bases in length[3–8]. Paired-ion-exchange chromatography is capable of separating very long detritylated oligonucleotides, but requires corrosion-resistant equipment and subsequent desalting[9]. The alternative, and previously the only technique applicable to longer oligonucleotides, is electrophoretic separation of the crude mixture by polyacrylamide gel electrophoresis (PAGE) followed by electroelution[10,11]. The latter procedures have some drawbacks, as they are labour intensive and time consuming, show a low yield due to incomplete recovery from the gel and may require radioactive labelling. In addition, PAGE does not lend itself to automation and large scale preparations. Here we report a reversed-phase (RP) HPLC procedure for the purification and separation of very long 5′-O-dimethoxytrityl(DMT)-derivatized oligonucleotides, and demonstrate its resolving power for several oligonucleotides of 88–143 nucleotides in length.

EXPERIMENTAL

Equipment

Five oligodeoxyribonucleotides (Table I) were generated by solid phase phosphoramidite chemistry on an Applied Biosystems (Weiterstadt, F.R.G.) Model 381A

0021-9673/89/$03.50

TABLE I

OLIGODEOXYRIBONUCLEOTIDES

Length	*Base composition*				*Base composition (%)*				*GC/AT Ratio*
	A	*C*	*G*	*T*	*A*	*C*	*G*	*T*	
88-mer	4	32	31	21	4.5	36.4	35.2	23.9	1.4
90-mer	21	31	33	5	23.3	34.4	36.7	5.6	2.5
110-mer	28	30	34	18	25.5	27.3	30.9	16.4	1.4
124-mer	31	36	29	28	25.0	29.0	23.4	22.6	1.1
143-mer	24	42	43	34	16.8	29.4	30.1	23.8	1.5

DNA synthesizer. Liquid chromatography was carried out on a corrosion-resistant Hewlett-Packard (Waldbronn, F.R.G.) Model 1090M HPLC system equipped with a PV5 gradient mixer, workstation and diode-array detector. The RP ODS-Hypersil C_{18} column (250 mm × 46 mm; 5 ± 0.1 μm spherical particles; pore size 9 ± 0.2 μm) was obtained from R. Melz (Berlin, F.R.G.). For quantitative evaluations, a Model 1701 LS scintillation counter and a Model DU-65 spectrophotometer (both from Beckman Instruments, Munich, F.R.G.) equipped with a 30-μl microcuvette were employed.

Reagents

β-Cyanoethyl-derivatized phosphoramidite synthesis columns (width 500 Å, 0.2-μmol scale) and reagents for DNA synthesis were obtained from Applied Biosystems and used as specified by the supplier. The argon (Linde, Berlin, F.R.G.) used for pressurizing the solvent-delivery system compartment was of 99.99998% purity. Reagents and solvents for chromatographic procedures were of HPLC grade (Merck, Darmstadt, F.R.G.). Reagents for PAGE were obtained from Bio-Rad (Munich, F.R.G.). Adenosine [γ-^{32}P]triphosphate (ATP, specific activity 3000 μCi/mmol) was from New England Nuclear (Dreieich, F.R.G.), and T4 nucleotide kinase was from Bethesda Research Laboratories (Karlsruhe, F.R.G.).

Automated oligodeoxyribonucleotide synthesis

The 381A synthesizer was programmed to run 75-step cycles on a 0.2-μmol scale according to the Applied Biosystems 381A user manual (version 1.23). The coupling efficiency was monitored spectrophotometrically at 498 nm after cleavage and 1:10 (v/v) dilution in 0.1 *M* toluenesulphonic acid of the DMP protecting group. The oligonucleotide product was cleaved from the synthesis support by incubation with 1.5 ml 25% ammonia at room temperature for 20 min. This procedure was repeated three times with fresh ammonia. The pooled ammonia aliquots containing the synthesis products were incubated at 60°C overnight to remove exocyclic amines from deoxyadenosine, deoxycytidine and deoxyguanosine, and lyophilized after evaporation of the ammonia.

Chromatographic procedure

For analytical separations, 5–10 μl ($A_{260\ nm}$ = 1) of the crude oligonucleotide

extract were precipitated with ethanol (96%), resuspended in water and applied to the column. Subsequent preparative experiments were scaled up by a factor of 10. The solvents were 100 m*M* triethylamine–acetic acid (pH 7.0) (buffer A) and 80% acetonitrile in 100 m*M* triethylamine–acetic acid (pH 7.0) (buffer B). Oligonucleotides of up to 90 bases in length were separated by employing isocratic conditions of 10% buffer B for 5 min followed by a linear gradient up to 45% buffer B generated within 25 min. For oligonucleotides between 90 and 143 bases in length, the elution procedure was divided into five steps: (i) 5 min of isocratic conditions (10% buffer B); (ii) a linear increase to 18% of buffer B within 5 min; and (iii) for the elution of the desired final product, a slow rise in concentrations of buffer B from 18 to 25% within 30 min. In order to elute the bulk of shorter tritylated oligonucleotides, (iv) the concentration of buffer B was increased to 45% within the next 5 min, and (v) kept isocratic for another 5 min prior to returning to the starting condition. Both the flow-rate (0.75 ml/min) and column temperature (70°C) were kept constant for all separations. The detection wavelength was 260 nm with a band width of 4 nm. A reference wavelength (470 nm, band width 130 nm) was subtracted to minimize the background.

5′-DMT-oligonucleotides in chromatographically obtained fractions as well as the crude synthesis mixture (for PAGE) were deprotected with 0.05 ml 80% acetic acid for 15 min at room temperature. A 0.05-ml volume of 96% ethanol was added prior to lyophilization. For 5′-labelling, lyophilized oligonucleotides were redissolved in 100 m*M* Tris–HCl–10 m*M* $MgCl_2$–5 m*M* dithiothreitol–0.1 m*M* spermidine (pH 7.6), 8 units of T4 polynucleotide kinase and 4 μCi [γ-^{32}P]ATP. The reaction was allowed to proceed at 37°C for 1 h.

PAGE

Samples were kept at 100°C in 95% deionized formamide containing 10 m*M* EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue for 3 min, chilled on ice and separated on 8% polyacrylamide slab gels (18 cm × 16 cm) containing 8 *M* urea at 27 mA in 50 m*M* Tris-base–50 m*M* boric acid–1.5 m*M* EDTA (pH 8.7). After electrophoresis the gels were subjected to autoradiography and sealed into plastic bags for subsequent recovery of the material. Gel slices representing appropriate bands were cut out and embedded in a 1% agarose gel for electroelution onto a NA-45 ion-exchange membrane (Schleicher and Schuell, Dassel, F.R.G.). Immobilized DNA was released from the membrane by incubation at 65°C in 20 m*M* Tris-HCl–0.1 m*M* EDTA–1 *M* NaCl (pH 8.0) and precipitated with 95% ethanol. The pellet was washed twice in 80% ethanol (−20°C), and finally dissolved in water. The Cerenkov radiation of ^{32}P was monitored in the gel slice, on the NA-45 membrane and in the liquid phase. To facilitate demonstration of the resolution quality, some gels were fixed in 10% methanol–12% acetic acid (v/v) and dried prior to autoradiography.

RESULTS

Using automated solid-phase phosphoramidite chemistry an average coupling efficiency of 99.1% was obtained for the production of oligonucleotides of up to 143 nucleotides in length. However, even this technique results in the generation of shorter oligonucleotides outnumbering the desired oligonucleotide species and requiring an high resolution purification procedure able to keep pace with the speed of auto-

mated DNA synthesis. The most rapid procedure available today for the purification of oligonucleotides is RP-HPLC prior to removal of the 5'-DMT protecting group[7,12]. However, in RP-HPLC, increasing the length of the oligonucleotides decreases the influences of the hydrophobic DMT protecting group, thus resulting in shorter retention times. In addition, an increased formation of aggregates is noted. These influences severely impair the resolution of longer oligonucleotides from slightly shorter sequences when published linear gradients are used.

The base composition of the oligonucleotides employed in this study is listed in Table I. The sequences were free of internal "hairpin" structures. Initially, we applied previously established chromatographic conditions[3] to the purification and separa-

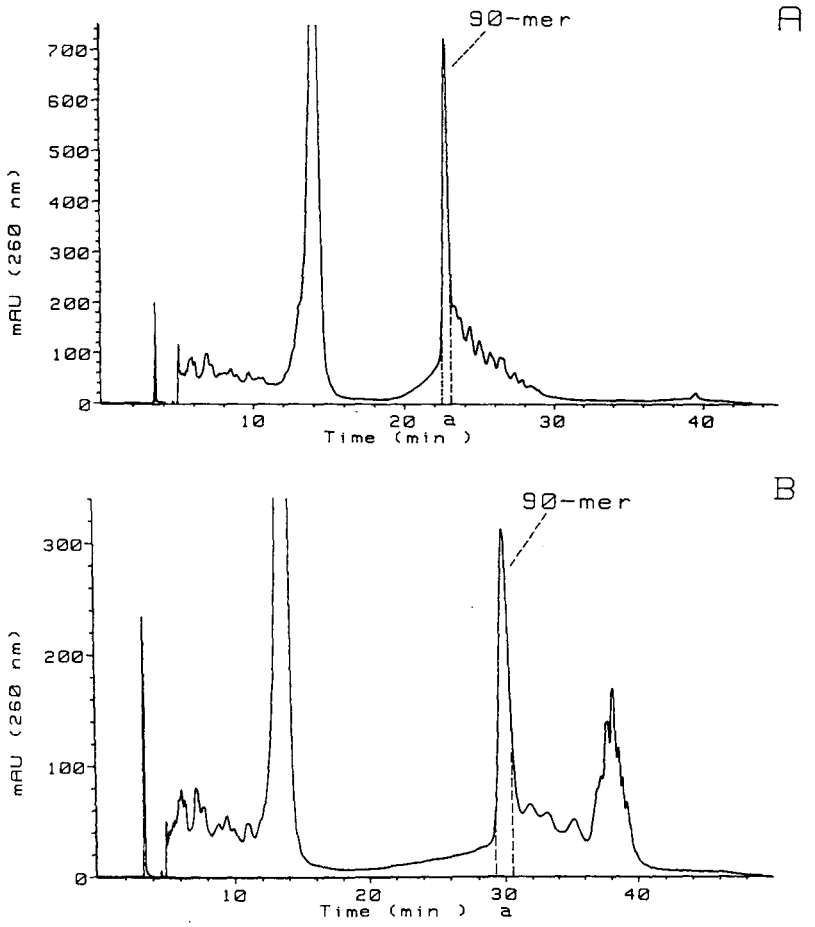

Fig. 1. Chromatogram of a 90-mer oligonucleotide (0.2-μmol synthesis) using (A) the linear gradient and (B) the five-step gradient as described in Experimental. Peaks eluted within 5 and 14 min consisted of deprotected oligomers and liberated protecting groups. Chromatogram (B) shows an extended separation of the 90-mer to approximately 85-mer range of DMT-protected oligonucleotides, thus allowing a more precise collection. The bulk of smaller oligomers was eluted between 37 and 39 min. Fraction a exclusively contained the 90-mer DMT product in PAGE.

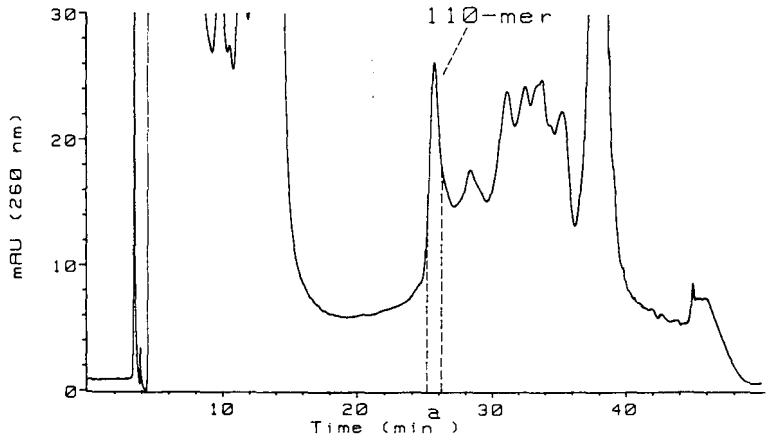

Fig. 2. Chromatogram of a 110-mer oligonucleotide (0.2-μmol synthesis) using a five-step gradient. Fraction a corresponds to lane a in Fig. 4.

tion of 88- and 90-mer oligonucleotides, but were unable to obtain a reasonable resolution. The separation improved when the column temperatures was raised to 70°C (Fig. 1A), and even further when the step gradient described above was applied (Fig. 1B). Oligonucleotides beyond 90 bases in length consistently required this five-step gradient for satisfactory results.

Typical chromatograms of synthetic 110- and 143-mer oligonucleotides (Figs. 2 and 3) demonstrate an elution profile allowing easy collection of the relevant fraction. In order to test the resolution quality, we subjected the contents of the fractions indicated to PAGE after detritylation and ^{32}P-end-labelling. In parallel, half the amount of crude extract applied to the column was detritylated and radiolabelled after ethanol precipitation. The first peak of tritylated oligonucleotides consisted

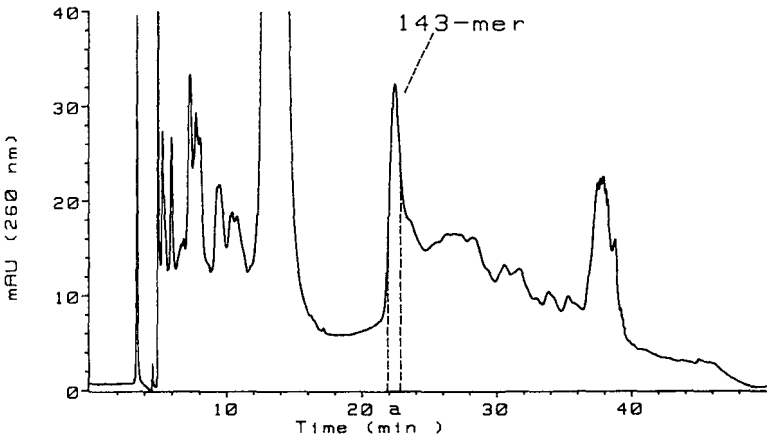

Fig. 3. Chromatogram of a 143-mer oligonucleotide (0.2-μmol synthesis) using a five-step gradient. Fraction a corresponds to lane a in Fig. 4.

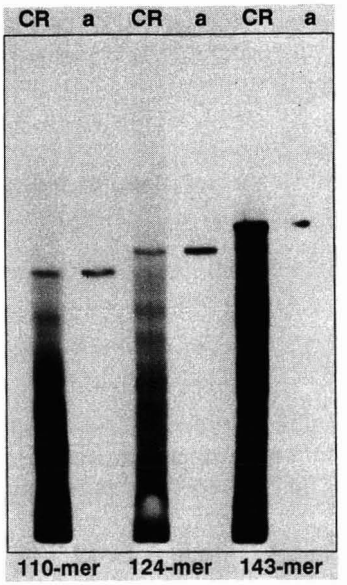

Fig. 4. Autoradiography following PAGE of ^{32}P-end-labelled RP-HPLC-purified oligonucleotides and the corresponding crude extracts of synthesis (CR). In case of the 110- and 143-mer products, the lanes correspond to the fractions indicated in Figs. 2 and 3; for the 124-mer (chromatogram not shown) lane a corresponds to the product peak fraction. In each case 0.5 $A_{260\ nm}$ units of crude extract were applied to the gel. Exposure time: 90 min.

entirely of the largest oligonucleotide species in comparison to the total synthesis mixture (Fig. 4). Used for gene synthesis and subsequent sequence determination, the 110-, 124- and 143-mer oligonucleotides conformed to the desired nucleotide sequence.

For estimating the yield of the chromatographic procedure for the 143-mer oligonucleotide, one $A_{260\ nm}$ unit was applied to the column. Subsequently, the mate-

TABLE II

OLIGONUCLEOTIDE PRODUCT YIELDS AFTER RP-HPLC AND PAGE PURIFICATION

	RP-HPLC	*PAGE*
Crude material		
amount applied ($A_{260\ nm}$)	1.17	1.17
Separated fraction/band		
cpm before electroelution	–	50 200
$A_{260\ nm}$	0.015[a]	0.006[a]
cpm after electroelution and reelectrophoresis	–	30 500[b]

[a] The yield for RP-HPLC is calculated as 0.6 μg, *i.e.*, 1.3% of the amount applied and for PAGE/electroelution 0.24 μg, *i.e.*, 0.5% of the amount applied.

[b] The loss of material during electroelution is 40% as determined by scintillation counting under identical conditions (material embedded in gel slices of similar dimensions).

rial from the collected peak was detritylated, end-labelled and subjected to PAGE. In parallel, 0.5 $A_{260\ nm}$ units of ^{32}P-labelled crude product were separated by PAGE and the material of the longest band was electroeluted and reelectropheresed. The results of this experiment are given in Table II and indicate for RP-HPLC a yield about 2.5 times higher than that of the PAGE/electroelution procedure.

DISCUSSION

The application of conventional C_{18}-functionalized RP-HPLC columns with modified conditions enables a rapid and efficient purification of oligonucleotides of up to 143 nucleotides in length. Both analytical and preparative chromatograms can be obtained in less than 90 min. The yield obtained with this procedure is considerably higher than that of PAGE with subsequent electroelution. Therefore, one single chromatographic experiment may yield material sufficient for most biological applications without tedious methods of sample preparation. Furthermore, up to 10 A_{260} units of products from automated DNA synthesis may be separated in a single step. RP-HPLC has the advantage over other HPLC procedures, that long time-consuming gradients and the use of anions can be avoided. We expect that separation of even longer oligonucleotides can be obtained in highly purified form with the procedure described (i) because there was still sufficient retention time between the early eluting bulk of by products and the 5'-DMT-protected oligonucleotides, and (ii) because of the very slow increase in the acetonitrile concentration in the relevant elution period.

ACKNOWLEDGEMENT

We thank the Bundesministerium für Forschung und Technologie for support in the form of a grant No. 01GA8501. This work is part of the Ph.D. thesis of M.H.

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Journal of Chromatography, 477 (1989) 427–433
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 627

Note

Development of a polymer-based reversed-phase high-performance liquid chromatographic stability indicating assay for U-78 608, an iron complexing monocarbam antibiotic

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U-78 608 belongs to a new class of β-lactam antibiotics known as the monocarbams (Fig. 1). The monoanionic form of 3-hydroxy-4(1H)-pyridinone, a moiety found in U-78 608, has been shown to form 1:1, 2:1 and 3:1 ligand–substrate complexes with Fe^{3+} (ref. 1). This candidate has shown remarkable activity toward *Pseudomonas*[2–4], presumably because of its ability to complex iron[5]. While the ability to chelate iron appears critical to the anti *Pseudomonas* activity of U-78 608, it also complicates the chromatographic analysis of the compound.

This report discusses the development of a reversed-phase high-performance liquid chromatographic (RP-HPLC) stability indicating assay for the separation of U-78 608 from impurities and degradation products. To avoid trace metal column effects commonly observed with silica-based stationary phases[6], a polymer-based column was used. The assay has been utilized to determine the pH/degradation rate profile of U-78 608.

EXPERIMENTAL

Chemicals

Ethylenediaminetetracetic acid (EDTA) disodium salt was purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). All other chemicals were reagent grade and water was deionized and double-distilled. U-78 608 was obtained from the Chemical Research Preparation Unit of Upjohn (Kalamazoo, MI, U.S.A.).

