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CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin and Science Citation Index)

Contents (continued)

ANNOUNCEMENT

ANALYTICAL METHODS
AND PROBLEMS IN BIOTECHNOLOGY

**An International Symposium, Noordwijkerhout, The Netherlands,
17-19 April 1984**

*(Organized under the auspices of the Section on Analytical Chemistry
of the Royal Netherlands Chemical Society and the Netherlands
Biotechnological Society)*

Scope of the Symposium

The development of analytical methods for biotechnological applications
is an area of growing importance. Analytical methods currently available
are now being adapted for practical use in biotechnological research,
development and industrial production. But a large gap remains to be
bridged between experts in analytical methodology and experts in
biotechnology.
The purpose of the Symposium is to outline the problems faced in this
field and to describe the rapid developments taking place. It is aimed at
an interdisciplinary audience of those involved in industrial and academic
biotechnology, as well as at analytical chemists themselves.

Scientific Programme

The Symposium is expected to cover a wide and representative range of
current research activity on all aspects of analytical chemistry related to
biotechnology. Analytical tools will be presented for process control in
industrial biotechnology, for environmental biotechnology, and for
fundamental research. The Programme will consist of invited plenary
lectures, invited and submitted research papers and discussion sessions.
The official language will be English and the following sessions
are planned: Analytical Strategies, Analytical Techniques, Process
Control.

Further Information

Those wishing to receive the first circular, which will include the topics of
the Symposium, should contact: W.A. Scheffers, Symposium Analytical
Methods and Problems in Biotechnology, Delft University of Technology,
Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft,
The Netherlands. Tel: (015)-78 24 11.

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HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY OF LOW-MOLECULAR-WEIGHT LIGNINS AND MODEL COMPOUNDS

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SUMMARY

An experimental high-performance size exclusion chromatographic procedure to obtain apparent molecular weights and their distribution for low-molecular-weight lignins was developed. Polystyrenes and lignin model compounds were utilized for calibration of the molecular weights. Values found in this study for weight and number average molecular weights for steam-exploded aspen lignins agree well with values reported by other workers using different solvent systems and calibration methods for similar lignins. In addition, possible associative effects were estimated by using one model compound with a free phenolic group and a carbonyl group, acetovanillone. The mobile phase and concentration range were optimized to minimize associative effects. The procedure used proved to be reproducible and to give good resolution of paucidisperse components. An examination of the application of Kirkwood–Riseman theory to the elution behavior of low-molecular-weight polystyrenes and lignin model compounds, is also included.

INTRODUCTION

Lignins[1-6] are irregular phenylpropane polymers which comprise, together with cellulose and hemicelluloses, the cell wall of plant materials. Their precursors are the compounds 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, 4-hydroxy-3,5-dimethoxycinnamyl (sinapyl) alcohol and *p*-hydroxycinnamyl (*p*-coumaryl) alcohol. The proportions of each alcohol depend on the species and on the location in the cell wall. In this work we investigated hardwood lignins which are composed mainly of polymers of coniferyl and sinapyl alcohols. The major type of bond present links the central carbon atom (*β*-position) of the side chain to the aryl group through an ether (*β*-O-4-alkyl aryl ether), although many other types of bonds are also found linking monomeric precursors. Unlike other natural polymers, lignins cannot be degraded to

give structurally intact precursors. Due to the presence of many reactive sites in the molecule, hydrolysis reactions are often coupled to condensation reactions.

Lignins are the major renewable source of phenolic compounds. There are methods of separation of the three major polymeric components of wood which lead to lignin polymers of low molecular weight, high solubility in simple organic solvents and high reactivity[7,8]. Such materials may be of industrial importance in the future. A more detailed knowledge of the relationship between the structural features of lignins and their physical and chemical properties is a prerequisite for commercial application, whether as a source of phenolic compounds or as a replacement for phenol in thermosetting resins. The understanding of the macromolecular properties of lignins requires a detailed knowledge of the molecular weights of these polymers and of their distribution[9,10].