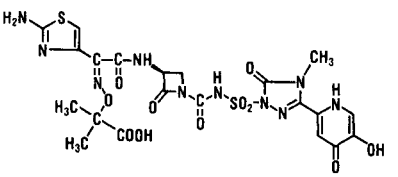

Fig. 1. Structure of U-78 608.

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UV absorbance

Ultraviolet scans of U-78 608 (free acid) in purified water and 0.01 *M* $(NH_4)_2HPO_4$ (pH 8.0)–methanol (80:20, v/v) from 190 to 400 nm were performed using a Perkin-Elmer (Norwalk, CT, U.S.A.) Lamda V UV–VIS spectrophotometer. Sample concentrations of approximately $3 \cdot 10^{-5}$ *M* were used, with the respective solvents as reference solutions.

Ionization constants

The dissociation constants for U-78 608 were determined potentiometrically using a Brinkman (Westbury, NY, U.S.A.) Metrohm 672 automatic titrator. The bulk drug was dried *in vacuo* (*ca.* 27 Torr) at room temperature over 24 h. The material was then weighed and diluted with 0.1 *M* sodium chloride to yield a final concentration of $3.7 \cdot 10^{-4}$ *M*. Duplicate samples were titrated at room temperature with standardized 0.01 *M* sodium hydroxide solution.

HPLC equipment and conditions

A DuPont (Wilmington, DE, U.S.A.) series 8800 quaternary gradient liquid chromatographic system, Perkin Elmer ISS-100 autosampler and IBM (Danbury, CT, U.S.A.) PC/XT with Nelson Analytical (Cupertino, CA, U.S.A.) series 2600 chromatography software were used in conjunction with a Hamilton (Reno, NV, U.S.A.) PRP-1®, 5-μm, 150 mm × 4.1 mm I.D. HPLC column and Brownlee (Santa Clara, CA, U.S.A.) PRP-GU®, 10-μm, 30 mm × 4.6 mm I.D. guard column. The injection volume was 40 μl with UV detection at 280 nm. All components were at room temperature.

Atomic absorption

HPLC fractions from the analysis of U-78 608 were collected and analyzed by atomic absorption for iron content. A Varian (Walnut Creek, CA, U.S.A.) AA-875 spectrophotometer (fitted with Fe hollow cathode lamp), GTA-95 graphite tube atomizer and PSD95 PGRMBL sample dispenser were used for the analysis.

Solution state stability study

Samples of U-78 608 were diluted with low ionic strength ($\mu = 0.01$) buffers[7] ranging from pH 2 through 10. The final concentration of U-78 608 was approximately 10^{-4} *M*. The solutions were stored in glass vials at 25.0 ± 0.1°C and periodically assayed. Rate constants were reported from HPLC stability data through roughly two half-lives. Confidence intervals reported for the rate constants were determined from the slopes of first-order kinetic plots.

RESULTS AND DISCUSSION

UV absorbance

Fig. 2 (curve A) shows UV maxima at 220, 250 and 280 nm for U-78 608 in water (pH 4.5). Ultraviolet detection at 280 nm was chosen for the HPLC analysis since this maximum was not shifted by pH adjustment to pH 8 and the addition of 20% methanol (curve B). Furthermore, the absorption of EDTA below 254 nm prohibited the use of a lower wavelength.

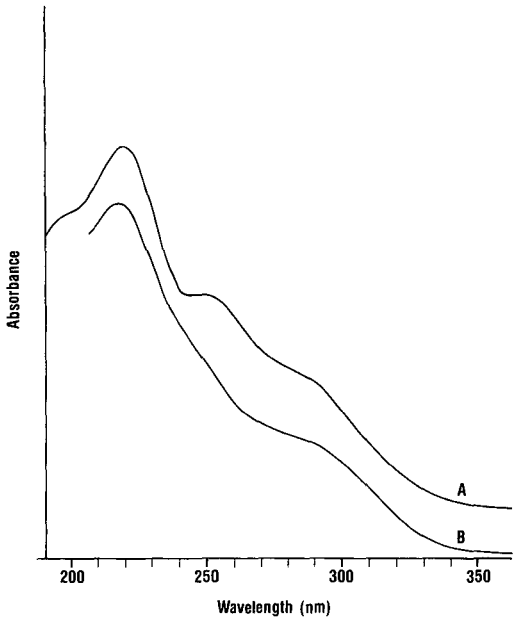

Fig. 2. Ultraviolet scans of U-78 608 in (A) water (pH 4.5) and (B) 20% methanol (pH 8.0).

Potentiometric titration

U-78 608 contains three functional groups capable of losing a proton (carboxylate, amide and hydroxyl). Potentiometric titrations were performed at an ionic strength (μ) of 0.1 to provide information about the species present as a function of pH. This information was useful in choosing the proper chromatographic approach. Fig. 3 displays a potentiometric titration curve for U-78 608. Two endpoints

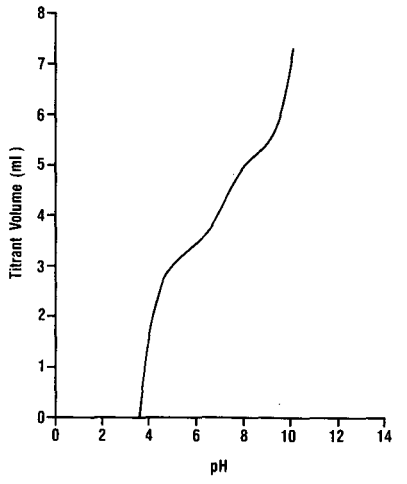

Fig. 3. Potentiometric titration curve of U-78 608 at an ionic strength of 0.1.

TABLE I

ACID DISSOCIATION CONSTANTS OF U-78 608

Univariate 95% confidence intervals in parentheses.

pK_a	*Thermodynamic*	*Apparent* (μ = 0.1 *M*)
1	2.72 (10.9%)	3.17 (3.79%)
2	5.11 (1.96%)	4.76 (1.89%)
3	8.81 (1.25%)	8.21 (1.34%)

were detected at roughly two and three equivalents of base. The apparent and thermodynamic dissociation constants of U-78 608 were determined by fitting the titration data using a least squares non-linear curve fitting program[8] (Table I). Based on these pK_a values, preliminary studies utilized ion-suppression and ion-pairing chromatography.

Early HPLC development

U-78 608 is considered to be a chelating agent because of the hydroxypyridinone moiety. Since conventional silica-based packings are known to contain residual amounts of metal ions[6], a polymeric reversed-phase packing was used throughout the development program. Initially, various mixtures of acetonitrile (10–30%) and pH 5.0 acetate buffer were tested as an isocratic mobile phase. At high organic concentration, U-78 608 eluted near the void volume with little resolution. At low organic composition, the peak was retained on the column (capacity factor, k' = 2.2), but the shape was extremely asymmetrical (tailing).

The peak tailing observed may have been due to either secondary equilibrium (*i.e.*, ionization) or metal complexation. The titration data (see Fig. 3) suggest that the compound was present primarily as the dianion at pH 5 in aqueous solutions. With the addition of organic modifier, the apparent pK_a values should be higher than the values measured in aqueous conditions. Thus, if the peak asymmetry noted above was due to secondary equilibrium, reversed-phase ion suppression would appear an appropriate method of chromatography for increasing retention time without tailing. Various mixtures of methanol (25–30%) and pH 3.0 citrate buffer were tested. The drug peak was adequately retained (k' = 2.0) although asymmetrical (tailing). A more acidic mobile phase may have reduced the tailing, however, it was likely that acid catalyzed hydrolysis of the β-lactam ring would have created a stability problem.

An alternate approach to reduce the tailing was ion-pair chromatography. Therefore, an isocratic mixture of tetrabutylammonium phosphate in phosphate buffer (pH 7–8) and methanol (10–30%) was tested. The drug peak either eluted near the void volume with insufficient resolution or when further retained was broad with tailing.

Competitive metal complexation

The failure of both ion-suppression and ion-pairing chromatography suggested that the peak broading might be due to metal complexation. HPLC systems which minimize mobile phase contact with stainless-steel are available, however, it was

decided to add EDTA to the mobile phase to compete for any polyvalent metal ions which may be causing the peak asymmetry. A binary mobile phase consisting of (A) 0.005 *M* EDTA and 0.01 *M* $(NH_4)_2HPO_4$ (pH 8.0), and (B) 100% methanol was used. A linear gradient from 100% A to A–B (70:30) over 20 min yielded a symmetrical peak for U-78 608 with a capacity factor of approximately 9. The column was reequilibrated in 10 min at 100% A. This assay was utilized for the remainder of the present study.

Assay linearity, precision and sensitivity

The assay was linear from 5 to 650 μg/ml U-78 608 with a correlation coefficient of 0.998. The precision (coefficient of variation) of six replicate injections of $1 \cdot 10^{-4}$ *M* solutions of U-78 608 was 1.2%. The chromatographic profiles analyzed from two column packing lots were found to be similar. The minimum detectable limit was approximately 0.5 μg/ml at 280 nm.

Interference studies

Stressed samples of U-78 608 in acidic, neutral and alkaline media, as well as aqueous mobile phase, were assayed over time to determine where any potential

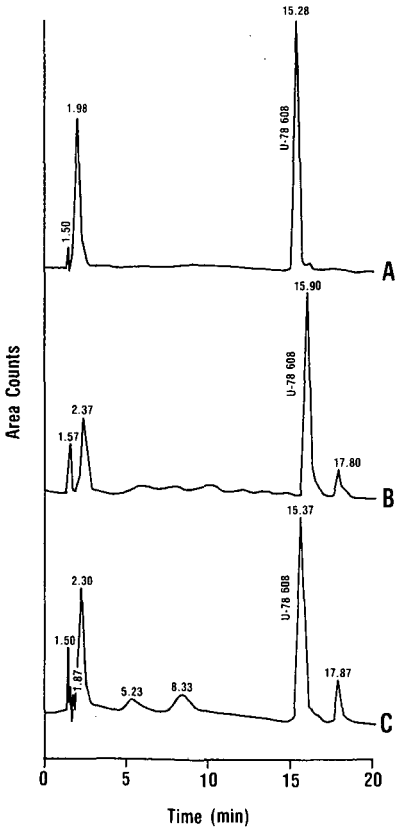

Fig. 4. Typical HPLC chromatograms of degraded U-78 608 under (A) neutral and alkaline conditions (pH 5–10), (B) acidic conditions (pH < 5), and (C) high temperature (> 45°C).

degradation products may elute, and to separate these if necessary from the parent compound. All degradation products appeared well resolved from the parent compound as shown in Fig. 4. After 5 h at pH 9 and 25°C, U-78 608 appeared to degrade to one major degradation product (Fig. 4A). Similar chromatograms were obtained for slightly acidic, neutral and alkaline samples (pH 5–10). Under acidic conditions (<pH 5), additional degradation products were observed (Fig. 4B, pH 3.0, 13 days). Elevated temperatures (>45°C) also resulted in the formation of multiple degradation products. Fig. 4C shows U-78 608 has degraded to multiple products after 48 h at pH 5 and 56°C.

Since base catalyzed hydrolysis of the β-lactam ring was suspected, the stability of samples prepared in aqueous mobile phase (pH 8.0) was of concern. A limited stability study of U-78 608 in the aqueous mobile phase at 25°C was conducted. The pseudo-first order rate constant (and 95% confidence interval) was calculated at 0.117 (10.55%) h^{-1} which corresponds to a t_{90} (time to 10% degradation) of approximately 1 h. Therefore, negligible on column degradation of U-78 608 was expected.

Atomic absorption

Since U-78 608 is suspected of being a strong metal binder, atomic absorption was utilized to determine if any of the observed peaks were due to iron–drug complexes. Sample fractions of the major peaks were found to contain no significant difference in iron concentration than the pure mobile phase. This suggests that U-78 608–iron complexes are not present in the assay.

pH degradation rate profile

The pseudo-first order rate constants (k) at 25.0°C for U-78 608 are plotted as a function of pH in Fig. 5. The maximum stability for U-78 608 in solution at 25°C was near pH 5 (indicated by the minimum in the curve). At pH 5.0, the rate constant with 95% confidence interval and t_{90} were calculated at 0.024 $days^{-1}$ (8.8%) and 4.3 days, respectively.

Above pH 7, the degradation appears to proceed primarily by specific base catalysis. The slope of log k as a function of pH from pH 7 to 10 was approximately

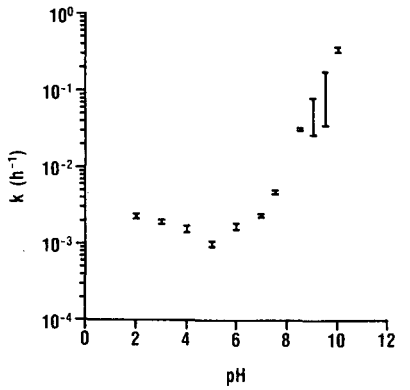

Fig. 5. Solution degradation rate profile for U-78 608 at 25°C and an ionic strength of 0.01 (error bars represent 95% confidence intervals).

0.7, compared to the anticipated slope of unity. Since U-78 608 has a dissociable proton with a pK_a value of 8.81 (see Table I), it appears that ionization of the compound in the pH 7–10 region introduces a small shoulder in the pH to stability curve resulting in an apparent slope of less than unity.

ACKNOWLEDGEMENT

The authors wish to acknowledge J. DeZwaan (Physical and Analytical Chemistry, The Upjohn Company) for his help with the atomic absorption measurement.

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Journal of Chromatography, 477 (1989) 434–438
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 640

Note

Application of analytical high-speed counter-current chromatography to the isolation of bioactive natural products

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Counter-current chromatography (CCC), based on the principle of liquid–liquid partition, has gained increasing popularity and a number of efficient systems have been developed for CCC performances[1,2]. Ito's planet centrifugal system represents one of the most convenient methods in fractionation of a variety of natural products, peptides and synthetic organic molecules[3–5]. The high resolving power of the system has been exemplified in the separation of antibiotics from actinomycete fermentation products[6,7]. Recently, further improvement of the system with a 0.85 mm I.D. multilayer coiled column and an operational speed of 2000 rpm has generated a highly efficient system. Its analytical capability has been demonstrated in the separation of plant alkaloids, plant indole hormones and herbicides[5,8,9]. In general, the observed resolution and speed are comparable to those of high-performance liquid chromatography (HPLC).

The study of bioactive principles from *Schisandra rubriflora* Rhed et Wils, a traditional Chinese herbal medicine for the treatment of chronic hepatitis, has led to the identification of nine lignans[10] (Fig. 1). Because of structural similarities among these bioactive lignans, the isolation of individual lignans for pharmacological evaluations has been a major challenge, particularly in the case of schisanhenol, which cannot be resolved from its acetate even with an analytical reversed-phase HPLC system (see Fig. 2).

In this paper, the analytical and semipreparative capabilities of the high-speed analytical CCC system are further demonstrated in the fractionation of bioactive schisanhenol and schisanhenol acetate. It is evident that analytical high-speed CCC provides a novel method complementary to HPLC for analytical applications.

EXPERIMENTAL

Apparatus

The apparatus employed is a newly developed analytical high-speed planet cen-

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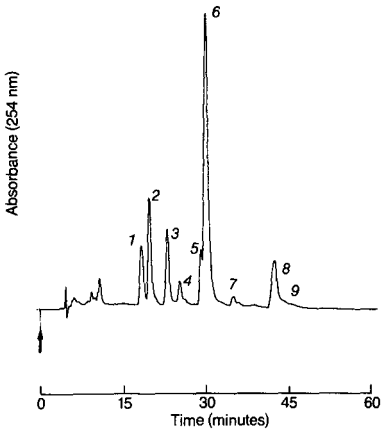

Fig. 1. HPLC trace of bioactive lignans from *Schisandra rubriflora* Rhed et Wils. Column: Zorbax-ODS, 250 × 4.6 mm I.D.; mobile phase: methanol–water (75:25); sample: ethanolic extract of *S. rubiflora* Rhed et Wils kernels; detection: UV absorbance at 254 nm. Peaks: 1 = wuweizisu C; 2 = (−)-rubschisandrin; 3 = rubschisantherin; 4 = deoxyschisandrin; 5 = schisanhenol acetate; 6 = schisanhenol; 7 = schisanhanol B; 8 = gomisin O; 9 = pregomisin.

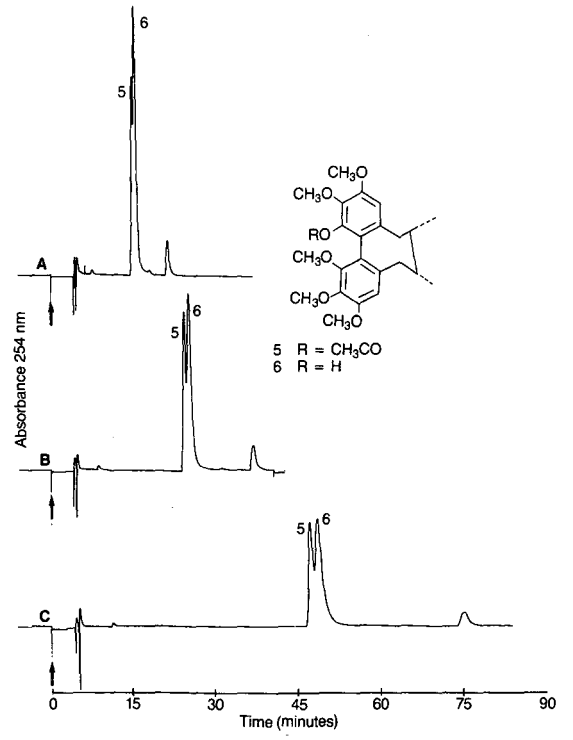

Fig. 2. HPLC traces of schisanhenol (6) and schisanhenol acetate (5). Column and detection as in Fig. 1; mobile phases: methanol–water (80:20) (A), (75:25) (B) and (70:30) (C).

trifuge with a 5-cm revolutional radius and a 0.85-mm-diameter multilayer coiled column. The details of this apparatus have been described in ref. 9. The holder shaft is equipped with a plastic planetary gear which is coupled to an identical stationary sun gear rigidly mounted on the central axis of the centrifuge. This gear coupling produces a desired synchronous planetary motion of the column holder. The holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity. As described elsewhere[4], this particular type of planetary motion permits the flow tubes to rotate around the central axis of the centrifuge without twisting, thus facilitating continuous elution of the mobile phase through the rotating column. The revolutional speed of the centrifuge is continuously adjustable up to 2000 rpm with a speed control unit.

The multilayer coiled column was prepared by winding a long piece of PTFE tubing (0.85 mm I.D.) onto the holder with a 5-cm hub diameter, making multiple coiled layers. The β value (the ratio between the helical radius, r, and the revolutional radius, R:$\beta = r/R$) varied from 0.5 at the internal terminal to 0.8 at the external terminal. The total column capacity of the multilayer coil measured approximately 39 ml.

The HPLC system consisted of a Model 6000A pump, a Model 46K injector and a Model 440 UV detector (all from Waters Assoc.). HPLC separations were performed on a 25 cm × 4.6 mm Zorbax-ODS column (DuPont).