The lignins investigated in this work were obtained from a process called steam explosion. The basic method was discovered by Mason[11] and is applied commercially to the manufacture of hardboards. More recently, however, it was found that the steam explosion method could be applied in the separation of the polymeric components of hardwoods such that the resulting cellulosic fraction was almost totally nutritive to ruminants[12] or could easily be converted by enzymatic or acid hydrolysis into glucose[13]. In this method, chips of hardwood are subjected to steam at pressures of 500–1000 p.s.i., and temperatures of around 200°C, for very short residence times (less than 1 min), after which the steam pressure is suddenly released, turning the chips to a mass of brown fibers. Chemically this is an acid hydrolysis process, the acid (mainly acetic) being generated by the hydrolysis of the acetyl groups of the xylan hemicelluloses. Coupled to the chemical process, the mechanical shock aids the depolymerization and enables most of the repolymerization reactions to be avoided. The product is a finely divided mass of fibers consisting of depolymerized and now water-soluble hemicellulose, aqueous-alcohol-soluble lignin and low-molecular-weight, but crystalline, cellulose[14,15].

The products of steam explosion have been characterized by several researchers. Marchessault and St.-Pierre[15] have investigated lignin obtained by methanol extraction of moist (50 % water) exploded aspen (*Populus tremuloides*), followed by aqueous fractional precipitation. These authors characterized the lignin using elemental analysis, infrared, proton and carbon-13 NMR of unacetylated materials, as well as size exclusion chromatography. Chum et al.[16] have investigated lignin obtained by ethanol extraction of moist exploded aspen after removal of the hemicelluloses by aqueous extraction. A complete functional group analysis (phenolic hydroxy, aliphatic hydroxy, methoxy, carbonyl) based on chemical analyses and on quantitative carbon-13 NMR of the acetylated materials was described. Also, Glasser et al.[17] have reported the characterization of a sodium hydroxide-extracted lignin from steam exploded aspen.

Size exclusion chromatography (SEC) has been employed to yield information on the molecular weights of lignins and their distribution[9,10]. In many instances, data from different authors in different solvent systems do not agree. McCarthy and co-workers[18,19] have investigated associative effects, which have been found to mask the molecular weight distribution. Such effects and a resulting bimodal elution profile were eliminated by increasing the ionic strength of the solvent, *e.g.*, by adding LiCl or LiBr to dimethylformamide or aqueous sodium hydroxide as mobile phase. Obi-

aga and Wayman[20] have also improved the calibration procedures for lignins, using equilibrium ultracentrifugation in conjunction with SEC. However, compared to high-performance SEC (HPSEC), the procedures mentioned above are quite time consuming.

MATERIALS, METHODS AND THEORETICAL

Instrumentation

The HPSEC of lignins and elution standards was performed on a Beckman/Altex 100A solvent pumping system with a Beckman/Altex Model 420 system computer control and manual injection using a Beckman/Altex Model 210 injection valve (Beckman/Altex, Berkeley, CA, U.S.A.). Detection was made at 280 nm with a Waters Model 450 variable wavelength detector. A bimodal column system was constructed by connecting in series three μSpherogel columns (300 × 8.0 mm I.D.) obtained from Rainin Instrument Company (Woburn, MA, U.S.A.). These columns were connected to the injection port in the order of increasing pore size, 100, 100 and 500 Å. The mobile phase consisted of ultra-high-purity dioxane and chloroform from Burdick & Jackson Labs., (Muskegon, MI, U.S.A.). Because of the likelihood of peroxide formation, the dioxane was used fresh for each chromatographic series. The mobile phase was mixed in the appropriate proportions, filtered and deaerated by bubbling with helium before chromatography. The mobile phase flow-rate was 1.0 ml/min, with UV detector attenuation at 0.02–0.40 a.u.f.s. Chromatography was performed with 50-μl injections of 0.2–0.4 % (w/v) lignin solutions. Elution data were recorded on the Beckman/Altex Model C-R1A data processor with electronic integration. The hard copy elution diagrams were manually entered into the computer memory and stored on tape using a Tektronix Model 4052A computer (Tektronix, Beaverton, OR, U.S.A.) and digital tablet. Extended BASIC computer programs were written to allow superposition of chromatographic data and elution data plotted with molecular-weight calibration.

Elution standards and model compounds

The polystyrene standards were obtained from Beckman/Altex. Acetovanillone (AV) (4-hydroxy-3-methoxyphenylethanone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was purified by recrystallization. 1-Benzyloxy-2-methoxybenzene (BMB) (O-benzylguaiacol) was prepared from guaiacol and benzyl chloride (Aldrich) following the procedure of Leopold[21], and was recrystallized from hexane. [1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanone] (BMME) (ω-guaiacylacetovanillone-O-benzyl ether) was prepared by boiling under reflux in acetone ω-bromoacetovanillone-O-benzyl ether (obtained by bromination of O-benzylacetovanillone), guaiacol and potassium carbonate for 1.5 h. The mixture was added to water, and the emulsion extracted with chloroform. The combined chloroform extracts were washed with 1 M sodium hydroxide solution to remove excess of unreacted guaiacol. After removal of the solvent under reduced pressure, the remaining oil crystallized upon standing. Purification was carried out by recrystallization from acetone or ethyl acetate (calc. for $C_{23}H_{22}O_5$; C 73.0%, H 5.82%, OCH_3 16.4%. Found: C 72.05%; H 5.82%; OCH_3 15.9%). 1-(4-Benzyloxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propan-1-one (BMHMP) was ob-

tained by reacting the previous compound, BMME, with formaldehyde and purifying it according to Weinstein and Gold[22].

Lignin samples

The aspen lignin used in this work was isolated from steam-exploded aspen wood supplied by Iotech Corp. (Canada). Aspen chips were subjected to steam explosion at 240°C for 55 sec of residence time. A modification of the procedure described by Marchessault and St.-Pierre[15] was used to separate the lignin fraction from the other polymers and contaminants (extractives). Water extraction for 3 h removed the soluble hemicellulosic fraction (wood:water ratio 11:1). After filtration, the insoluble lignocellulosic fraction was washed twice with water, and the solid stirred with ethanol under nitrogen (same wood:liquid ratio). The filtrate and ethanol washings were combined, and the ethanol removed under reduced pressure. The yield of ethanol-extracted steam-exploded aspen lignin (EESEAL) was 18 % based on the original dry weight of the wood (78 % yield based on Klason lignin). The crude lignin extract was purified by continuous carbon tetrachloride extraction to remove the fatty acids and alcohols which were also extracted by ethanol. The statistical functional group distribution of this lignin was: $C_{9.0}H_{6.7}O_{1.5}(OH)_{1.2}(OCH_3)_{1.2}$[16].

The EESEAL was subjected to fractionation based on differential solubility as a function of pH. EESEAL (3 g) was dissolved in 150 ml of methanol containing 0.1 M tetraethylammonium perchlorate (supporting electrolyte for prior electrochemical studies) at pH 7.0 or 12.0 (adjusted by adding sodium methoxide). The solutions were stirred under nitrogen for 2 h. The methanolic solution was added to 300 ml of deaerated aqueous 0.5 M hydrochloric acid at about 2°C. The very fine precipitate of the acid-insoluble (AI) fraction was then isolated by centrifugation and the supernatant was retained. The acid-insoluble fraction was washed several times by resuspension in water followed by centrifugation and drying in a vacuum desiccator over P_2O_5. The supernatant solution was saturated with KCl and then extracted three times, with ethyl acetate (100 ml each time). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried with $MgSO_4$ and the solvent evaporated under reduced pressure. The final product, the acid-soluble lignin fraction, was a glassy brown material. Yields of the acid-insoluble lignins were between 73 and 67 %, whereas the yields of the acid-soluble lignins were 14 and 25 %, for fractionations carried out at pH 7 and 12, respectively.