Reagents and materials

Organic solvents used for preparation of the two-phase solvent systems, including *n*-hexane and ethanol, are glass-distilled chromatographic grade purchased from Burdick & Jackson Labs., Muskegon, MI, U.S.A. Experiments were performed with the two-phase system *n*-hexane–ethanol–water (6:5:5 v/v/v), which gives better peak resolution than the 6:5:1 solvent system. The two-phase solvent system was prepared by thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature followed by filtration through a 5-μm filter and degassing. The samples of *Schisandra rubriflora* Rhed et Wils were kindly provided by Professor Y. Y. Chen (Institute of Materia Medica, Chinese Academy of Medical Sciences). The ethanolic extract of the kernels of *S. rubriflora* was filtered and concentrated to provide the crude sample for HPLC analysis. The semipurified sample for CCC analysis containing mainly schisanhanol acetate and schisanhanol was obtained from preparative silica gel column chromatography.

Methods

The analytical CCC was performed with an analytical high-speed planet centrifuge system equipped with a multilayer coil column of 0.85 mm I.D. and at a revolutional speed of 1500 rpm.

In each separation the multilayer coiled column was first filled with the upper phase solvent as the stationary phase. The mobile phase solvent (lower phase) was then pumped into the column head inlet while the apparatus was run at a revolutional speed of 1500 rpm. After equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (0.15 mg in 0.2 ml of lower mobile phase) was injected through the head inlet. The effluent from the tail outlet of the column was continuously monitored with a UV detector at 254 nm and fractionated into test tubes with an LKB fraction collector.

RESULTS AND DISCUSSION

The capabilities of high-speed analytical CCC have been successfully demonstrated in the separation of plant alkaloids, plant indole hormones and herbicides[9]. A recent study of bioactive lignans from *S. rubriflora* Rhed et Wils has identified nine lignans[10] (Fig. 1). These bioactive lignans are similar in structure, thus it is difficult to obtain individual lignans in pure form for pharmacological evaluations. As shown in Fig. 2, the schisanhenol and its acetate could not be totally resolved even with a reversed-phase analytical HPLC system (12 000 plates) using several solvent systems; *i.e.*, methanol–water (80:20), (75:25) or (70:30). It is postulated that the hydroxyl group in schisanhenol is ideally situated for intramolecular hydrogen bonding with a neighboring methoxy group and that therefore the resulting hydrogen-bonding complex behaves similarly to its acetate in terms of adsorption and desorption process while passing through the reversed-phase HPLC column. This is also supported by the observed reverse sequence of elution in which the acetate eluted first followed by schisanhenol under reversed-phase HPLC conditions.

On the other hand, the application of analytical CCC which eliminates the complications arising from solid supports and based on the partition coefficients of the two molecules has resulted in a baseline separation of schisanhenol and its acetate (Fig. 3). The semipreparative capability of the analytical CCC system is also evidenced in the sample size which is 25 times higher than that generally used in HPLC.

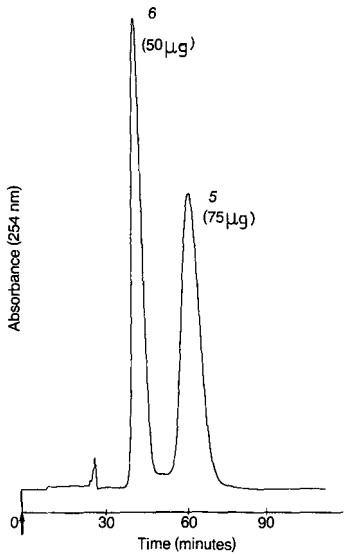

Fig. 3. High-speed analytical counter-current chromatographic separation of schisanhenol (6) and schisanhenol acetate (5). Solvent system: *n*-hexane–ethanol–water (6:5:5), mobile phase: lower phase, volume retention ratio: upper phase:lower phase = 19 ml:20 ml, flow-rate: 0.8 ml/min; column pressure: 165 p.s.i.; detection: UV absorbance at 254 nm.

CONCLUSIONS

The complete resolution of the bioactive lignans schisanhenol and schisanhenol acetate demonstrates that high-speed analytical CCC can be a complementary method to HPLC for analytical and semipreparative applications. The major advantages are (1) high resolution and speed, (2) avoidance of complications arising from solid adsorbance, (3) total recovery of bioactivity and sample, (4) inexpensive operation, and (5) analytical and semipreparative applications.

ACKNOWLEDGEMENT

We express our appreciation to Dr. Robert Bowman of National Heart, Lung and Blood Institute for the loan of some equipment.

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Journal of Chromatography, 477 (1989) 439–447
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 642

Note

Reversed-phase high-performance liquid chromatographic procedure for the determination of maysin in corn silks

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The resistance of certain corn (*Zea mays* L.) genotypes to the corn earworm, *Heliothis zea* (Boddie), has been associated with the toxicity of their cornsilks[1–3]. Elliger *et al.*[4] showed that the activity of "Zapalote Chico" cornsilks could be attributed to one major flavonoid called maysin identified as 2″-*O*-α-L-rhamnosyl-6-*C*-6-deoxy-*xylo*-hexos-4-ulosyl)luteolin (Fig. 1). Waiss *et al.*[5] and Wiseman and co-workers[6,7] demonstrated that the activity of maysin and of Zapalote Chico cornsilks was true antibiosis. Waiss *et al.*[5] developed a spectrophotometric method for the measurement of maysin in silk extracts. Widstrom *et al.* used this method of maysin analysis to determine genetic variability in maysin contents of various maize populations[8] and maize grown in diverse environments[9]. However, considerable variation in maysin content was found in these studies. Recently, Wiseman *et al.*[10] reported extremely variable levels of maysin from year to year in resistant corn lines subjected to laboratory feeding bioassay studies with corn earworm larvae. No correlation between maysin concentration and larval weights was apparent among lines with varying levels of maysin.

It appeared that the discrepancy was due to the maysin analysis, as it was initially recognized that only half the absorbance intensity at 352 nm for corn silk extracts was actually due to maysin[5]. Consequently, it was essential to develop a more specific chromatographic method for maysin in corn silks. As maysin is not commercially available, it was necessary to develop an isolation procedure to obtain (from Zapolote Chico silks) a pure maysin standard.

EXPERIMENTAL[a]

Materials

Solvents were Burdick & Jackson (Muskegon, MI, U.S.A.) "distilled-in-glass" grade. Chrysin (5,7-dihydroxyflavone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was recrystallized from methanol.

High-performance liquid chromatography (HPLC)

Reversed-phase HPLC was performed with a Hewlett-Packard 1084B liquid chromatograph, using an Altex Ultrasphere ODS column, 25 cm × 4.6 mm I.D. The solvent gradient was: linear from methanol–water (20:80) (solvent A) to methanol (solvent B) in 40 min, with a flow-rate of 1 ml/min and a 12-min recycle time. Both solvents contained 0.1% orthophosphoric acid. The column effluent was monitored at 340 nm (reference wavelength, 550 nm).

Sample collection and preparation

To obtain a representative sample, silks (one silk bundle from an individual ear) from five different plants were combined, weighed and placed in amber wide-mouth jars. The silks were covered with 500 ml methanol and the jars were sealed with PTFE-lined caps and kept frozen until analysis. A second five-silk composite was obtained for determination of moisture content of the silks, which were dried at 100°C to constant weight. The silk–methanol sample was warmed to room temperature and 20 ml of a solution of the internal standard, chrysin (17 mg/20 ml methanol), were added. After ultrasonication of the sample for 30 min, an aliquot was filtered through a Nylon 66 membrane filter (0.45 μm, Micron Separations, Westboro, MA, U.S.A.) into an autoinjector vial and a 20-μl aliquot was analyzed by HPLC.

Isolation of maysin

Solvent extraction. Maysin was isolated from Zapalote Chico [selection ZC 2451 No. (P)(C3)] grown at the facilities of the USDA-ARS Southern Grain Insects Lab., Tifton, GA, U.S.A. Approximately 3230 g of fresh Zapalote Chico silks (representing about 910 silks) were blended with 10 l of methanol in a Waring Blender and stored at 0°C, prior to transport to our laboratory. After warming to room temperature, the silk–methanol mixture was ultrasonicated for 30 min and then filtered through filter paper. The resulting methanol extract, now also containing the water from the fresh silks, was concentrated on a rotary evaporator to a final volume of approximately 1650 ml. The resulting aqueous solution was then extracted with methylene chloride (3 × 500 ml) to remove lipids. The remaining solution was divided into three equal parts for ease in handling. Each portion was extracted with ethyl acetate (3 × 500 ml) and the ethyl acetate extracts were pooled. The residual aqueous solutions were then mixed with equal volumes of acetonitrile, which gave a totally miscible mixture. The solutions were stored at 0°C overnight to effect separation of the

[a] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

water and acetonitrile layers. The acetonitrile layers were decanted, pooled and added to the previous ethyl acetate extracts. The combined ethyl acetate and acetonitrile extracts were concentrated on a rotary evaporator to give 25.68 g of a dark reddish, syrupy residue.

Silicic acid chromatography. This residue was dissolved in 400 ml of methanol–ethyl acetate–acetonitrile–isooctane (1:1:1:1) and mixed with 60 g of silicic acid (Mallinckrodt, 100 mesh, washed with methanol, dried at 150°C for 16 h). After evaporation of the solvent from the sample–silicic acid mixture, the coated silicic acid was slurried with methylene chloride and placed on top of a 300-g silicic acid column (4 × 70 cm), packed in methylene chloride. The column was eluted with the following solvents (%, v/v): 2 l methylene chloride, 1.5 l ethyl acetate–methylene chloride (25:75), 1 l ethyl acetate–methylene chloride (50:50), 1 l ethyl acetate–methylene chloride (75:25), 1 l ethyl acetate, 5.5 l acetone–ethyl acetate (10:90), 4.5 l acetone–ethyl acetate (20:80), 2 l acetone–ethyl acetate (40:60), 4 l acetone–ethyl acetate (50:50), 5 l acetone, 1 l acetone–methanol (50:50), 1 l methanol. The 10% and 20% acetone in ethyl acetate fractions were combined and on evaporation yielded 8.26 g of material that was approximately 80% pure maysin. The first 1.5 l of acetone–ethyl acetate (10:90) from the silicic acid column contained 89% pure maysin in 3.77 g of material.

Reversed-phase chromatography. The silicic acid–maysin fraction was then purified by C_{18} reversed-phase chromatography. The packing material from a Waters (Milford, MA, U.S.A.) PrepPak 500 C_{18} reversed-phase cartridge was repacked into a smaller glass Cheminert LC column (54 × 2.54 cm) (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The smaller diameter column afforded better resolution of the maysin and conserved solvents. The column was washed with methanol and then recycled to water as the initial solvent. Samples of 1 g of the silicic acid–maysin fraction were dissolved in 3 ml of methanol–water (25:75) and applied to the column with a 4-ml loop injection valve. The column was operated at a flow-rate of 2 ml/min. Gradient elution, utilizing two Altex Model 110 pumps and Model 420 programmer (Beckman Instruments, Altex Division, San Ramon, CA, U.S.A.), was employed. The column effluent was monitored at 340 nm (Altex Model 153 UV detector), and 8-ml fractions were collected (Mini-Fractionator, Gilson Medical Electronics, Middleton, WI, U.S.A.). Two different solvent programs were tested.

The first solvent program used a linear gradient: 100% water to 100% methanol over 24 h. A 1-g sample of the silicic acid–maysin yielded 372 mg of maysin of >98% purity and 220 mg of maysin of >95–98% purity.

The second solvent program utilized a linear gradient of methanol–water (50:50) to methanol–water (75:25) over 400 min, then a linear gradient of methanol–water (75:25) to 100% methanol over 200 min. A 1-g amount of the silicic acid–maysin fraction gave 700 mg of >80% maysin which, when rechromatographed under the same conditions, gave 600 mg of >95% pure maysin.

The isolated maysin was recrystallized from acetonitrile–methanol (approx. 1:1, v/v). It gave a decomposition point of 200–210°C (lit.[4], decomposition point of 225°C, crystallized from acetone–methanol). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Model AM spectrometer (250 MHz) in [$^{2}H_6$]dimethyl sulfoxide at ambient temperature.

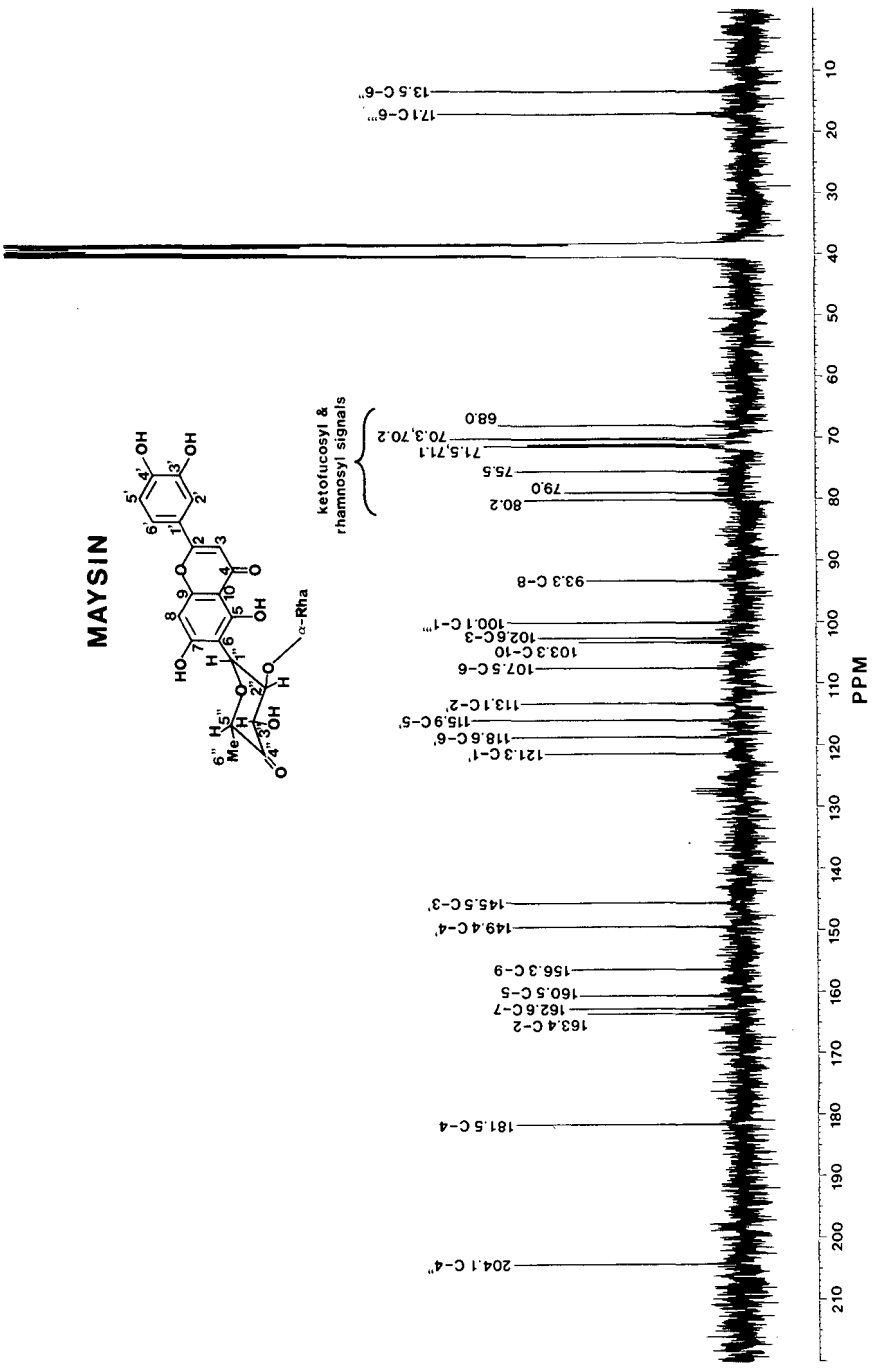

Fig. 1. ^{13}C NMR spectrum of isolated maysin ([$^2H_6$]dimethyl sulfoxide).

RESULTS AND DISCUSSION

Our initial screenings of methanol extracts of corn silks demonstrated the utility of reversed-phase HPLC for analyses of the flavonoids of silk. However, for quantitative analysis, we required a pure sample of maysin. Accordingly, pure maysin was isolated from silks of Zapalote Chico by a combination of solvent partitioning, silicic acid column chromatography, and preparative reversed-phase chromatography. The isolated maysin was shown to be pure by HPLC and its ^{1}H NMR spectrum matched that in the literature[4].

The ^{13}C NMR spectrum of isolated maysin is given in Fig. 1, while chemical shift assignments of maysin and related flavonols are given in Table I. Assignments were made by comparison with those of 6-*C*-glucosylluteolin and 2″-*O*-rhamnosyl-8-*C*-glucosylapigenin (2″-*O*-rhamnosylvitexin)[11]. These agreed with ^{13}C spectra of maysin assignments provided by Elliger[12]. The ^{13}C chemical shift at 100.1 ppm in the maysin spectrum (Fig. 1) was assigned to the C-1 carbon of rhamnose based on analogous chemical shifts of rhamnose in 2″-*O*-rhamnosylvitexin (Table I).

The commercially available aglycone, chrysin (5,7-dihydroxyflavone), was found to be an acceptable internal standard. Methanol was used to dilute concentrated silk samples to retain chrysin in solution. An HPLC chromatogram of the separation of chlorogenic acid, maysin and chrysin is given in Fig. 2. Excellent peak shapes were obtained with the Altex Ultrasphere ODS column and a linear gradient from methanol–water (20:80) to 100% methanol. In our system (HP 1084, UV monitor set at 340 nm), the response factor of maysin relative to chrysin was found to be 1.10. Although other HPLC UV detectors may give slightly different responses, HPLC quantitation of maysin, assuming a unitary UV response equal to chrysin, should be an acceptable method.

Representative HPLC chromatograms of high-, medium- and low-maysin-containing corn silks are given in Fig. 3. Adequate separation of maysin from other flavonoids was obtained. As some variability in silk maysin levels was found among individual plants of a variety, it was decided to produce a representative sample by combining five silk bundles for analysis. Quantitative analyses of corn silks of varying maysin content (Table II) showed quite a large variation in maysin content among the various entries. Zapalote Chico, as expected, contained the highest level of maysin in its silks. Annual teosinte, (*Zea mays* L., ssp. *mexicana*) an insect-resistant primitive corn, also contained substantial quantities of maysin. However, a number of entries, that had been selected for corn earworm resistance in field trials, were found to contain very low levels of maysin. Thus, entries 3 and 14 were resistant to corn earworm attack, but were found to have widely different levels of maysin. Therefore, the resistance of entries 10–15 and 17 (Table II) on field plants may involve other chemical or physical factors, as opposed to the true antibiosis exhibited by Zapalote Chico (entry 1).

Maysin analyses data by the previously reported spectrophotometric method (UV analysis) did not correlate well with observed corn earworm resistance. Maysin levels by the reported HPLC method were often found to be contrary to the previous UV method values. However, the HPLC values correlated much more closely with the observed corn earworm resistance. It is thought that interfering compounds in the UV method resulted in erroneously high maysin levels of certain genotypes. For

TABLE I

^{13}C NMR CHEMICAL SHIFT ASSIGNMENTS OF MAYSIN AND RELATED FLAVONOLS ([$^2H_6$]DIMETHYL SULFOXIDE)

Carbon assignment[a]	*Maysin*	*Isoorientin*[b] *(6-C-glucosylluteolin)*	*2″-O-Rhamnosyl-8-C-glucosyl-apigenin*[b]
C-4″	204.1		
C-4	181.5	181.7	181.9
C-2	163.4	163.5	163.9
C-7	162.6	163.0	162.1
C-5	160.5	160.5	160.5
C-9	156.3	156.0	155.7
C-4′	149.4	149.5	161.0
C-3′	145.5	145.6	115.8 (C-3′,5′)
C-1′	121.3	121.3	121.5
C-6′	118.6	118.8	128.9 (C-2′,6′)
C-5′	115.9	115.9	
C-2′	113.1	113.2	
C-6	107.5	108.7	98.2
C-10	103.3	103.3	104.1
C-3	102.6	102.7	102.3
C-1‴	100.1		100.2
C-8	93.3	93.4	104.3
C*-5″	80.2	81.3	81.7
C*-3″	79.0	78.8	79.8
C*-2″	75.5	70.4	75.0
C*-1″,3‴	{ 71.5 71.1	72.9 (C-1″)	71.4 (C-1″,3‴)
C*-2‴,4‴	{ 70.3 70.2	70.1 (C-4″)	70.3 (C-4″,2‴,4‴)
C-5‴	68.0		68.1
C-6‴	17.1		17.6
C-6″	13.5	61.3	

[a] See Fig. 1; * indicate tentative assignments.