Mobile phase selection

The mobile phase chosen was a binary system of dioxane and chloroform. These solvents are considered good in high pressure liquid chromatography (LC) because of their low viscosity, low UV cutoff (< 240 nm) and a low vapor pressure. They are also known to be compatible with cross-linked styrene–divinylbenzene copolymer packing materials like μSpherogel[23]. Also, the solvent strength and solubility parameters are similar to those of benzene[24], a known "good" solvent for polystyrene[25]. It was imperative that the mobile phase chosen was a good solvent for both the polystyrenes and the lignins (see Discussion). Lignin solubitility was examined as the parameter of choice for the dioxane–chloroform proportion. Dioxane and chloroform solutions were prepared in ratios from 10:90 to 90:10 (v/v), in steps of 10 %; the maximum solubility of the lignin crystals was obtained between 70:30 and 50:50. The 50:50 mixture was chosen as the mobile phase.

Calculation of column parameters

The elution volume external to the column bead pores is defined as V_0, and that volume accessible to all molecules is V_t. The volume occupied by the pores is $V_i = V_t - V_0$ and the volume displaced by the bead material, V_s, is equal to the geometrical column volume, V_g, minus V_t[26]. For the chromatography system discussed here these parameters were: V_t = 35.80 ml, V_0 = 15.98 ml, V_g = 41.90 ml, V_i = 19.82 ml and V_s = 6.1 ml.

Elution theory

The classical approach to the treatment of elution behavior by SEC is to attempt to correlate some function of molecular weight f(M), or molecular size, to a function of the solute elution volume, f(V). The elution volume parameter is thought to reflect, in part, the geometry and volume of the pores in the column packing material. When defined as K_d, this parameter is equal to $(V_e - V_0)/(V_t - V_0)$[26]. The elution volume parameter $F(v)$, defined as

$$F(v) = (V_e^{1/3} - V_0^{1/3})/(V_t^{1/3} - V_0^{1/3})$$

has been suggested by Himmel and Squire[27,28] for native proteins eluting from TSK semi-rigid organic gels. Here the gel pores are described as an assembly of cones, crevices and cylinders. When considered simply, the parameter f(M) may be chosen as log M or $M^{1/3}$. Data calculated from several empirical relationships based on these parameters are shown in Table II.

Treatment of small molecule elution

The correlation of gel elution data with small molecules to some appropriate parameter of molecular size is both difficult and poorly addressed in the literature. Molecules of very small size (less than M = 300) that are rigid and similar in shape can be treated according to a constant molecular parameter such as the molecular weight, molecular volume[29] or the longest axis length. The latter approach was shown to work well with polynuclear aromatics[30]. However, as a result of the complexity of the solution conformation of polystyrenes greater than distyrene, a direct examination of the molecular dimensions is difficult. When small molecules and oligomers are non-rigid and non-uniform in molecular conformation, as well as in the degree of and the effect of solvation and solvent draining, these parameters must be considered in evaluating elution behavior.

As a first approximation, polystyrenes of degree of polymerization, DP < 200 appear to be excellent SEC models for the low-molecular-weight lignins. Both series of polymers are polyaromatic with similar solubilities. The basic chemical repeating unit of polystyrene is styrene (M = 104). However, the widely recognized unit for computing polymer dimensions for polystyrene is the C–C single bond[31]. The basic unit length, l_{av}, is then 1.54 Å. Since there are two of these units per styrene monomer, the unit molecular weight is $M_0 = M/2 = 52$. The value σ is defined as equal to M/M_0 and is the number of mass elements (or bonds) per polymer chain.

The statistical theory of linear polymer systems has been widely discussed[31] and allows the calculation (by summing dimensions over all possible conformations) of important macromolecular parameters such as the average end-to-end distance,

h_{av}, and the radius of gyration, R_G. The radius of gyration is an excellant molecular parameter on which to compare the elution behavior of unlike solutes by SEC[32]. Furthermore, the effective hydrodynamic radius, R_e, may provide a further improvement in elution prediction as this parameter includes the contribution of the associated solvent to R_G. Here, $R_e = \xi_f R_g[33]$.

We will develop this parameter R_e for polystyrenes of $2 < DP < 30$ ($4 < \sigma < 60$), as this is the range of interest here for SEC elution behavior. From the Kirkwood–Riseman[34–36] theory the frictional coefficient, f, described for random coil macromolecules is dependent upon, in part, the frictional contributions of each mass element, ζ; then

$$f = \frac{(3\pi^{1/2}/8)6\pi\eta R_G}{1 + (9\pi^{3/2}\eta R_G)/4\sigma\zeta} \tag{1}$$

where η is the viscosity of the solvent.

The term $(9\pi^{3/2}\eta R_G)/4\sigma\zeta$ is assumed small in the treatment of large polymers (*i.e.*, $\sigma \geqslant 100$); however, we must include it to consider the low-molecular-weight polymers of interest here. If one assumes the frictional contribution of each element is $6\pi\eta l_{av}$, then

$$f = \frac{(3\pi^{1/2}/8)6\pi\eta R_G}{1 + 0.153\,(\pi/\sigma)^{1/2}\,\alpha\beta/l_{av}} \tag{2}$$

where α is the interaction parameter which describes the segment effective exclusion volume and β is the effective length per segment (usually $\approx 3l_{av}$). Also $f = 6\pi\eta R_e$, where R_e is the effective hydrodynamic radius, therefore:

$$R_e = \frac{(3\pi^{1/2}/8)R_G}{1 + 0.153\,(\pi/\sigma)^{1/2}\,\alpha\beta/l_{av}}$$

The relation parameter, ξ_f, is equal to R_e/R_G. For high polymers ($\sigma > 1000$), $\xi_f = 0.665$[34]. However, for the low DP oligomers here, we find:

$$\xi_f = \frac{0.665}{1 + 0.271\alpha\beta/\sigma^{1/2}l_{av}} \tag{3}$$

For polystyrenes in ideal solvents, $l_{av} = 1.54$ Å, $\beta = 5.02$ Å and $\alpha = 1$[25]. Eqn. 3 becomes:

$$\xi_f = \frac{0.665}{1 + 0.564/\sigma^{1/2}} = \frac{0.665\,\sigma^{1/2}}{0.564 + \sigma^{1/2}} \tag{4}$$

Continuing[37], we can write

$$R_G = \alpha\beta\sigma^{1/2}/6^{1/2} \tag{5}$$

and:

$$R_e = \frac{(3\pi^{1/2}/8)\alpha\beta\sigma^{1/2}/6^{1/2}}{1 + 0.153\,(\pi/\sigma)^{1/2}\,\alpha\beta/l_{av}} \tag{6}$$

Collecting terms and solving for R_e, we obtain:

$$R_e = \frac{\sigma}{1/l_{av} + (\sigma^{1/2}/0.271\alpha\beta)} \tag{7}$$

For polystyrenes in all solvents we expect $l_{av} = 1.54$ Å and $\beta \approx 3\,l_{av} \approx 4.62$ Å. A specific form of eqn. 7 is:

$$R_e = \frac{1.25\,\alpha\sigma}{0.814\,\alpha + \sigma^{1/2}} \tag{8}$$

For polystyrenes in ideal solvents, $\alpha = 1$ and:

$$R_e = \frac{1.25\,\sigma}{0.814 + \sigma^{1/2}} \tag{9}$$

The interaction parameter α is also equal to AM^x where x is not greater than 0.10 and A is determined experimentally. For polystyrenes in a good solvent[25], $\alpha = 0.521\,M^{0.086} = 0.734\sigma^{0.086}$. Eqn. 8 becomes:

$$R_e = \frac{0.918\sigma^{1.086}}{0.597\sigma^{0.086} + \sigma^{0.5}} = \frac{0.0125\,M^{0.586}}{(0.424/M^{0.414}) + 0.139} \tag{10}$$

Ideally, values for α must be determined experimentally. Here, values have been estimated from literature data for polystyrenes in solvents similar to that chosen for this study. It is also important to note that portions of Kirkwood–Riseman theory are based on the earlier work of Flory[38], where the system is considered ideal ($\alpha = 1$) and ξ_f is constant only for polymers of $\sigma > 1000$. It has been suggested[33], however, that the limit of fit is unknown and may apply to very low values of σ.