[b] From ref. 11.

example, Coe G12, apparently high in UV-interfering compounds, gave UV maysin values more than twice as large as Zapalote Chico and about fourteen times that of Stowell's Evergreen. The HPLC values (Table II) indicate that Zapalote Chico has about three times the maysin content of Stowell's Evergreen and Coe G12 which are similar in maysin content. However, relative maysin contents of Zapalote Chico and Stowell's Evergreen (genotypes with negligible UV-interfering compounds) by UV analysis indicate that the maysin content of Zapalote Chico was 2.5 to 6 times larger than for Stowell's Evergreen. The values for HPLC in Table II indicated 3.4 times as much maysin in Zapalote Chico silks as those for Stowell's Evergreen, a value not drastically different from the UV analyses.

The HPLC-determined maysin values are, therefore, expected to relate much better to the antibiosis type of resistance than the previously used UV-absorbance values. The primary reason for this expectation is that problems encountered with silks having other compounds, that interfere with the UV absorbance of maysin, have

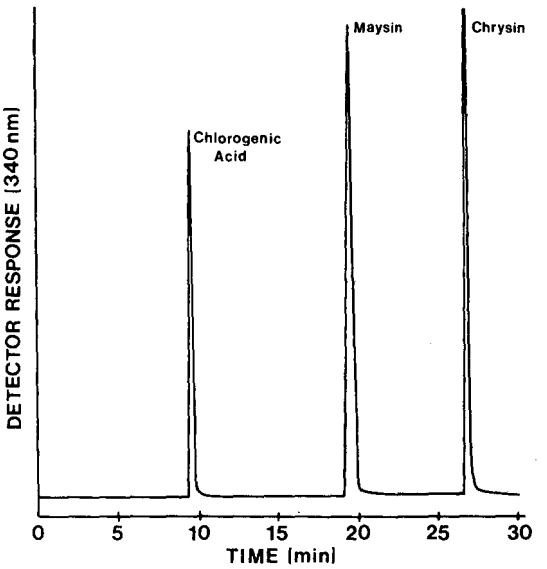

Fig. 2. HPLC of standards: chlorogenic acid, maysin and chrysin (internal standard).

TABLE II

MAYSIN CONTENT OF THE SILKS OF SEVERAL CORN ENTRIES AS DETERMINED BY HPLC ANALYSIS

Corn entry	*Maysin level (% of silks)*[a]	*Relative maysin content*
1 Zapalote Chico 2451	6.30	100.0
2 Annual teosinte	5.49	87.1
3 MAS	3.72	59.0
4 RFC-RI(C7)	3.66	58.1
5 SGP-M10	2.15	34.1
6 Coe G12	2.14	34.0
7 CC-M10	2.05	32.5
8 GT115	1.94	30.8
9 Stowell's Evergreen	1.84	29.2
10 10LDD Sel Rec	1.13	17.9
11 SwtCD Sel Rec-RM1	0.20	3.2
12 RFC-RMI-D1 Sel	0.17	2.7
13 GT119	0.15	2.4
14 DARS	0.10	1.6
15 DDSB	0.08	1.3
16 Coe G10	0.03	0.4
17 DDSA	0.01	0.2

[a] Percent dry weight.

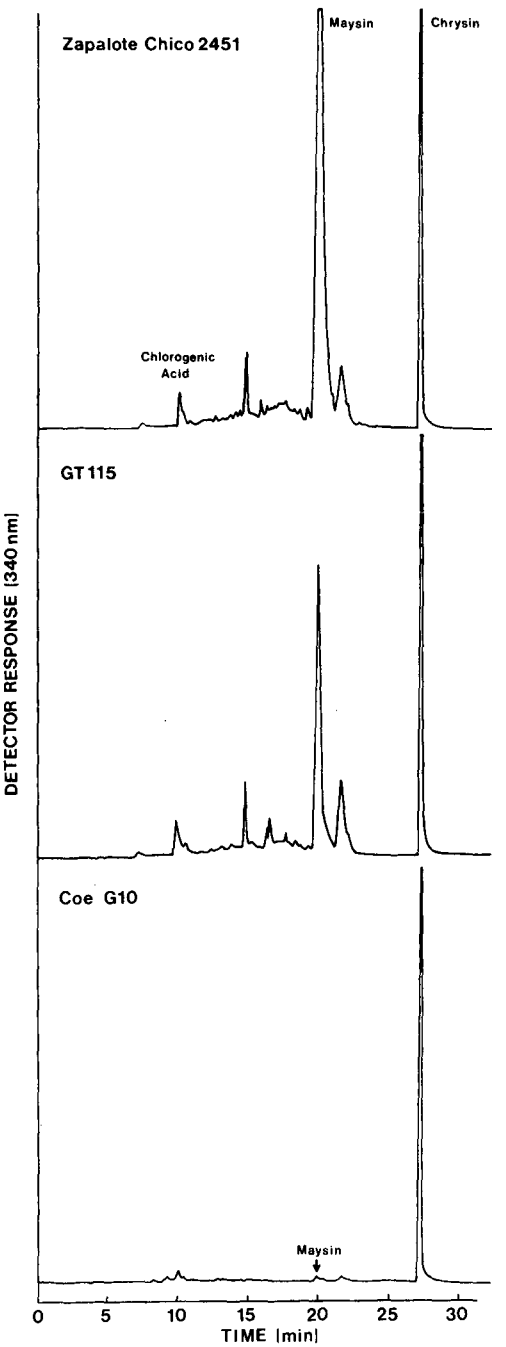

Fig. 3. HPLC of selected corn silk extracts.

been eliminated. Since the corn entry Stowell's Evergreen (Table II) is not considered a very high corn earworm-resistant line, it appears that in order to impart corn earworm resistance due to maysin content, silk levels of maysin above 2% dry weight may be needed.

ACKNOWLEDGEMENT

We would like to thank Dr. Carl A. Elliger for copies of the ^{1}H and ^{13}C NMR spectra of maysin and derhamnosylmaysin.

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Journal of Chromatography, 477 (1989) 448–453
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 563

Note

Application of fast protein liquid chromatography for the isolation of vertebrate casein kinase-1

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(First received January 19th, 1989; revised manuscript received April 12th, 1989)

The purification of casein kinase-1 from various sources has received considerable attention during the last decade due to the pleiotropic effect of this enzyme on metabolic processes[1,2]. Traditional methods of isolation include several chromatographic steps[3–5], which made the whole procedure laborious and time-consuming and reduced either the yield of the enzyme or its activity. This communication presents a simplified method for the isolation of casein kinase-1 from animal sources which uses the advantages of high-performance liquid chromatography (HPLC) of proteins.

EXPERIMENTAL

Apparatus

A fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) was used, consisting of two P-500 pumps, an MV-7 injection valve with 10- and 50-ml Superloops for sample injection, an LCC-500 chromatography controller, an UV-1 monitor with an HR-10 flow cell (optical path 10 mm), equipped for detection at 280 nm, a FRAC-100 fraction collector and REC-482 dual pen recorder. Pre-packed chromatography columns of Mono Q HR 5/5 (5 cm × 5 mm I.D.) and Mono S HR 5/5 (Pharmacia) were used for HPLC.

Reagents

Triethanolamine hydrochloride (TEA–HCl), phenylmethylsulphonyl fluoride (PMSF) and sodium dodecyl sulphate (SDS) were obtained from Serva, [γ-^{32}P]ATP from Isotop (Tashkent, U.S.S.R.), casein from Calbiochem, 2-mercaptoethanol and Coomassie Blue R-250 from Loba-Chemie; other salts and reagents were of analytical purity.

Protein in crude extracts and chromatographic fractions was assayed by the method of Bradford[6].

Enzyme test

Casein kinase activity was assayed in 20-μl aliquots of each fraction by the transfer of radiolabelled phosphate from ATP to dephosphorylated casein in 0.1 ml of a standard incubation mixture containing 20 m*M* TEA–HCl, pH 7.8, 100 m*M* sodium

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chloride, 5 m*M* magnesium chloride, 6 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.1 m*M* PMSF, 10% glycerol, 0.1 m*M* [γ-^{32}P]ATP (specific activity 1000 cpm/pmol), 60 μg of casein and enzyme. Reactions were initiated by the addition of enzyme and incubated for 30 min at 34°C. Measurement of ^{32}P incorporation is described in refs. 3 and 4. One unit of enzyme activity is defined as the amount of kinase that catalyzes the transfer of 1 pmol of phosphate from ATP to casein in 1 min under the conditions described above.

Isolation of enzyme

Fresh rabbit liver and ripening oocytes of *Rana temporaria* frog were quick-frozen and stored in liquid nitrogen. Samples of 10 g were homogenized in 100 ml of buffer A containing 20 m*M* TEA–HCl, pH 7.8, 100 m*M* sodium chloride, 5 m*M* magnesium chloride, 6 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.5 m*M* PMSF, 10% glycerol in a rotary blendor for 5 min at 1000 rpm and additionally in a Potter Elvehjem tissue grinder (20 strokes). Cell debris was removed by centrifugation at 15000 *g* for 20 min. To the crude extracts obtained, finely powdered ammonium sulphate was slowly added to 45% saturation at 4°C with constant stirring. After 1 h the precipitates formed were collected by centrifugation at 20000 *g* for 1 h and dissolved in sodium chloride-free buffer A so that the final ammonium sulphate concentration was 0.2 *M*. This solution was then centrifuged at 150000 *g* for 1 h and the precipitate was removed. The supernatants were diluted by addition of an equal volume of buffer A without sodium chloride and each was batch-adsorbed to 20 ml of phosphocellulose P_{11} equilibrated in buffer A. After 1 h the resin was collected by filtration, washed four times with 50-ml aliquots of buffer A, transferred to a column (20 × 1.5 cm) and eluted with a 100-ml linear gradient of 0.1–1.2 *M* sodium chloride in buffer A. Casein kinase-1 from both sources was eluted at 0.44–0.5 *M* sodium chloride in a volume of 20 ml. The casein kinase-I peak from phosphocellulose was collected, diluted to 0.05 *M* sodium chloride and was applied to a Mono Q HR 5/5 column, equilibrated with buffer A containing 0.05 *M* sodium chloride at a flow-rate of 1 ml/min. The enzyme was eluted as an individual peak in a volume of 5 ml by a linear gradient of sodium chloride (0.05–0.6 *M*) in buffer A at a flow-rate of 1 ml/min. The casein kinase-1 peak from Mono Q was collected and applied to a Mono S HR 5/5 column. The enzyme was eluted as an individual peak in a volume of 1–2 ml by a linear gradient of 0.1–0.6 *M* sodium chloride at the same flow-rate. The casein kinase-1 peak from Mono S was collected, diluted to 0.2 *M* sodium chloride and applied to a 0.5-ml column of Blue-Sepharose (Pharmacia), equilibrated with buffer A at a flow-rate of 3 ml/h. The column was washed with 2 ml of buffer A containing 0.2 *M* sodium chloride and the enzyme was eluted in a volume of 1 ml by buffer A containing 1.5 *M* sodium chloride and 10 m*M* ATP, then glycerol was added to 50% and the enzyme stored at −18°C without any loss of activity for at least 4 months.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[7] in 10% gels with a Pharmacia GE-4 electrophoresis apparatus and an EPS 400/500 power supply. Gels were stained with 0.05% Coomassie Blue R-250 in 50% trichloroacetic acid.

RESULTS AND DISCUSSION

The first stage of the enzyme purification from extracts is a modification of the procedure developed for the isolation of casein kinase-1 from calf thymus[4]. Enzyme is recovered by batch adsorption to phosphocellulose from ribosome-free supernatants and is separated from casein kinase-2 by gradient elution (Fig. 1).

Further purification of casein kinase-1 was achieved by chromatography on Mono Q and Mono S. FPLC on Mono Beads ion exchangers provides the major purification steps in the procedure described (Table I). The enzyme binds quantitatively to the Mono Q at *ca.* 0.05 *M* sodium chloride and is eluted at *ca.* 0.12 *M* sodium chloride (Fig. 2a,b). The peak of casein kinase activity which elutes at *ca.* 0.38 *M* sodium chloride reflects the presence of trace amounts of casein kinase-2 in the preparation. The next step in the purification is chromatography on Mono S. Casein kinase activity is eluted as a single narrow peak at *ca.* 0.28 *M* sodium chloride coincident with the protein peak (Fig. 2c and d).

The final stage of the procedure is chromatography on Blue-Sepharose which serves to concentrate and to purify the enzyme (Table I). Both an high salt concentration and ATP are required to elute casein kinase-1 from resin.

As is seen from the present data, the chromatographic behaviour of enzymes from both sources on ion exchangers used is almost identical. The final preparations contain casein kinase-1 with a specific activity of 570–650 units/μg which is homogeneous according to SDS-PAGE (Fig. 3). The M_r values of the enzymes from oocytes and liver are 34 000 and 37 000 respectively. The total yield of the enzyme from the both sources is about 20% (Table I).

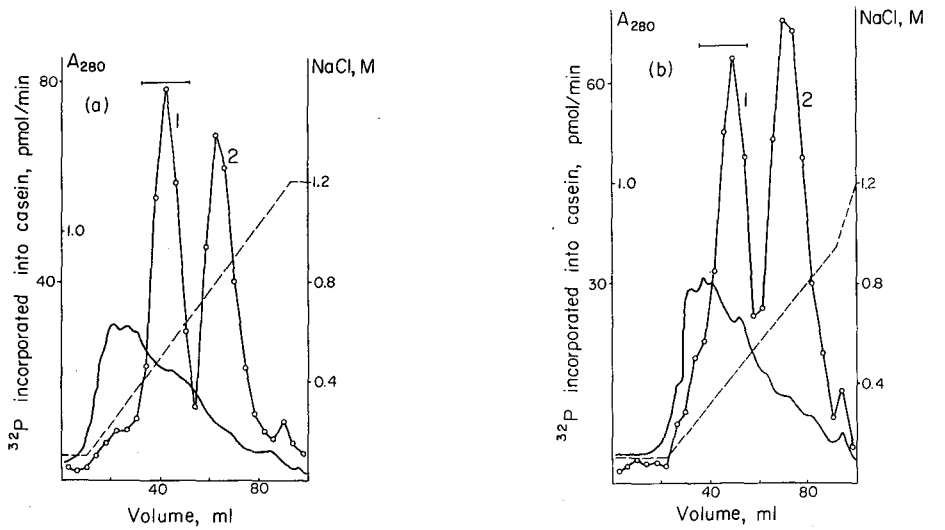

Fig. 1. Chromatography of ribosome-free extracts from rabbit liver (a) and frog oocytes (b) on phosphocellulose columns. The fractions were assayed for casein kinase activity as described in Experimental (○—○). Absorbance was monitored at 280 nm, 2.0 absorbance units full scale (a.u.f.s.). The sodium chloride concentration is indicated by the dashed line. Pooled fractions are indicated by the bar. Peaks: 1 = casein kinase-1; 2 = casein kinase-2.

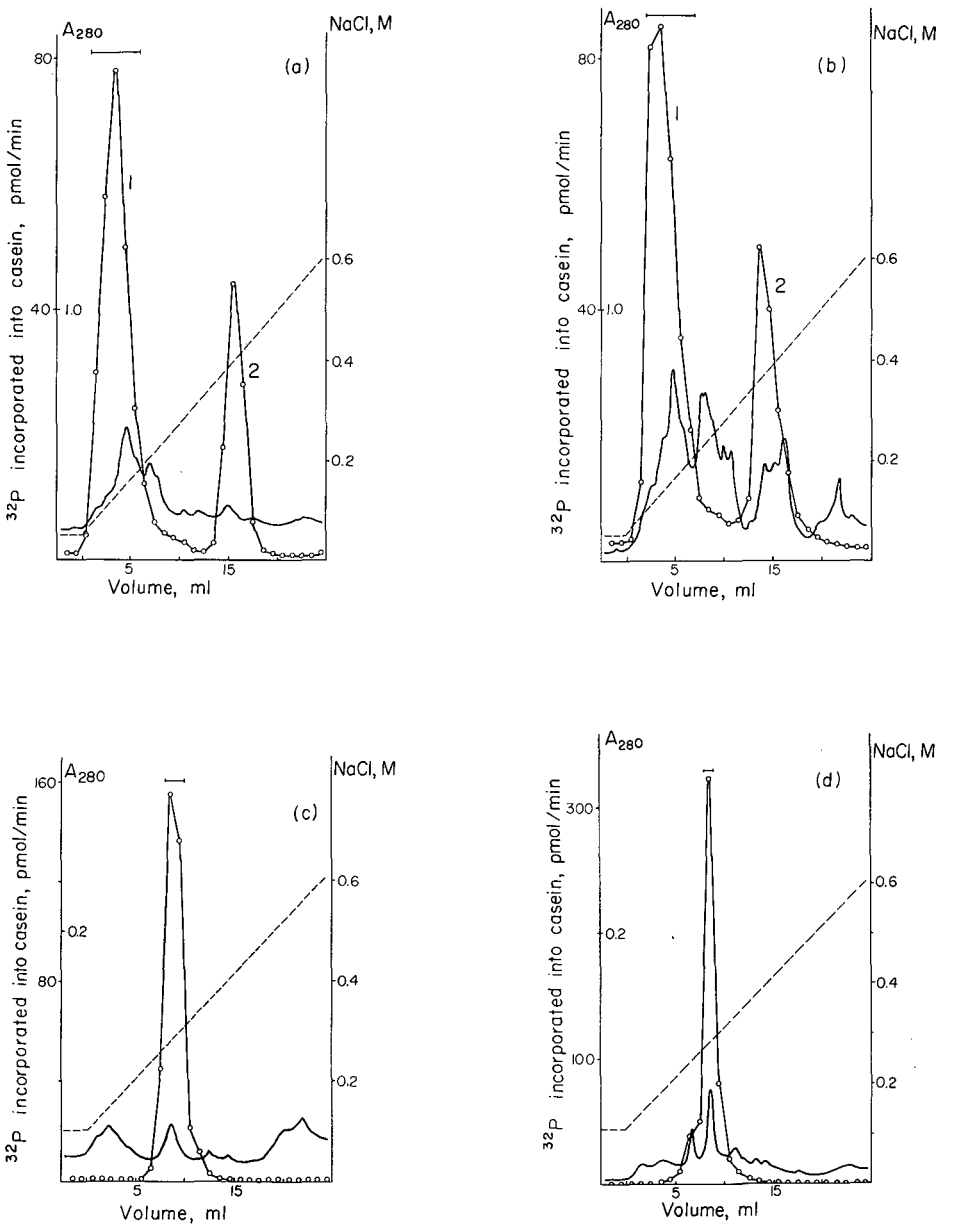

Fig. 2. Chromatography of casein kinase-1 from rabbit liver on Mono Q (a) and Mono S (c) HR 5/5 columns and casein kinase-1 from frog oocytes on Mono Q (b) and Mono S (d) HR 5/5 columns (see Experimental). The fractions were assayed for casein kinase activity as described in Experimental (○—○). The sodium chloride concentration is indicated by the dashed line. Absorbance was monitored at 280 nm, 0.5 a.u.f.s. Pooled fractions are indicated by the bar. Peaks as in Fig. 1.

TABLE I
PURIFICATION OF CASEIN KINASE-1 FROM ANIMAL EXTRACTS

Source	*Step*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*
Rabbit liver	Ribosome-free extract	570.4	139 750[a]	240
	Phosphocellulose P_{11}	8.25	47 440	5750
	Mono Q column	0.47	14 650	31 170
	Mono S column	0.046	14 730	320 220
	Blue-Sepharose	0.021	13 650	650 000
Frog oocytes	Ribosome-free extract	427.5	150 300[a]	350
	Phosphocellulose P_{11}	13.2	36 600	2770
	Mono Q column	0.63	17 250	27 380
	Mono S column	0.055	16 930	307 820
	Blue-Sepharose	0.027	15 340	568 150

[a] This value represents a summary activity of mixed casein kinases in the extract.

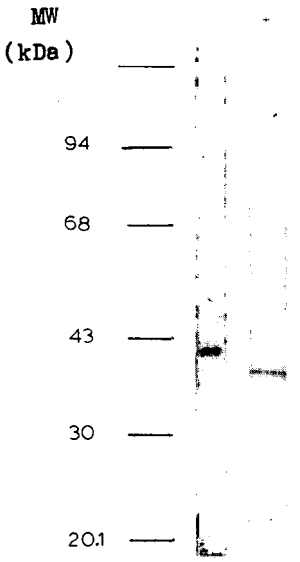

Fig. 3. SDS-PAGE of casein kinase-1 from rabbit liver (left), 0.5 μg, and from frog oocytes (right), 0.4 μg.

CONCLUSION

The procedure developed uses the advantages of Pharmacia Mono Beads ion exchangers Mono Q, Mono S and the FPLC system and takes two working days to obtain highly active homogeneous casein kinase-I in good yield starting from animal tissue. Purification of the enzyme, according to the conventional procedure of Dahmus[4], in my hands resulted in half the yield and takes 4 days to obtain pure enzyme. Analytical scale Mono Q and Mono S HR 5/5 columns gave good results also

on a semi-preparative scale and in combination with phosphocellulose and Blue-Sepharose are suitable for the purification of hundreds of micrograms of casein kinase-1 from animal tissues.

ACKNOWLEDGEMENT

I thank A. D. Wolfson for technical assistance.

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Journal of Chromatography, 477 (1989) 454–457
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 567

Note

High-performance liquid chromatographic improvement of the Young racemization test

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The Young racemization test, introduced to peptide chemistry in 1964[1], was for many years one of the most important and widely used tests for evaluation of the quality of peptide reagents, optimization of the conditions of peptide synthesis reactions and for studying the mechanism of peptide bond formation. According to the test the degree of racemization for the model reaction (where Bz is benzoyl)

$$2\text{Bz-L-Leu} + 2\text{GlyOEt} \rightarrow \text{Bz-L-Leu-GlyOEt} + \text{Bz-D-Leu-GlyOEt}$$

was determined by measuring the optical rotation of the product; the lower the optical rotation, the higher is the degree of racemization. There are disadvantages in using the Young test, such as low sensitivity (detection limit of racemate ranges from 1 to 3%) and its susceptibility to contamination of side products causing errors in the measurement of the optical rotation. Some improvements decrease the detection limit to 0.001%, by use of isotopically labelled compounds[2], and eliminating the by-product interaction[3]. Despite their potential usefulness, the improvements have not gained general acceptance due to the high cost of the isotope method and the tedious procedures involved. However, the Young test still has great potential because there are only a few racemization tests, including the Young test, which offer the possibility of studying racemization phenomena in peptide synthesis without the interference from asymmetric induction.

In this paper we report an improvement of the Young test by employing high-performance liquid chromatography (HPLC) on a chiral stationary phase for the resolution and quantitation of protected enantiomeric peptides in the reaction mixture. With this simplified procedure the analysis is rapid, sensitive (detection limit 0.01% of racemate) and precise. Furthermore this method may be used for the determination of the degree of racemization not only for the Young model but also for other protected peptides.

EXPERIMENTAL

Apparatus and reagents

An Hewlett-Packard chromatograph Model 1090A equipped with diode array detection (DAD) and controlled by an HP 85B workstation was used for quantitative

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analysis. The chiral column was prepared by passing a solution of 3,5-dinitrobenzoyl-L-leucine (DNB-L-Leu) (Aldrich) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Fluka) through a stainless-steel column (100 mm × 4.6 mm I.D.) packed with Nucleosil APS 100-5 (J.J.'s Chromatography)[4]. Tetrahydrofuran (THF) was distilled from above Na–K alloy, alcohols were dried over molecular sieves and *n*-hexane (Reachim) was used as obtained.

The coupling reaction

The reactions on a 0.25-mmol scale were carried out in THF at 25.0°C for 24 h in thermostatted vessels with magnetic stirring. Amino acid components, coupling reagents and the additive, except triethylamine, were added as solids. The initial concentration of the reagents was 0.050 *M*.

RESULTS

The recent discoveries of the possibilities of resolution and quantitation of enantiomeric protected dipeptides using commercially available HPLC chiral columns (easy to prepare in any chemical laboratory also)[5–7] prompted us to modify the Young racemization test and to convert it into a general method of studying peptide bond formation. Thus the HPLC modification of the Young test reported here not only simplifies the test and increases its accuracy but makes possible the determination of the degree of racemization in the synthesis of many peptides other than the Young model containing C-terminal glycine, thus providing much more information. DNB-L-Leu grafted on the aminopropyl silica (Pirkle phase) appeared the best of a few stationary phases tested in our laboratory for separations of the ethyl benzoyl-

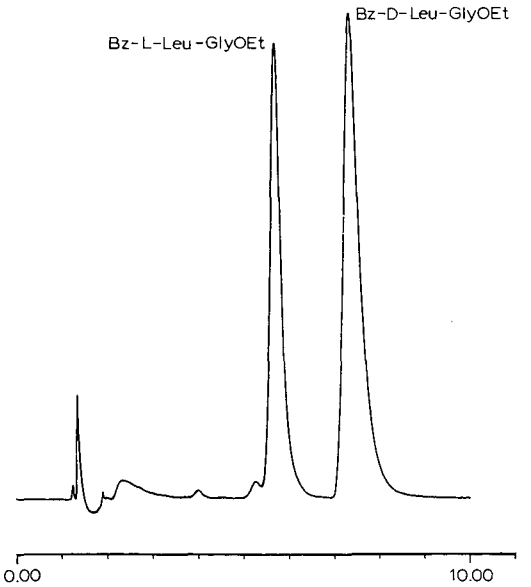

Fig. 1. Chromatogram of the reaction mixture: Bz-LeuOH + HCl · GlyOEt/BPO-Cl. Column: DNB-Leu-silica, 100 mm × 4.6 mm. Mobile phase: ethanol–*n*-hexane (3:97), flow-rate 1.0 ml/min. Detection: 254 nm.

TABLE I

SEPARATION FACTORS, α, AND RESOLUTIONS, R_s, FOR ENANTIOMERS OF DIPEPTIDES Bz-DL-AA-Gly-OEt, AND PARTITION FACTORS, k', FOR THE FIRST ELUTING L-ENANTIOMER

For chromatographic conditions see Fig. 1.

Amino acid	*3% Ethanol*			*5% Ethanol*			*5% Isopropanol*			*35% THF*		
AA	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s
Ala	7.98	1.33	0.40	4.46	1.31	2.25	8.51	1.33	1.43	3.25	1.31	1.14
Val	3.62	1.25	0.72	2.04	1.24	1.59	3.43	1.26	1.17	2.10	1.20	0.79
Leu	3.61	1.41	0.48	2.15	1.38	2.20	3.20	1.49	1.76	1.77	1.35	1.17
Phe	6.91	1.41	0.56	3.99	1.38	2.28	7.23	1.44	1.71	2.46	1.34	1.14

amino acid glycinates (Fig. 1). On the Pirkle phase the separation factor, α, of the protected enantiomeric dipeptides seems to be almost independent of the mobile phase modifier concentration (Table I). Moreover the change of the modifier from a protic (ROH) to an aprotic (THF) solvent does not significantly influence the α value. This is consistent with the charge-transfer interaction model according to which the prevalent enantiomer recognition takes place at the stationary phase. On the other hand, the resolution, R_s, is far better in the mobile phase containing a protic modifier mainly due to the improved peak symmetry. The repeatability of the method was good ($\pm 0.5\%$ for D-enantiomer and $\pm 0.6\%$ for L-enantiomer) and the detection limit was found to be 0.5 nmol (0.01%).

The present method was employed to 1,3-dicyclohexylcarbodiimide–1-hydroxybenzotriazole (DCC–HOBt), EEDQ and bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) mediated peptide synthesis (Table II). The racemization degree and the yield of the products can readily be determined almost instantly (analysis time less than 10 min) using simple isocratic HPLC equipment. The reaction mixture was injected directly on the HPLC column thus avoiding any disadvantages commonly accompanying the Young test.

TABLE II

COMPARISON OF SOME COMMON COUPLING REAGENTS IN THE REACTION Bz-L-Leu + HCl · GlyOEt + n · NEt_3 IN THF AT 25°C

Coupling method	n	*Yield (%)*		*Racemization (%)*
		D-isomer	*L-isomer*	
DCC–HOBt	1	4.8	85.2	10.7
EEDQ	1	2.1	65.6	6.2
BOP-Cl	3	12.8	47.5	42.5

ACKNOWLEDGEMENTS

This work was supported by Polish Academy of Sciences research grant CPBP 01-13. We are grateful to Dr. Maurice Manning for stimulating discussions and essential help in the preparation of the manuscript.

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Journal of Chromatography, 477 (1989) 458–462
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 576

Note

Separation and determination of 8β-hydroxyasterolid and perlolyrine in *Codonopsis pilosula* by reversed-phase high-performance liquid chromatography

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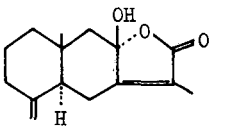
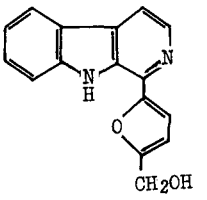
Codonopsis pilosula (Franch.) Nannf., known as "Dangshen", is a traditional tonic medicine in China and is widely used in place of *Panax gingseng* in the treatment of shortness of breath accompanied by palpitation, lassitude and physical weakness.

There have been many reports on the investigation of the chemical ingredients and various kinds of compounds have been isolated from this herbal medicine. However, few methods for the determination of active compounds have been reported.



A. 8β-hydroxyasterolid



B. perlolyrine

This paper deals with a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for separation and determination of 8β-hydroxyasterolid (A) and perlolyrine (B) respectively. 8β-Hydroxyasterolid is the first sesquiterpenoid lactone isolated from *C. pilosula*, and perlolyrine, as we reported before[1], is the first alkaloid isolated from this plant. It is very interesting that perlolyrine exists both in *C. pilosula* and in *Panax gingseng*, since the former has long been used as a substitute for the latter and similar ingredients have not yet been found in both plants. 8β-Hydroxyasterolid shows antiinflammatory properties[2] and perlolyrine belongs to the β-carboline alkaloids, which produce activity toward the benzodiazepine and γ-aminobutyric acid (GABA) receptors[3]. Due to these reasons, a method for determining their contents in different species of Codonopsis was needed. Because of the great difference in their contents in specimens, different HPLC conditions for compounds A and B have been designed.

0021-9673/89/$03.50

EXPERIMENTAL

Materials

8β-Hydroxyasterolid was isolated from *C. pilosula* (cultivated in Lucheng, Shanxi province, China) using a silica gel column (120 cm × 5 cm I.D.) eluted under low pressure with light petroleum (b.p. 30–60°C)–ethyl acetate (6:1) and then recrystallized from chloroform. The purified lactone comprised colourless platelet crystals, m.p. 168–169°C; $[\alpha]_D^{25} = +105.34°$ (*c* 0.83, $CHCl_3$). Its IR spectrum showed an hydroxyl group (3390 cm^{-1}), an α,β-unsaturated lactone group (1750, 1705 cm^{-1}) and a methene group (3090, 1650, 900 cm^{-1}). The mass spectrum showed a $(M)^+$ peak at *m/e* 248 and fragments at 230, 220, 215, 147, 44 (base peak). The UV spectrum had a maximum absorption at 224 nm in methanol. These data and the ^1H NMR spectrum were identical with those reported previously[4].

Perlolyrine was synthesized from 2-hydromethylfurfural and tryptophan according to the literature[5] and used as a reference substance for HPLC. It comprised yellow needle crystals, m.p. 183°C (subliming at 150°C) and its UV, mass and ^1H NMR spectra as well as the HPLC retention time were identical with those of the natural product.

Methanol was of analytical grade. Silica gel was obtained from Qingdao Oceanic Chemical Co.

The Codonopsis plant materials were collected from Shanxi, Sichuan provinces, etc. and identified by associate Professor Yao Damu and Professor Zhao Dawen of our Institute. The sample of Panax gingseng was provided by associate Professor Yan Kedong of the same Institute.

Apparatus

The equipment comprised a M 6000A pump, an U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a SPD-1 UV–VIS detector and a Chromatopac C-R1B data processor (Shimadzu, Kyoto, Japan).

HPLC conditions

A μBondapak C_{18} column (300 mm × 4 mm), 10-μm particles, was employed. The mobile phases were methanol–water, (60:40, v/v) for compound A, (58:42) for B. The flow-rate and column temperature were 1.0 ml/min and 25°C, respectively. The detector was operated at 220 nm for A and 290 nm for B with a sensitivity of 0.08 a.u.f.s. Chart speed 2.5 mm/min.

Sample preparation

Compound A. Methanol (50 ml) was added to 2 g of powdered plant material in a stoppered flask and placed in an ultrosonic bath for 30 min. The extract was filtered and the filtrate was evaporated to dryness on a rotatory evaporator under reduced pressure. The residue was dissolved in 20 ml of water and extracted with chloroform (20 ml × 3). The extract was evaporated to dryness. The residue was dissolved in exactly 2.0 ml of methanol and filtered with a Millipore FH membrane. A 5.0-μl volume of the filtrate was injected for HPLC.

Compound B. Methanol (60 ml) was added to 6 g of powdered plant material and placed in an ultrasonic bath for 30 min. The extract was filtered and the filtrate

was evaporated to dryness. The residue was dissolved in 30 ml of 5% HCl. After elimination of lipids with light petroleum (20 ml × 2), the acidic aqueous layer was neutralized with NH_4OH and extracted with diethyl ether (20 ml × 4). The ether layers were collected, washed with a small amount of water and evaporated to dryness. The residue was dissolved in a small amount of methanol and spotted on a silica G plate (0.5 mm, 10 cm × 10 cm, prepared manually) and then developed by chloroform–methanol (9:1). A bright blue-green fluorescent band appeared on the plate. It was eluted with chloroform–methanol (1:1) and the eluate was allowed to evaporate to dryness. The residue was dissolved in precisely 0.5 ml of methanol, filtered with a Millipore FH membrane and 20.0 μl of the filtrate were subjected to HPLC.

The large amount of sugar in *C. pilosula* is the main interference in the HPLC of 8β-hydroxyasterolid. Therefore, elimination of sugar is an inevitable step. A great deal of lipid, besides sugar, also interferes with the detection of perlolyrine. These components were removed from the samples employed in the estimation of perlolyrine, which were concentrated appropriately by thin-layer chromatography (TLC) prior to HPLC. This treatment also protects the chromatographic column, thus prolonging its useful life.

Calibration graphs were constructed from the results of each of five consecutive injections. Stock solutions were prepared by dissolving 1.60 mg of compound A and 1.04 mg of B separately in 2 ml of methanol. The reference standard solutions were obtained by diluting in methanol (0.08 μg/μl for A and 0.013 μg/μl for B) and processed as described above.

RESULTS AND DISCUSSION

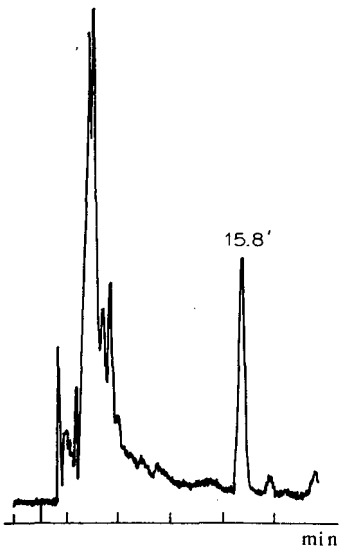
Figs. 1 and 2 show chromatograms of compound A and B in a sample. Satisfactory results were obtained.

The calibration graphs for A and B showed good linearity in the ranges of 160–800 and 26–78 ng respectively.

The recoveries of A and B were 101.6 and 101.0% respectively.

The contents of 8β-hydroxyasterolid in 10 samples, 6 cultivated and 4 wild, are presented in Table I. Rather significant variations both in quality and quantity were observed between cultivated and wild samples. For wild samples, the HPLC peak of the lactone was not found in *C. subglobosa* and in *C. tangshen*. In *C. pilosula* var. *modesta* collected in Songpan, Sichuan, the trace amount of the lactone was too low to be detected. In a sample of same species collected in Lanping, Sichuan, however, the content of the lactone was the highest among all of the samples concerned. Among cultivated samples, some variations were also observed.

The contents of perlolyrine in 11 samples, 10 of Codonopsis as above and 1 of Panax gingseng, are also shown in Table I. Rather significant variations were observed. The HPLC peak of perlolyrine was not found for *C. pilosula* var. *modesta* Nannf. (growing wild in Lanping, Sichuan) and for *C. subglobosa*. Trace amounts of the alkaloid were detected in *C. pilosula* cultivated in Gansu, Inner Mongolia and Shanxi provinces. Significant variation was also observed between the samples of the same species from different sources, *i.e.*, in two samples of *C. pilosula* var. *modesta* Nannf., growing wild in Lanping and Songpan respectively, not far away from each other geographically.



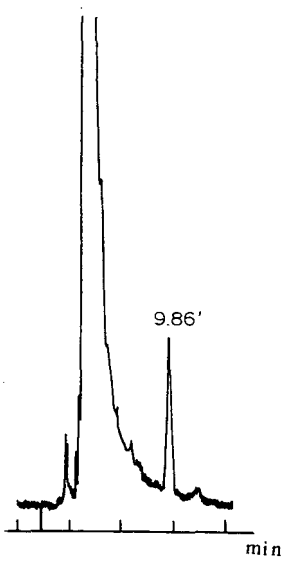


Fig. 1. HPLC trace of 8β-hydroxyasterolid.

Fig. 2. HPLC trace of perlolyrine.

The content of perlolyrine in Panax gingseng is higher than that in all Codonopsis plants concerned except *C. tangshen*.

The contents of 8β-hydroxyasterolid and perlolyrine in all the samples showed similar variations. These would be useful for quality control of the medicine.