It now becomes important to compare "actual" values of R_e found through molecular volumetric estimation with those found from Kirkwood–Riseman treatment (eqn. 10). As this is only possible for reasonably small oligomers, we will use PS 1 and PS 2.

Steam exploded lignin is believed to be essentially linear (DP < 5) since acid hydrolysis cleaves the branching α-ether bonds[7]. This generates a significant problem for calculation of statistical solution conformations for high DP lignins, however, as the concept of end-to-end distance is altered[39]. Also, the question of appropriate values for M_0 is not insignificant. For the lignin model compounds BMB, BMME and BMHMP, eqn. 3 must be used to describe ξ_f. R_e is then calculated from R_G. If we assume that l_{av} for these compounds is also 1.54 Å (the C–C bond distance) then $\beta \approx 3\,l_{av} \approx 4.62$ Å. As a first approximation, we will assume that for BMB, BMME and BMHMP the number per molecule of C–C or C–O single bonds separat-
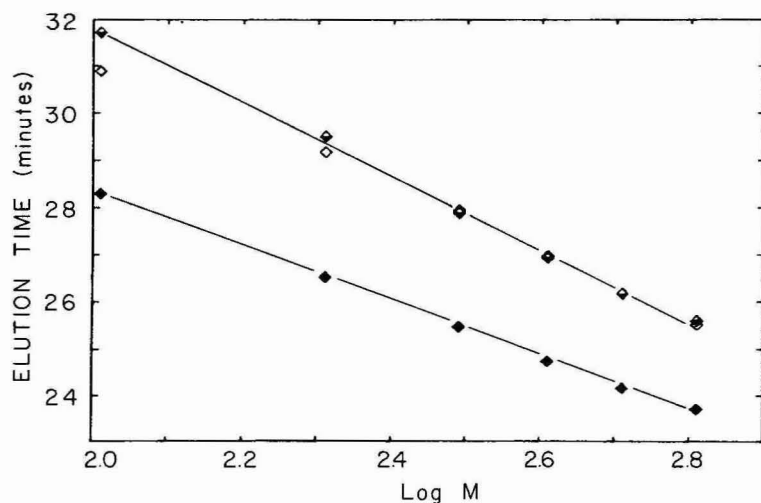
Fig. 1. Comparison of elution time and log M of low-molecular-weight polystyrenes at a flow-rate of 1.0 ml/min in different solvent systems. Closed squares, open squares and half-open squares correspond to data from dioxane–chloroform (10:90, 50:50 and 90:10, v/v).

TABLE I

ELUTION STANDARDS

No.	Name	$M \times 10^{-3}$	$V_e = t_e$ (min)
1	PS	3000.00	15.97
2	PS	275.0	15.95
3	PS	148.00	15.98
4	PS 288	30.10	16.05
5	PS 87	9.000	17.95
6	PS 27	2.820	20.50
7	PS 9	0.936	24.35
8	PS 7	0.728	24.15
9	PS 6	0.624	25.60
10	PS 5	0.520	26.18
11	PS 4	0.416	26.94
12	BMHMP	0.408	26.38
13	BMME	0.378	27.45
14	AV 2	0.332	25.44
15	PS 3	0.312	27.89
16	BMB	0.214	29.08
17	PS 2	0.208	29.18
18	AV 1	0.166	27.60
19	PS 1	0.104	30.89
20	Benzene	0.078	33.10
21	Methanol	0.034	33.78
22	water	0.020	35.80

TABLE II

DATA FROM LINEAR REGRESSION

Function	Relative error*	Correlation** coefficient
$\log M$ vs. K_d	0.100	−0.9967
$M^{1/3}$ vs. $K_d^{1/3}$	0.134	−0.9957
$M^{0.50}$ vs. $K_d^{1/3}$	0.361	−0.9838***
$M^{1/3}$ vs. $F(v)$	0.320	−0.9767***
$M^{1/3}$ vs. K_d	0.395	−0.9643***

* Calculated as the standard deviation, $\sigma = [1/(N - 1)]^{1/2} [(M_{calc.} - M_{true})/M_{true}]^{1/2}$.

** Calculated for the linear portion of the elution curve (Nos. 4–19, except AV 1 and AV 2).

*** Distinct curvature is observed.

ing the aromatic rings is equal to σ. This argument is similar to that developed for polystyrenes (above). For BMB, BMME and BMHMP, σ and M_0 are: 3 and 71, 7 and 54 and 7 and 58 respectively. We will also compare values of R_e found for lignin model compounds (BMB, BMME and BMHMP) from direct molecular volumes to values of R_e for polystyrenes found from statistical treatment. Calculations from molecular models (Framework Molecular Models; Prentice-Hall, NY, U.S.A.) led to the estimation of the molecular volume of model compounds with $M < 400$. Assuming these small oligomers may be treated as spheres, the geometrical radius is $R = (3V/4\pi)^{1/3}$ and the radius of gyration is defined as $R_G = (3/5R^2)^{1/2}$ (ref. 40). R_e is then found from R_G and ξ_f.

RESULTS

Polystyrene oligomers of DP 1–6 were chromatographed in three solvent systems for a comparison of column packing swelling and elution aberrations. These solvent systems were dioxane–chloroform (10:90, 50:50 and 90:10, v/v). Examination of these elution data (Fig. 1) shows that changes in elution time are minimal with over 50 % dioxane mixtures. This may reflect an equilibrium condition for solvent interaction with the styrene–divinylbenzene copolymer gel, resulting in pore size stability. Dioxane–chloroform (50:50, v/v) was therefore chosen as the mobile phase.

The elution behavior of 14 polystyrene standards and 5 model compounds is presented in Table I. These data were plotted as various functions of molecular weight (M, $M^{1/3}$, $M^{0.50}$) vs. functions of elution volume [K_d, $K_d^{1/3}$, $F(v)$]. Calculated data from the five plots showing the highest correlation coefficients are included in Table II. A standard deviation of 10 % in M was found for $\log M$ vs. K_d. This function, and $M^{1/3}$ vs. $K_d^{1/3}$, showed no evidence of curvature throughout the included volume. The other three functions showed severe curvature over this molecular weight range. A plot of $\log M$ vs. K_d for the data in Table I is shown in Fig. 2. The solid squares indicate the elution of the model compound acetovanillone, which exhibits association. This anomalous behavior is examined in the Discussion. The excluded volume appears to be 15.98 ml from the chromatographic data of the three high-molecular-weight polystyrenes examined. The estimation of V_t was made from the detection of water in water-saturated mobile phase injected as a sample. Also,
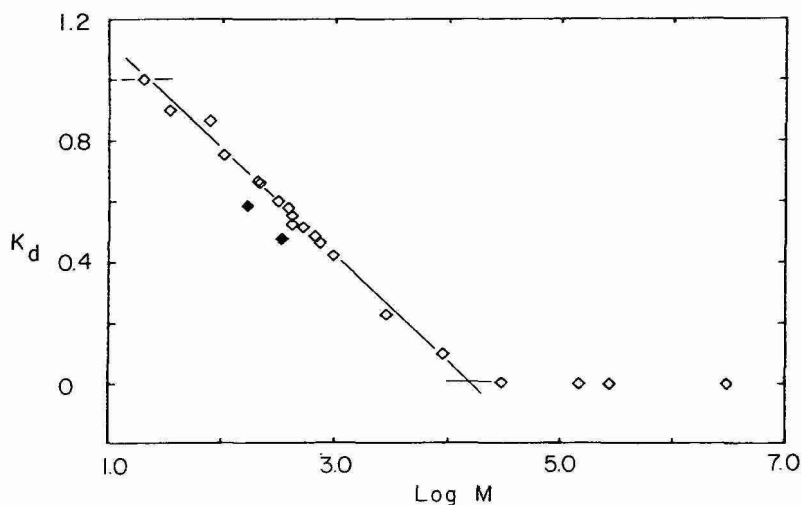
Fig. 2. Plot of log M and elution volume parameter, K_d. Open squares from left to right are the elution standards (1–22) from Table I. The linear regression equation for these data is $K_d = 1.47 - 0.324 \log M$. Closed squares represent acetovanillone monomer and dimer.

elution at this volume, 35.80 ml, was observed routinely with lignins and some model compounds.