TABLE I

CONTENTS OF 8β-HYDROXYASTEROLID AND PERLOLYRINE

No.	*Source*	*Plant*	*Content of 8β-hydroxyasterolid (‰)*	*Content of perlolyrine (‰)*
1	Lucheng, Shanxi		0.052	Trace
2	Lucheng, Shanxi		0.054	0.00020
3	Lucheng, Shanxi	*Codonopsis pilosula*	0.053	0.00015
4	Lucheng, Shanxi	(Franch.) Nannf.	0.069	0.00050
5	Gansu		0.048	Trace
6	Inner Mongolia		0.013	Trace
7	Songpan, Sichuan	*Codonopsis pilosula*	Trace	0.00013
8	Lanping, Sichuan	var. *modesta* Nannf.	0.093	–
9	Ganzi, Sichuan	*Codonopsis subglobosa*	–	–
10	Wuxi, Sichuan	*Codonopsis tangshen* Olive.	–	0.0019
11	Jilin	*Panax gingseng*		0.0010

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Journal of Chromatography, 477 (1989) 463–466
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 571

Note

Identification and quantitation of rifamycins by reversed-phase high-performance liquid chromatography

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Tuberculosis and leprosy are among the major communicable diseases in the developing countries. It is estimated that every year about 10 million people develop tuberculosis and there are about 12 million people suffering from leprosy[1]. In the chemotherapy of both these diseases one very successful drug is rifampicin[2–5]. Rifampicin is a semisynthetic derivative of the microbial metabolites belonging to the ansamycin group of antibiotics, the rifamycins[6]. Among the new drugs being explored for a better treatment of mycobacterial infections, rifamycin derivatives have great potential[7].

Microbial production of these rifamycins yields a mixture of many closely related members, among which the rifamycins B, O, S and SV are of commercial importance. Although some methods of identification[6,8–10] and quantitation[11] exist, none really offers complete analysis. Using high-performance liquid chromatography (HPLC) a method which effectively separates and quantitates these rifamycins (Fig. 1) has been achieved.

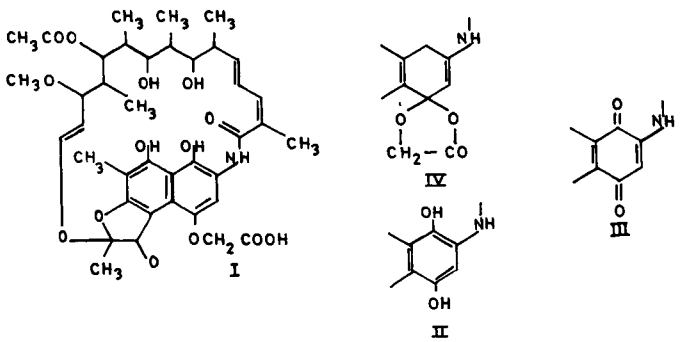

Fig. 1. Structures of rifamycin: I = B; II = SV; III = S and IV = O.

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0021-9673/89/$03.50

EXPERIMENTAL

Reagents and chemicals

The rifamycin standards B, S and SV were obtained by courtesy of Professor J. Nuesch (Ciba Geigy, Basle, Switzerland) and rifamycin O from Dr. Egidio Marchi (Alfarecerche, Rome, Italy). *Nocardia mediterranei* (ATCC 21271) was obtained from the American Type Culture Collection, U.S.A. All reagents and chemicals used were of analytical grade.

Chromatographic conditions

The chromatographic apparatus consisted of a Beckman 342 dual-pump HPLC system equipped with a Model 165 detector set at 254 nm. The detector signals were recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The rifamycins were separated on a reversed-phase, Altex Ultrasphere ODS column (particle size 5 μm; 25 cm $\times$ 4.6 mm). A rotary injection valve with a 20-μl injection loop was used and the column temperature was ambient. Mobile phase: solvent A was 0.05 *M* ammonium formate in deionized water adjusted to pH 7.2 by addition of 0.1 *M* NaOH; solvent B was HPLC grade methanol (LKB Biochrom, Cambridge, U.K.). Both solvents were passed through a 0.2-μm Millipore filter and degassed before use. The solvent programme was a 10-min linear gradient from 50 to 75% solvent B in A, and then held at 75% B for 15 min. At the end of each experiment the initial conditions were reestablished and the column was equilibrated for 10 min. The flow-rate was 1.0 ml/min in all experiments.

Standards and sample preparation

Standard solutions of the rifamycins B, O, S and SV were prepared separately in ethyl acetate. Each rifamycin component was chromatographed individually in triplicate to determine the exact retention time. The components were later mixed in appropriate proportion and subsequently subjected to HPLC with varying parameters till all the four rifamycins were clearly resolved. For the identification of the rifamycins produced by *Nocardia mediterranei* ATCC 21271[12,13] a 5-ml aliquot of the beer was withdrawn from the fermenter and acidified to pH 4.5 with 5 *M* sulphuric acid. After acidification, 1 ml of ethyl acetate was added and then shaken vigorously on a vortex mixer for 1 min. It was then centrifuged at 1000 *g* for 5 min and an aliquot of the ethyl acetate extract analysed.

RESULTS AND DISCUSSION

To determine the optimum conditions for the separation of the rifamycins, an isocratic methanol–0.05 *M* ammonium formate (60:40, v/v) system was first tried. This system took about 50 min to give well separated rifamycin components, with the rifamycin O peak tending to tail. In order to shorten the elution time, a gradient system was devised (as described in Experimental). This gave a distinct separation in 20 min. Fig. 2a shows a typical chromatogram of a mixture (200 ng each) of the four different rifamycins B, SV, S and O clearly resolved on the Ultrasphere ODS column. Having established the separation method, the ethyl acetate extract from a fermentation broth of *Nocardia mediterranei* ATCC 21271, a known producer of rifamycin

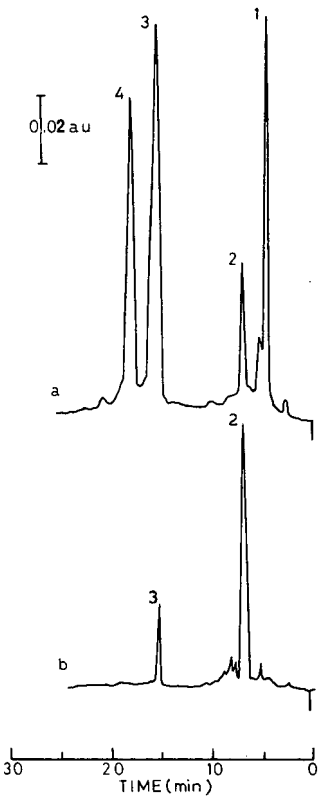

Fig. 2. HPLC of (a) 200 ng each of standard rifamycins (peaks: I = B; II = SV; III = S; IV = O) and (b) of an ethyl acetate extract from *N. mediterranei* fermentation broth (peaks: II = SV; III = S).

SV^{12}, was injected. The results presented in Fig. 2b clearly show that the broth which had a substantial amount of rifamycin SV (1.6 mg/ml) also had a significant amount (0.2 mg/ml) of rifamycin S. To estimate the recovery and precision of the method, five replicate injections of control broth spiked with 200 ng/ml of each of the rifamycins

TABLE I

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF RIFAMYCINS FROM SPIKED CONTROL BROTH

Each value represents the mean ± C.V. (%) of five determinations per concentration.

Rifamycin	*Concentration spiked (ng/ml)*		
	200	*400*	*800*
B	198.2 ± 4.5	401.0 ± 0.8	800.6 ± 0.8
SV	196.7 ± 5.0	393 ± 1.1	792 ± 0.4
S	192.7 ± 0.7	391 ± 1.3	791.8 ± 0.3
O	200.3 ± 2.1	401.5 ± 1.8	804.2 ± 1.0

were made. Recovery values of 99, 98, 96 and 100% for rifamycin B, SV, S and O were obtained. These values were fairly constant over a wide range of concentration. The coefficient of variation (C.V.) was less than 5% for both inter and intra assay for all the rifamycins spiked at a concentration of 200 ng/ml (Table I).

Calibration graphs generated in the concentration range 50–2000 ng showed good linearity with a correlation coefficient of 0.993 for rifamycin B, 0.988 for rifamycin SV, 0.998 for rifamycin S and 0.999 for rifamycin O. Based on a signal-to-noise ratio of more than 3, the limit of detection is *ca.* 20 ng of each rifamycin in an injection volume of 20 μl.

The method presented here will be of use in the accurate analysis of fermentation broths and would not only reveal the quantity but also the presence of other rifamycins produced at a particular stage. It can also be adapted to study other intermediates of the biosynthetic pathway of the rifamycins.

ACKNOWLEDGEMENTS

The authors thank A. K. Shukla for his helpful technical assistance and Themis Chemicals, Bombay, for partial financial support.

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Journal of Chromatography, 477 (1989) 467–470
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 594

Note

Improved method for the separation of methylolmelamines by high-performance liquid chromatography

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Exudation of formaldehyde and melamine from cups made of melamine resin has been reported using water or 4% acetic acid as a solvent for extraction[1–4]. On the other hand, it is well known that melamine reacts with formaldehyde to form methylolmelamines (N-hydroxymethylmelamines)[5–8] and the resulting methylolmelamines have been separated by high-performance liquid chromatography (HPLC)[5–7]. The techniques used were not suitable as regards the sensitivity and resolution for the determination of very small quantities of methylolmelamines. We now report a rapid and sensitive reversed-phase HPLC assay which permits the microanalysis of methylolmelamines.

EXPERIMENTAL

Materials

Formalin (37% formaldehyde solution), acetonitrile and acetic acid were obtained from Wako (Osaka, Japan), and melamine from Tokyo Kasei (Tokyo, Japan).

HPLC

A Model 5A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with UV spectrophotometric detector set at 235 nm was operated at room temperature for qualitative and quantitative analyses. A stainless-steel reversed-phase HPLC column (250 mm × 4.6 mm I.D.) was prepared with Develosil ODS-5 (Nomura Chemical, Japan). Elution was carried out with acetonitrile–acetic acid–water (5:0.5:94.5, v/v) for highly substituted methylolmelamines and with acetonitrile–acetic acid–water (1:0.5:98.5, v/v) for the separation of low substituted methylolmelamines. The flow-rate was 0.8 ml/min.

Preparation of standard methylolmelamines mixture

A methylolmelamines mixture was prepared according to Tomita's method[5] as follows; 0.16 *M* formaldehyde and 0.033 *M* melamine in 0.05 *M* phosphate buffer pH 9.0, were mixed in equal quantities and the mixture was allowed to stand for 120 h at 28°C.

0021-9673/89/$03.50

Preparation of sample solutions

Cups of volume 200 ml, made of melamine resin, were used. An 100-ml portion of water or 4% acetic acid was poured into the cups. The cups were covered with watch-glasses, then heated in an electric range for 2.5 min and 10 μl of the extracts were injected into the chromatograph.

RESULTS AND DISCUSSION

All of the nine methylolmelamines, including two isomers of di-, tri- and tetramethylolmelamine, were separated by HPLC and each species isolated was identified by NMR according to Tomita[5]. An addition reaction between melamine and formaldehyde was carried out under the same conditions as those in his report and a similar chromatogram was obtained from the reaction mixture as shown in Fig. 1. Each peak was identified by comparing the corresponding peaks on both chromatograms, that reported by Tomita and ours. The retention time increased in the order of the number of methylol substituents on the amino groups of melamine.

The pH of the mobile phase was the first parameter studied. The intensity of the absorption of melamine at 235 nm increased when the pH was lowered[1]. The same phenomenum was observed for methylolmelamines. Addition of acetic acid suppressed tailing of the peaks on the chromatogram and improved their resolution. The peak height increased remarkably with increasing acidity, which made possible trace analysis. The detection limit was less than 10 ng/ml for melamine with a 20-μl injection. No significant variation in sensitivity was observed in the range of 0.1–1.0%

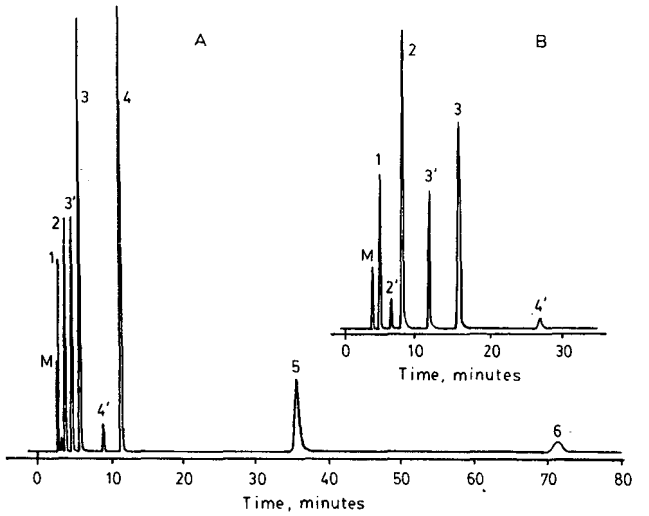

Fig. 1. Elution profile of an equilibrium methylolmelamines mixture obtained from the reaction between 0.16 *M* formaldehyde and 0.033 *M* melamine. HPLC conditions: column, ODS-5 (250 mm × 4.6 mm I.D.); detector, UV (235 nm); mobile phases: A, acetonitrile–acetic acid–water (5:0.5:94.5, v/v); B, acetonitrile–acetic acid–water (1:0.5:98.5, v/v); flow-rate, 0.8 ml/min. Peaks: M = Melamine; 1 = monomethylolmelamine; 2 = N,N′-dimethylolmelamine; 2′ = N,N-dimethylolmelamine; 3 = N,N′,N″-trimethylolmelamine; 3′ = N,N,N′-trimethylolmelamine; 4 = N,N,N′,N″-tetramethylolmelamine; 4′ = N,N,N′,N′-tetramethylolmelamine; 5 = pentamethylolmelamine; 6 = hexamethylolmelamine.

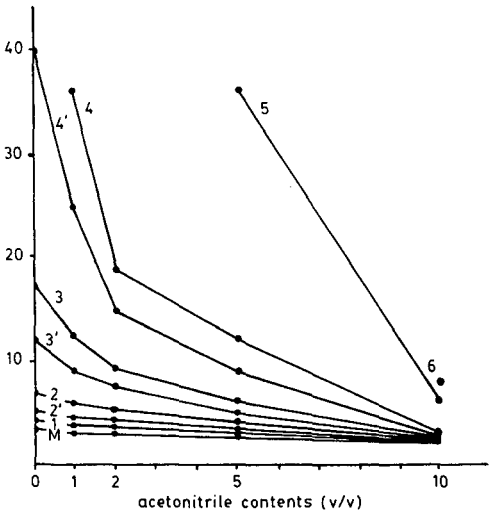

Fig. 2. Dependence of retention times on the acetonitrile content in the mobile phase containing 0.5% (v/v) of acetic acid. Compounds numbered as in Fig. 1.

(v/v) of acetic acid. Next, the dependences of the retention times of methylolmelamines on the acetonitrile content were studied between 0 and 10% (v/v), maintaining acetic acid content constant at 0.5% (v/v) for a good separation of interesting methylolmelamines. The results in Fig. 2 clearly show that the acetonitrile content had a strong effect on the retention times.

The sensitivity for melamine was of the same order of magnitude as that obtained by Inoue *et al.*[1], while their paper made no mention of methylolmelamines. In regard to methylolmelamines, the present method offered distinct advantages in sensitivity and resolution over Tomita's[5], where neutral media were used as the mobile phase and a differential refractometer was used as the detector.

The method proposed was applied to the determination of melamine and methylolmelamines in extracts from cups made of melamine resin and the results are presented in Table I. A typical chromatogram is shown in Fig. 3. Exudation of melamine and monomethylolmelamine was observed. The peak height was used for quantification and compared with that from a standard melamine solution. The

TABLE I

MELAMINE AND MONOMETHYLOLMELAMINE (ppm) IN SAMPLE SOLUTION

Sample solutions were prepared as described in the text.

Cup	*Solvent*	*Melamine*	*Monomethylolmelamine*
A	Water	0.85	0.03
B	Water	0.24	0.02
C	4% Acetic acid	4.20	0.32
D	4% Acetic acid	0.83	0.07

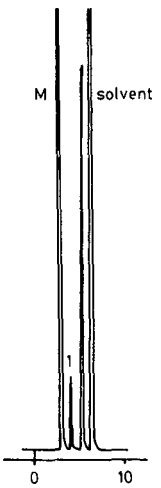

Fig. 3. A chromatogram of a sample solution in 4% acetic acid. Time scale in minutes.

amount of monomethylolmelamine was calculated by assuming that its absorption intensity is close to that of melamine. The presence of monomethylolmelamine was identified for the first time in extracts from cups made of melamine resin.

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Journal of Chromatography, 477 (1989) 471–473
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 633

Note

Chiral separation of the optical isomers of the antimalarial drug halofantrine

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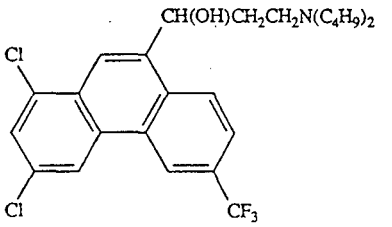
(Received May 9th, 1989)

1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-N,N-dibutylaminopropan-1-ol (**1**) is a very effective antimalarial agent[1,2]. It is generally known as halofantrine and is currently being marketed by SK&F as the hydrochloride under the trade name of Halfan for the acute treatment of most forms of malaria, even multiple-drug resistant strains.



(**1**)

1 has a chiral centre and the optical isomers have been resolved chemically by fractional crystallisation of the *d*-camphoric acid salts from a hexane–tetrahydrofuran solvent mixture[3].

For halofantrine to be used optimally as an antimalarial agent it is important to evaluate in detail any differences in the pharmacodynamics and the pharmacokinetics of the enantiomers of this drug. To achieve this, it is necessary to have available an analytical method that can distinguish between the two enantiomers with good sensitivity. In this article, we describe a new high-performance liquid chromatography (HPLC) procedure which allows the efficient resolution of these enantiomers using a chiral stationary phase.

EXPERIMENTAL

Chemicals

n-Hexane (Rathburn Chemicals), propan-2-ol, chloroform, ethanol, 2-butanol (BDH) and 99% triethylamine (Aldrich) were filtered through a Millipore Durapore 0.45-μm membrane filter and degassed with helium before use. Racemic halofantrine free base was prepared from halofantrine hydrochloride[4] by the following procedure.

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Halofantrine hydrochloride (13.2 g, 24.6 mmol) was vigorously stirred in 40% (w/v) sodium hydroxide solution (160 ml)/diethyl ether (300 ml) until a solution was formed ($\approx$ 1 h). The alkaline layer was separated from the diethyl ether and was further extracted twice with 150 ml of diethyl ether. The combined ether extracts were dried over magnesium sulphate and filtered. Evaporation of the diethyl ether gave an oil which solidified on addition of dimethyl sulphoxide. Final recrystallisation from nitromethane gave 7.3 g (81%) of **1**, m.p. 83–85°C. Analysis ($C_{26}H_{30}NOCl_2F_3$) calculated C 62.40, H 6.04, N 2.80, Cl 14.17; found C 62.33, H 5.95, N 2.67, Cl 14.41. Mass spectrometry, *m/e* 500 (M^+), 456, 142, 100 (base).

High-performance liquid chromatography

The HPLC method to separate the optical isomers of **1** was developed on a Perkin-Elmer Series 4 liquid chromatograph, equipped with a Perkin-Elmer ISS-100 autoinjector and a Kratos Spectroflow 783 variable-wavelength UV absorbance detector operated at 260 nm. The chiral column (250 × 4.9 mm I.D.) used for the enantiomeric separation was of the Pirkle type and the chromatographic support consisted of L-N-(3,5-dinitrobenzoyl)leucine covalently bound to 3-aminosilica (particle size 5 μm). This column, supplied by Hichrom, was operated at 0°C. The best separation of the enantiomers was achieved using *n*-hexane, chloroform and propan-2-ol (containing 1% triethylamine) in the ratio of 90:5:5 (v/v/v) and flowing at 0.2 ml min^{-1}. UV spectra were recorded on a Hewlett-Packard 1040A diode array detection system.

Specific rotation $[\alpha]_D^T$

$[\alpha]_D^T$ of collected fractions was determined in *tert.*-butyl methylether using a Perkin-Elmer 241 polarimeter set at the sodium D-line (589 nm). Optical rotation was determined in a cell of 100 mm pathlength, thermostatted at 25°C.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows the chiral resolution of the optical isomers of halofantrine using the conditions detailed in the Experimental Section. As expected, the UV absorbance of the separate enantiomers is identical to that of halofantrine itself. The (+)-enantiomer elutes first with a retention time of 24.3 min followed by the (+)-enantiomer at 26.1 min. The separation factor α is equal to 1.28.

A number of different chiral resolution chromatographic methods, utilising a variety of column supports (Chiralcel OC, OJ and OF, Resolvosil-BSA-7 and Cyclobond I) were used. These columns did not give any degree of separation. A number of different solvent system combinations were also tried on the L-leucine column. For example, replacing propan-2-ol with either ethanol or 2-butanol gave no enantiomeric separation. Moreover, the tertiary amino group of **1** appears to interact strongly with the silanol groups in the column packing so that excluding triethyl amine as a mobile phase additive[5] gave broader peaks, longer retention times and poor enantiomeric resolution.

Repeat injection and collection of two fractions, one from the beginning of elution of the first peak to the same apex and another from the second peak to the end of elution, gave (on evaporation of the solvent) sufficient quantities of the two

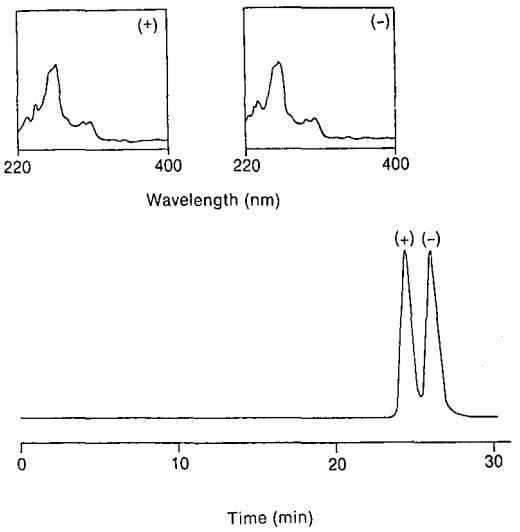

Fig. 1. Enantiomeric separation of the optical isomers of halofantrine **1**. Inserts show the UV spectra of the (+)- and (−)-enantiomers.

enantiomers for polarimetric analysis. This identified the first and second fractions as containing predominantly the (+)- and (−)-enantiomers, respectively. The optical rotation $[\alpha]_D^{25}$ of these fractions were measured as +38° and −24° respectively. These rotations are of a similar order of magnitude to those (about 41°) reported in the literature for the enantiomers of **1** resolved by complex formation with *d*-camphoric acid[3].

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Journal of Chromatography, 477 (1989) 474–476
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 566

Note

Quantitation of polydimethylsiloxane in pharmaceutical formulations by gel permeation chromatography

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(First received January 16th, 1989; revised manuscript received April 12th, 1989)

The analysis and quantitation of polydimethylsiloxane are routinely performed by infrared (IR) spectroscopy[1]. If the matrix is simple, reliable and straightforward results are generated. However, quantitation of polydimethylsiloxane in emulsions and oral suspensions requires careful sample handling and extensive background subtraction of the sample matrix[2,3].

For example, Fourier transform infrared (FTIR) spectroscopy has been used for the quantitation of dimethicone (a mixture containing polydimethylsiloxane and silicon dioxide) in lotions[3]. Despite the use of liquid–liquid extraction followed by column liquid–solid extraction prior to the FTIR analysis and matrix subtraction, a slightly positive bias due to matrix interference was obtained.

The molecular weight distribution of polysiloxane is commonly assessed by gel permeation chromatography (GPC) utilizing refractive index (RI) detection[4–6]. This technique, despite its selectivity for large molecules, has not been extensively used for quantitation of macromolecules. In this paper the usefulness of GPC-RI together with a simple work-up procedure for the quantitation of polidimethylsiloxane (PDMS) in complex matrices as emulsions is described.

EXPERIMENTAL

Chemicals

Chloroform, dichloromethane, *n*-hexane, toluene and methyl isobutyl ketone were of LiChrosolv or analytical grade (Merck, Darmstadt, F.R.G.).

As a standard, Antifoam M (Dow Corning, Glamorgan, U.K.) was used. The amount of PDMS in this standard was determined by IR spectroscopy[1].

Apparatus

The liquid chromatograph consisted of a Shimadzu LC-pump (Model LC-3A, Shimadzu, Kyoto, Japan) with a Shimadzu autoinjector (Model SIL-6A) and an ERMA refractive index detector (Model ERC-7512; Erma, Tokyo, Japan). A Shimadzu, Model C-R3A integrator was used.

Sample preparation

An 100 ± 10 mg emulsion was accurately weighed and dissolved in 10.0 ml

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methyl isobutyl ketone (MIBK). The sample was mixed on a Vortex mixer for about 30 s followed by ultrasonication for 15 min and centrifugation (600 *g*) for another 15 min. The supernatant was used as the sample solution.

Chromatographic conditions

The analytical column (300 mm × 7.7 mm) contained polystyrene–divinylbenzene, 10-μm particles, mixed pore size, M_w working range 1000–10^6 (PL-gel; Polymer Laboratories, Amerst, U.S.A.). The mobile phase toluene was delivered at a flow-rate of 1 ml/min. The volume injected was 30 μl.

RESULTS AND DISCUSSION

The extraction recovery from several solvents of different polarities was determined. The polydimethylsiloxane concentration found in the tested formulation was compared to the expected theoretical concentration, see Table I.

Of the solvents tested, quantitative recoveries were obtained from *n*-hexane and methyl isobutyl ketone. However, the precision was lower with *n*-hexane. Methyl isobutyl ketone, on the other hand showed both good recovery and precision and thus was the obvious choice.

The void volume, V_o, of the column was determined by use of an high-molecular-weight PDMS ($M_w > 1 \cdot 10^6$) and was found to be 5.5 ml. The total permeation

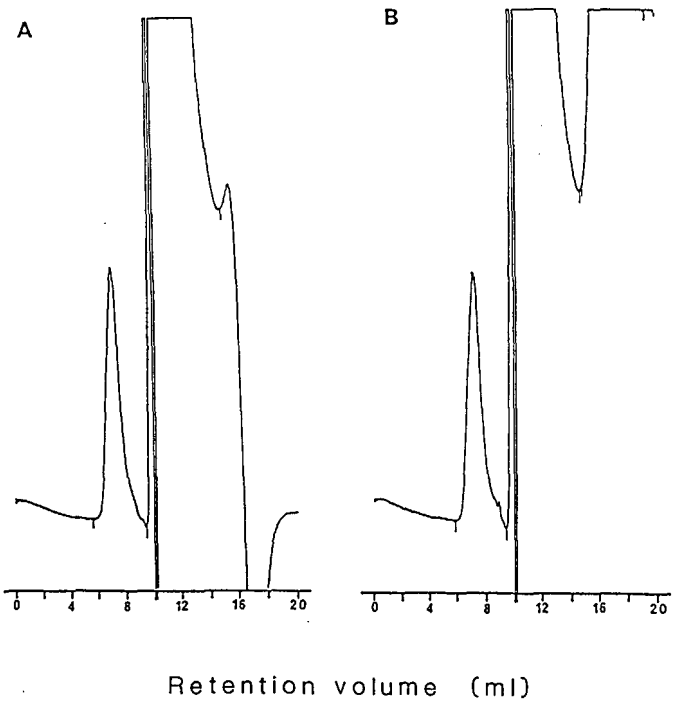

Fig. 1. Gel permeation chromatographic separation of (A) PDMS standard and (B) sample. The retention volume for the PDMS was 7.20 ml.

TABLE I

EXTRACTION RECOVERY OF POLYDIMETHYLSILOXANE

Solvent	*% Recovery*	*n*
Chloroform	76 ± 6	10
Dichloromethane	81 ± 3	4
Toluene	37 ± 7	2
n-Hexane	93 ± 10	42
Methyl isobutyl ketone	99 ± 2	11

volume, V_t, determined by use of chloroform (dissolved in the mobile phase) was found to be 13.5 ml. The retention volume, V_R, for the PDMS in the pharmaceutical samples was 7.20 ± 0.032 ml (n = 32) (see Fig. 1).

The general purpose mixed gel bed column was chosen in order to provide separation of PDMS from smaller as well as larger molecules, *i.e.*, carboxypolymethylene present in the emulsion. It was, however, experimentally established that carboxypolymethylene was not extracted to any significant amount. However, since the column provided a resolution, with good accuracy and precision, no further efforts to improve the separation of PDMS from the smaller molecules present in the emulsion were made.

The precision of the method was investigated at the level of 1 mg/ml (in MIBK). Eleven samples from the same formulation were analysed (double injections) and the relative standard deviation was found to be 1.9%. The calibration graph was linear in the concentration range 0.5–2.5 mg/ml. Typical regression coefficients were 0.9998–0.9999. The lower limit of detection at a signal-to-noise ratio of 3:1 was 1.5 μg injected on-column.

Gel permeation chromatography in combination with a simple work-up procedure provides a straightforward analysis method for PDMS in relative complex matrices such as emulsions. Notable, also is the ease in comparison to IR spectroscopy by which the analysis step can be automated by use of an autosampler.

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journal of chromatography news section

ANNOUNCEMENTS OF MEETINGS

7th INTERNATIONAL SYMPOSIUM ON PREPARATIVE CHROMATOGRAPHY, GHENT, BELGIUM, APRIL 8–11, 1990

The 7th International Symposium on Preparative Chromatography (PREP-90) will be held for the third time in Europe on April 8–11, 1990 in the Congress Centre of Ghent, Belgium. It is the continuation of the successful PREP meetings in Paris (1986), Baden-Baden (1988) and Washington.

These meetings have revealed the broad interest of the participants and this will be kept in mind on planning PREP-90. All aspects of preparative gas and liquid chromatography are welcome. The scientific programme will include general and specific survey lectures, oral and poster contributions, company presentations and discussion sessions.

All prominent companies active in preparative chromatography will participate in an exhibition of state-of-the-art equipment which will be held in conjunction with the poster presentations. Several workshop seminars are planned as well.

Furthermore, the unique settings in the city of Ghent are superbly appropriate for the organisation of an enjoyable and interesting social programme. The registration fee of the symposium will be BFr 15000. This will include complete documentation, coffee breaks, luncheons, refreshments, a welcome reception, a banquet and various other social activities.

Abstracts will be considered by the scientific committee and should be limited to one single-space typed page stating title, author(s), brief description and references. Please mention also oral or poster preference as well as a complete address and telephone number. The accepted communications will be published in a special issue of the *Journal of Chromatography*.

For further information, contact: Professor M. Verzele Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S4) B-9000 Ghent, Belgium Tel.: (091) 225715 (2287); fax: (091) 228321.

11th INTERNATIONAL SYMPOSIUM ON BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY AND ELECTROPHORESIS, TALLINN, U.S.S.R., APRIL 24–28, 1990

The 11th International Symposium on Biomedical Applications of Chromatography and Electrophoresis will be organized in Tallinn (Estonia) by the Scientific Council on Chromatography of the Academy of Sciences of the U.S.S.R. in cooperation with the Chromatography and Electrophoresis Section of the Czechoslovak Chemical Society and the Chromatography Group of the Society for Clinical Chemistry and Laboratory Diagnostics of the German Democratic Republic and Estonian Academy of Sciences.

All aspects of the theory and applications of chromatography and electrophoresis in biochemistry and medicine will be discussed. Special emphasis will be given to: applications of chromatography and electrophoresis in clinical diagnostics; analysis of drugs; drug monitoring and pharmacokinetic studies; sample preparation; new separation methods and instrumentation.

The programme will comprise lectures (invited plenary lectures and selected original contributions), posters and discussions. English is the symposium language. Authors desiring to present papers should submit a 150–250 word abstract by September 30th, 1989. The Scientific Committee will recommend a limited number of papers for publishing in a symposium issue of the *Journal of Chromatography, Biomedical Applications*. The recommended manuscripts will be, however, subjected to routine refereeing procedures.

The symposium fee is expected to be US$180 for active participants (board and lodging included).

For further details or registration contact: Dr. Ljudmila Kolomiets, Scientific Council on Chromatography, Institute of Physical Chemistry, Leninskii prospect 31, 117915 Moscow, U.S.S.R. Tel.: (095) 2320065; telex: 411029 pesum su.

INTERNATIONAL CONFERENCE ON CHIRALITY, CANCUN, MEXICO, JUNE 7–9, 1990

This conference will be held at the Sheraton Resort in Cancun, Mexico, June 7–9, 1990.

The three areas of emphasis for this conference are: enantioselective separations (both analytical and preparative): chiral recognition mechanisms: and the use and effect of these studies on the pharmaceutical and biomedical communities. The regulatory ramifications of the new enantioselective separation technologies will be addressed. Twenty-three of the world's leading researchers in these areas will present accounts of their latest work. In addition, representatives of regulatory agencies and the pharmaceutical industry will discuss their views for and against government regulation. Since attendance at this conference will be limited, all conferees should have ample opportunity to question the speakers and to discuss their work.

The attendance will be limited to 300 conferees. All conferees must have experience and/or a strong interest in this field as it pertains to their work, research, etc. It is expected that registration will be complete by April 1, 1990. To assure yourself a position at this conference please apply early.

All conferees are invited to present a relevant poster at this conference. Many of the speakers and their associates will be presenting posters as well. Abstracts (less than or equal to half a page, single spaced) should be sent to Ms. Mary Jo Richards at the address given below.

The registration fee for the conference is US$ 450.00. The room and board (mandatory for conferees) for three days and three nights is US$ 345.00 for a double room or US$ 465.00 for a single room.

For further details contact: Mary Jo Richards, University of Missouri-Rolla, Chemistry Department, 341 Schrenk Hall, Rolla, MO 65401, U.S.A. Tel.: (314) 341-4429; fax: (314) 341-2071.

1st INTERNATIONAL SYMPOSIUM ON ANALYTICAL CHEMISTRY, CHANGCHUN, CHINA, AUGUST 7–11, 1990

The 1st Changchun International Symposium on Analytical Chemistry, will provide a major forum for academic exchange on analytical chemistry. Key developments in the field will be highlighted by 10 well-known invited plenary speakers. Contributed presentations will be devoted to atomic spectrometry, automatic analysis, bioanalytical chemistry, chemometrics, chromatography, clinical chemistry and drug analysis, electroanalytical chemistry, environmental analysis, food analysis, magnetic resonance spectrometry, mass spectrometry, molecular spectrometry, surface analysis, teaching and education in analytical chemistry, trace analysis, X-ray spectrometry, etc.

There will be an exhibition of modern analytical instrumentation. A full social programme will complement the symposium agenda.

The symposium is sponsored by the State Education Commission of China, the Chinese Academy of Sciences and the Chinese Chemical Society and organized by Jilin University and Changchun Institute of Applied Chemistry of the Chinese Academy of Sciences. For further information and registration forms, please contact: Professor Qinhan Jin, Department of Chemistry, Jilin University, Changchun, Jilin 130021, China.

BUDAPEST CHROMATOGRAPHY CONFERENCE, BUDAPEST, HUNGARY, AUGUST 14–17, 1990

The Budapest Chromatography Conference, organized by the Hugarian Pharmacological Society and the Hungarian Chemical Society, will be held at the Building for Research and Teaching of Semmelweis University of Medicine, Budapest, Hungary, August 14–17, 1990.

The main topics of the conference are: theoretical aspects of chromatography; HPLC; supercritical-fluid chromatography; thin-layer chromatography; electrophoretic methods; instrumentation; stationary phases; quantitative evaluation; calculation methods and application of computers; chromatographic separation of amines, amino acids and peptides, proteins, nucleic acids, drugs and metabolites, and biologically active natural products; free communications.

Oral lectures and poster presentations will be accepted. Anyone wishing to contribute an original, unpublished work should submit an abstract of 100–500 words in English not later than August 31, 1989. Selected papers of the Budapest Chromatography Conference will be published.

An exhibition of chromatographic equipment, stationary phases, materials, solvents and books will be held during the conference.

For further details contact: Dr. Huba Kalász, Department of Pharmacology, Nagyvárad tér 4, H-1089 Budapest, Hungary.

2nd INTERNATIONAL SYMPOSIUM ON MICROCOLUMN SEPARATION METHODS, STOCKHOLM, SWEDEN, AUGUST 20–22, 1990

The 2nd International Symposium on Microcolumn Separation Methods, organized by the Analytical Division of the Swedish Chemical Society, will be held at the conference Hotel Aronsborg (40 km north of Stockholm), Sweden, August 20–22, 1990.

The development of miniaturized systems is presently the most dynamic field in the separation sciences. Techniques of capillary gas chromatography, microcolumn supercritical-fluid chromatography, microcolumn liquid chromatography and capillary electrophoresis have in the last few years proved their extraordinary usefulness. The inherent attractive feature of these techniques, namely the very high resolution, is especially relevant for very complex samples. Further, in applications where the sample volumes or amounts are limited miniaturized systems are the techniques of choice.

The symposium will highlight new detection strategies, hyphenated techniques, column technology, coupled column systems, sample introduction methods and contemporary applications.

Invited lecturers will give review and keynote lectures, and the scientific programme will further comprise short lectures as well as poster and discussion sessions.

The registration fee includes conference material and social activities. The fee will be approximately SEK 1900. A limited number of scholarships will be available for Ph.D. students on application.

For further details contact: Microcolumn Separation Methods, Attn. Eva Mattsson, The Swedish Chemical Society, The Analytical Division, Wallingatan 26B, S-111 24 Stockholm, Sweden.

6th INTERNATIONAL SYMPOSIUM ON ION EXCHANGE, BALATONFÜRED, HUNGARY, SEPTEMBER 3–7, 1990

The conference, organized by the Hungarian Chemical Society, will be held at the Lake Balaton (Balatonfüred), Hungary, September 3–7, 1990. The symposium will be organized immediately after the EUROANALYSIS VII (Vienna, Austria, August 25–31, 1990). The transportation from Vienna or from Budapest takes about 2 h by car or by train.

Ion exchange, is growing rapidly in popularity and importance in many fields of science like biotechnology, biochemistry, analytical chemistry, nuclear- and radiochemistry, hydrometallurgy, environmental science, etc. The aim of the symposium is to present a scientific programme combined with opportunity for discussion and the exchange of ideas and experience. The 4-day programme is divided into five thematically oriented sessions comprising invited, keynote and discussion lectures together with poster presentations.

Topics covered will include: synthesis of reactive polymers (resins, membranes and related materials); fundamental aspects of ion exchange (selectivity, kinetics); ion chromatography and other analytical applications (complexation, extraction, trace analysis, etc.); ion exchange in bioprocessing (biotechnology, biochemistry, purification, organic ion separations); application of ion exchangers in the process technology (nuclear and radiochemical technology, water treatment, hydrometallurgy etc.).

Manufacturers and suppliers of importance in the field on ion-exchange materials, column packings, ion-chromatography, technologies, equipments, literature and related areas are expected to participate.

For further details or registration contact: Professor J. Inczédy, Department of Analytical Chemistry, University of Veszprém, P.O. Box 158, H-8201 Veszprém, Hungary. Tel.: (3680) 22022; fax: (3680) 26016; telex: 32-397.

2nd INTERNATIONAL SYMPOSIUM ON CHIRAL DISCRIMINATION, ROME, ITALY, MAY 27–31, 1991

Molecular chirality, has recently received growing attention from biologists, microbiologists, pharmacologists, toxicologists etc. The study of molecular chirality and its consequences is revealing more and more how essential it is in the study of biological phenomena. Some of the aspects of chirality continue to be the subject of important "*ad hoc*" international symposia (*e.g.* the Rome Symposium on Separation of Enantiomers, October 1982; the series of International Symposia on Chiral Separations, held in the U.K., 1987, 1989; the Tübingen Meeting on Toxicology, April 1988), or of special sessions in the periodic symposia on chromatography (*e.g.* the biennial "International Symposia on Chromatography", the annual LC Symposia, etc.), and several symposia organized by American, Israeli, Japanese scientists.

Some preliminary suggested topics for this symposium are: enantiomeric separation by crystallization; kinetic resolution: chemical and microbiological; new high-performance methodologies for chiral separation; chiral stationary phases for analytical and preparative purposes; chiral discrimination mechanism: experimental and computer assisted investigation; relationship between stereochemistry and pharmacological activity; chiral recognition in pharmacokinetic processes; stereoisomeric purity of drugs; international regulatory issues for drugs.

The symposium format will include invited lectures and keynote lectures by distinguished international experts together with poster sessions and ample opportunity for discussion.

For further details contact: Professor C. Misiti or Professor F. Gasparrini, Laboratori di Chimica Organica, Facoltá di Farmacia, Universitá "La Sapienza", Piazzale Aldo Moro 5, 00 185 Rome, Italy. Tel.: (06) 4452900, fax (06) 49912780.

HPLC '91, 15th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, BASEL, SWITZERLAND, JUNE 3–7, 1991

HPLC '91, sponsored by The Swiss Association of Chemists, will be held in the Convention Center in Basel, Switzerland, June 3–7, 1991.

These series of symposia which alternate between Europe and the U.S.A. have become the most important meeting on liquid chromatography for the presentation of original scientific achievements within the most dynamically expanding techniques of separation science. Selected topics regarding the HPLC techniques and applications will be presented in plenary presentations, posters and discussions. All major companies in HPLC-instrumentation will participate in an exhibition. There will be time for informal contacts, exchange of ideas and experiences between participants.

The first circular and call for papers with more details will be available in May, 1990. The deadline for submission of abstracts will be November, 1990. If you are interested in attending HPLC '91 please write to the following address for more information and the first circular: Secretariat HPLC '91, Convention Center Basel, Congress Department, P.O. Box, CH-4021 Basel, Switzerland.

COURSES

ANALYTICAL CHEMISTRY SHORT COURSES, AMSTERDAM, THE NETHERLANDS

The following short courses will be organized by The Center for Professional Advancement in Amsterdam, The Netherlands during the autumn of 1989:
— High-Performance Liquid Chromatography: Fundamentals, Equipment and Operation; October 23–25, 1989; fee US$ 1205.
— Thermoanalytical Methods: Principles, Techniques, Applications and Instrument Demonstrations; October 23–26, 1989; fee US$ 1515.
— High-Performance Liquid Chromatography: Beyond the Basics; October 26–27, 1989; fee US$ 765.
— Characterization of Polymers: Their Analysis and Isolation; October 30–November 3, 1989; fee US$ 1690.
— Fourier Transform Infrared Spectroscopy; November 6–8, 1989; fee US$ 1205.
— Laboratory Information Management Systems (LIMS); November 6–9, 1989; fee US$ 1500.

Further details about the courses may be obtained from: The Center for Professional Advancement, Palestinastraat 1, 1071 LC Amsterdam, The Netherlands. Tel.: (020) 6623050; fax: (020) 797501; telex: 10662 cfpa nl.

BRADFORD ANALYTICAL COURSE ON CHIRAL SEPARATIONS IN HPLC, BRADFORD, U.K., APRIL 1–4, 1990

This four-day Bradford Analytical Course is designed to give a thorough training in the principles and practical applications of chiral separations in HPLC. Participants will have extensive opportunities to familiarise themselves with state-of-the-art chiral technology from many of the leading manufacturers in practical demonstrations.

The range of topics covered in lectures and tutorial discussion sessions will include: principles and implications of chirality in drugs and metabolites; design and utilisation of chiral column technology; development of chiral eluents; principles of stereoselective method optimisation; semi-prep and prep-scale chromatography; chiroptical techniques for method validation (ORD, CD, FT-NMR); applications in chiral quality control, chiral metabolism, therapeutic drug monitoring, clinical biochemistry; and trends in legislative implications for stereoselective drug design and control.

The course is presented by Professor Anthony F. Fell and Dr. Brian J. Clark. supported by a number of acknowledged international experts.

The (£455) includes full documentation. refreshments, lunch and course dinners. Accomodation for 3 nights in the Hall of Residence is £65 (to include bed and breakfast and 2 evening meals).

For further details or registration contact: Dr. Brian J. Clark, Pharmaceutical Chemistry, School of Pharmacy, University of Bradford, Bradford, BD7 1DP, U.K. Tel: (0274) 733466, ext. 585.

CALENDAR OF FORTHCOMING EVENTS

Oct. 1–4, 1989 Boston, MA, U.S.A.	**International Gel Permeation Chromatography Symposium** Contact: Lorraine Carter, Waters Chromatography Division of Millipore, 34 Maple Street, Milford, MA 01757, U.S.A. Tel.: (508) 478-2000.
Oct. 17–20, 1989 Tokyo, Japan	**10th International Symposium on Chromatography, CIS '89** Contact: Tadao Hoshino, Pharmaceutical Institute, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo 160, Japan. (Further details published in Vol. 456, No. 2.)
Oct. 22–27, 1989 Knoxville, TN, U.S.A.	**6th Symposium on Separation Science and Technology for Energy Applications** Contact: Dr. J.T. Bell, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6268, U.S.A. Tel.: (615) 574-4934 or 574-6795. (Further details published in Vol. 456, No. 2.)
Oct. 23–27, 1989 Mexico City, Mexico	**International Chromatography Meeting in Mexico** Contact: Dr. S. Ahuja, Ciba-Geigy Corporation, Old Mill Road, Suffern, NY 10901, U.S.A.
Oct. 29–Nov. 3, 1989 Rehovot, Israel	**8th International Symposium on Affinity Chromatography and Biological Recognition** Contact: E.A. Bayer and F. Kohen, AC&BR Secretariat, The Weizmann Institute, Institute of Science, Rehovot 76100, Israel. (Further details published in Vol. 448, No. 3.)
Nov. 6–8, 1989 Philadelphia, PA, U.S.A.	**9th International Symposium on High-Performance Liquid Chromatographic Separation of Proteins, Peptides and Polynucleotides** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 462.)
Nov. 15, 1989 London, U.K.	**Applications of Evolved Gas Chromatography** Contact: Dr. C.J. Keattch, Honorary Secretary of the Thermal Methods Group, Industrial and Laboratory Services, P.O. Box 9, Lyme Regis, Dorset DT7 3BT, U.K. Tel.: (02974) 2472.
Nov. 20–24, 1989 Eindhoven, The Netherlands	**Course on High-Performance Liquid Chromatography** Contact: H.A. Claessens, Technische Universiteit Eindhoven, Laboratorium voor Instrumentele Analyse, S.H. 2.15, Postbus 513, 5600 MB Eindhoven, The Netherlands.
Jan. 16–18, 1990 Fort Lauderdale, FL, U.S.A.	**6th International Symposium on Separation Science and Biotechnology** Contact: Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.

Jan. 29–31, 1990 San Francisco, CA, U.S.A.	**2nd International Symposium on High Performance Capillary Electrophoresis** Contact: Shirley Schlessinger, HPCE '90, Suite 1015, 400 East Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 475.)
Feb. 4–8, 1990 Bendigo, Australia	**18th Australian Polymer Symposium** Contact: Dr. J.D. Wells, Bendigo College of Advanced Education, Bendigo, Victoria 3550, Australia.
Feb. 22–23, 1990 Antwerp, Belgium	**Symposium on Hyphenated Techniques in Chromatography** Contact: VCV, Section Analytical Chemistry, c/o Dr. R. Smits, BASF Antwerpen, Scheldelaan, B-2040 Antwerp, Belgium. Tel.: (03) 5682831; telex: 31047 basant b.
March 5–9, 1990 New York, NY, U.S.A.	**41st Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Alma Johnson, Program Secretary, The Pittsburgh Conference, 300 Penn Center Blvd., Suite 332, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 475.)
March 7–9, 1990 Gothenburg, Sweden	**International Symposium on Characterization of Macromolecules used as Pharmaceutical Excipients** Contact: Symposium on Characterization of Macromolecules used as Pharmaceutical Excipients, The Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, 111 81 Stockholm, Sweden.
March 14–16, 1990 São Paulo, Brasil	**COLACRO III, 3rd Latin-American Congress on Chromatography** Contact: Dr. Fernando M. Lanças, Universidade de São Paulo, Instituto de Física e Química de São Carlos, 13560 São Carlos, SP Brasil. Tel.: (0162) 726222, ext. 275; telex: (16) 2374 FQSC BR. (Further details published in Vol. 475.)
March 27–June 7, 1990 Uppsala, Sweden	**Uppsala Separation School: Biochemical Separation Methods** Contact: Secretary Ulrika Jansson, Department of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden. (Further details published in Vol. 475.)
April 1–4, 1990 Bradford, U.K.	**Bradford Analytical Course on Chiral Separations in HPLC** Contact: Continuing Education Unit, University of Bradford, West Yorkshire BD7 1DP, U.K. Tel.: (0274) 733466, ext. 585.
April 3–5, 1990 Noordwijkerhout, The Netherlands	**ANATECH '90, 2nd International Symposium on Applications of Analytical Chemical Techniques to Industrial Process Control** Contact: Professor Dr. Willem E. van der Linden, Laboratory for Chemical Analysis-CT, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands (Further details published in Vol. 472, No. 2.)

April 8–11, 1990 Ghent, Belgium	**7th International Symposium on Preparative Chromatography** Contact: Professor M. Verzele, RUG-LOS, Krijgslaan 281 (S4), B-9000 Ghent, Belgium. Tel.: (091) 225715; fax (091) 228321.
Aug. 14–17, 1990 Budapest, Hungary	**Budapest Chromatography Conference** Contact: Intercongress IPV, Dózsa Gy. út 84/a, Budapest, H-1068 Hungary.
April 17–20, 1990 Barcelona, Spain	**2nd International Symposium on Applied Mass Spectrometry in the Health Sciences** Contact: Professor Emilio Gelpí, Palau de Congressos, Dept. de Convensions, Av. Reina Ma. Cristina s/n, 08004 Barcelona, Spain. (Further details published in Vol. 472, No. 2.)
April 17–20, 1990 Strasbourg, France	**20th International Roland W. Frei Memorial Symposium on Environmental Analytical Chemistry** Contact: Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-2123 Allschwil, Switzerland. Tel.: (41) 61-632789. (Further details published in Vol. 472, No. 2.)
April 19–21, 1990 Strasbourg, France	**4th Workshop on the Chemistry and Analysis of Environmental Hydrocarbons** Contact: Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-2123 Allschwill, Switzerland. Tel.: (41) 632789. (Further details published in Vol. 472, No. 2.)
April 24–26, 1990 Pécs, Hungary	**4th Symposium on the Analysis of Steroids** Contact: Professor S. Görög, c/o Chemical Works of Gedeon Richter Ltd., P.O. Box 27, H-1475 Budapest, Hungary. Tel.: (361) 574566, telex: 22-5067 RICH H, fax: (361) 473973. (Further details published in Vol. 472, No. 2.)
April 24–28, 1990 Tallinn, U.S.S.R.	**11th International Symposium on Biomedical Applications of Chromatography and Electrophoresis** Contact: Dr. Ljudmila Kolomiets, Scientific Council on Chromatography, Institute of Physical Chemistry, Leninskii Prospect 31, 117915 Moscow, U.S.S.R.
May 8–11, 1990 München, F.R.G.	**Biochemische Analytik 90, 12th International Conference on Biochemical Analysis** Contact: Ulrike Arnold, Anneli Höhnke, Nymphenburgerstrasse 70, D-8000 München, F.R.G. Tel.: (089) 1234500; fax: (089) 183258. (Further details published in Vol. 475.)
May 20–25, 1990 Boston, MA, U.S.A.	**HPLC '90, 14th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley, E. Schlessinger, Symposium Manager HPLC '90, 400 East Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (312) 527 2011. (Further details published in Vol. 472, No. 2.)
May 22–25, 1990 Dijon, France	**3rd European Meeting on Bio-Chromatography and Molecular Affinity** Contact: J.-P. Dandeu, Groupe Français de Bio-Chromatographie, Institut Pasteur, Unité d'Immuno-Allergie, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45688000. (Further details published in Vol. 456, No. 2.)

June 6–8, 1990 Snowbird, UT, U.S.A.	**3rd Symposium on Computer-Enhanced Analytical Spectroscopy** Contact: Peter C. Jurs, Department of Chemistry, Penn State University, University Park, PA 16802, U.S.A.
June 7–9, 1990 Cancum, Mexico	**International Conference on Chirality** Contact: Mary Jo Richards, University of Missouri-Rolla, Chemistry Department, 341 Schrenk Hall, Rolla, MO 65401, U.S.A. Tel.: (314) 341-4429.
June 12–15, 1990 Maastricht, The Netherlands	**SCA, 2nd Scientific Computing and Automation (Europe) Conference** Contact: SCA (Europe), c/o Reunion International, WG Plein 475, 1054 SH Amsterdam, The Netherlands. Tel.: (020) 165151; fax: (020) 890981. (Further details published in Vol. 475.)
July 9–11, 1990 Wrexham, U.K.	**Ion-Ex 90, International Conference and Industrial Exhibition on Industrial, Analytical and Preparative Applications of Ion-Exchange Processes** Contact: Ion-Ex 90, Conference Secretariat, Faculty of Research and Innovation, The North East Wales Institute, Connah's Quay, Deeside, Clwyd CH5 4BR, U.K. Tel. (0244) 817531, ext. 276 or 234, telex: 61629 NEWI G, fax: (0244) 822002. (Further details published in Vol. 464, No. 2.)
Aug. 7–11, 1990 Changchun, China	**1st Changchun International Symposium on Analytical Chemistry** Contact: Professor Qinhan Jin, Department of Chemistry, Jilin University, Changchun, Jilin 130021, China.
Aug. 20–22, 1990 Stockholm, Sweden	**2nd International Symposium on Microcolumn Separation Methods** Contact: The Swedish Chemical Society, The Analytical Division, Wallingatan 26B, S-111 24 Stockholm, Sweden.
Aug. 26–31, 1990 Vienna, Austria	**Euroanalysis VII, 7th European Conference on Analytical Chemistry** Contact: Professor Dr. M. Grasserbauer, c/o Interconvention, Austria Center Vienna, A-1450 Vienna, Austria. Tel.: (43) 222-2369/647; telex: 111803 icos a, Fax: (43) 222-2369/648. (Further details published in Vol. 445, No. 1.)
Aug. 26–31, 1990 Prague, Czechoslovakia	**10th International Congress on Chemical Engineering, Chemical Equipment Design and Automation** Contact: Congress CHISA '90, P.O. Box 857, 111 21 Prague 1, Czechoslovakia. Telex: 121114 chp c.
Sept. 3–7, 1990 Balatonfüred, Hungary	**6th International Symposium on Ion Exchange** Contact: Professor J. Inczédy, Department of Analytical Chemistry, University of Veszprém, P.O. Box 158, H-8201 Veszprém, Hungary. Tel.: (3680) 22022; fax: (3680) 26016; telex: 32297.

Sept. 23–28, 1990 Amsterdam, The Netherlands	**18th International Symposium on Chromatography** Contact: 18th International Symposium on Chromatography, RAI Organisatie Bureau Amsterdam bv, Europaplein 12, 1078 GZ Amsterdam, The Netherlands. Tel.: (31-20) 549 1212; telex: 13499 raico nl; Fax: (31-20) 464469. (Further details published in Vol. 464, No. 2.)
Oct. 19–23, 1990 Adelaide, Australia	**27th Meeting of the International Association of Forensic Toxicologists** Contact: V.J. McLinden, Chemistry Center (WA), 125 Hay Street, Perth, Western Australia 6000, Australia. (Further details published in Vol. 467, No. 2.)
Oct. 28–31, 1990 San Francisco, CA, U.S.A.	**ANABIOTEC '90, 3rd International Symposium on Analytical Methods in Biotechnology** Contact: Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 448, No. 3.)
Oct. 31–Nov. 2, 1990 Montreux, Switzerland	**7th Symposium on Liquid Chromatography–Mass Spectroscopy (LC–MS, MS–MS, SFC–MS)** Contact: M. Frei-Häusler, Strengigässli 20, CH-4123 Allschwil, Switzerland. (Further details published in Vol. 475.)
March 4–7, 1991 Les Diablerets, Switzerland	**4th Hans Wolfgang Nürnberg Memorial Workshop on Toxic Metal Compounds (Interrelation Between Chemistry and Biology)** Contact: Dr. Ernest Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland
May 27–31, 1991 Rome, Italy	**2nd International Symposium on Chiral Discrimination** Contact: Professor D. Misiti or Professor F. Gasparrini, Laboratori di Chimica Organica, Facoltà di Farmacia, Università "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy. Tel.: (06) 4452900; fax: (06) 49912780.
June 3–7, 1991 Basel, Switzerland	**HPLC '91, 15th International Symposium on Column Liquid Chromatography** Contact: Secretariat HPLC '91, Convention Center Basel, Congress Department, P.O. Box, CH-4021 Basel, Switzerland.
Aug. 21–24, 1991 Kumamoto, Japan	**5th International Conference on Flow Analysis** Contact: Professor Ishibashi, Department of Applied Analytical Chemistry, Faculty of Engineering 36, Kyushu University, Hokazaki, Higashiku, Fukuoka 812, Japan. (Further details published in Vol. 475.)
Sept. 4–6, 1991 Bilthoven, The Netherlands	**3rd Workshop on Chemistry and Fate of Modern Pesticides** Contact: Pesticides Workshop Office Dr. P. van Zoonen, RIVM, P.O. Box 1, 3720 Bilthoven, The Netherlands. (Further details published in Vol. 472, No. 2.)

PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	
Journal of Chromatography	461 462 463/1	463/2 464/1	464/2 465/1 465/2	466 467/1 467/2	468 469 470/1 470/2	471 472/1 472/2 473/1	473/2 474/1 474/2 475	476 477/1 477/2	478/1 478/2 479/1	The publication schedule for further issues will be published later
Bibliography Section		486/1		486/2		486/3		486/4		
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2	490/1 490/2	491/1	491/2	492 493/1	493/2 494	495

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

Dispatch. Before sending the manuscript to the Editor please check that the envelope contains three copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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