Acetovanillone (AV), a possible lignin degradation product, was subjected to a series of experiments designed to test for association behavior in this solvent system. The concentration dependence of association was found by injecting 50 μl of acetovanillone from serial dilutions in mobile phase, yielding 500–5 mM samples.
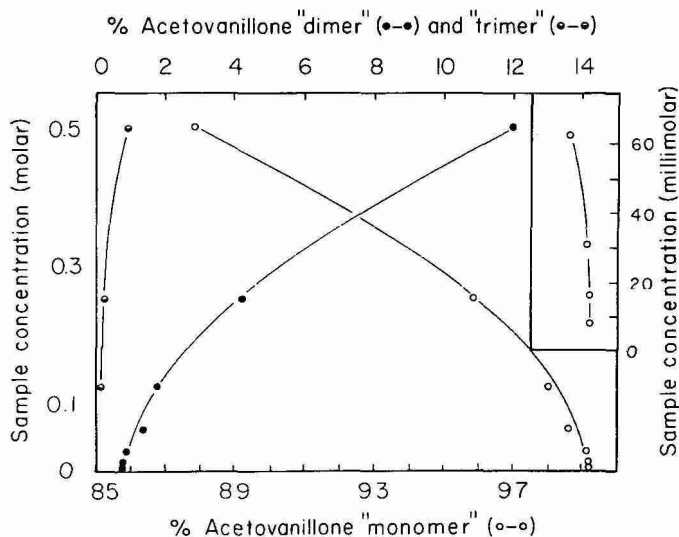
Fig. 3. Association study of acetovanillone from molar to millimolar sample concentration. The sample percentage was taken from electronic peak integration.

APPARENT MOLECULAR WEIGHT (× 10^{-1})
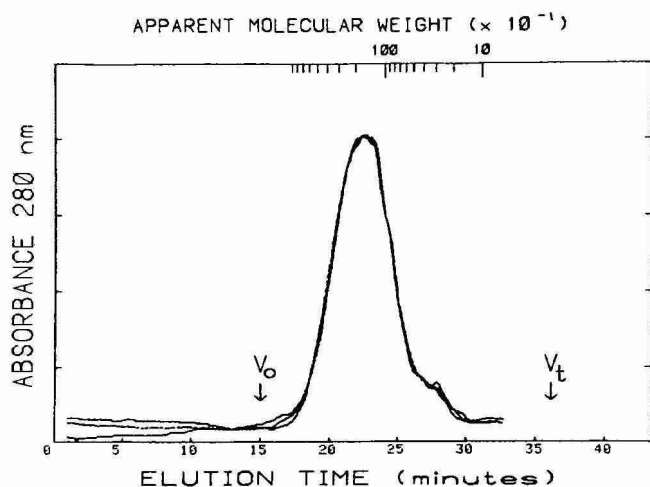
Fig. 4. The apparent molecular weight distribution and elution profile of EESEAL upon serial dilution in the dioxane–chloroform (50:50, v/v) solvent. Molecular weight calibration alignment is taken from the linear regression data in Fig. 2.

These data are shown in Fig. 3. It is apparent that AV is 99.3 % monomeric at sample concentrations below 10 mM.

The chromatography of EESEAL is shown in Fig. 4. This figure also illustrates the superposition of chromatograms from three serial dilutions of this lignin from 0.45 to 0.15 % (w/v). With an injection volume of 50 μl, the sample loading is 125–4.1 nmoles (assuming $M = 2000$). The excellent agreement in the elution curves indicates that chromatography was performed at sample loads low enough to eliminate associative effects. Lignin curves are presented with molecular calibration generated
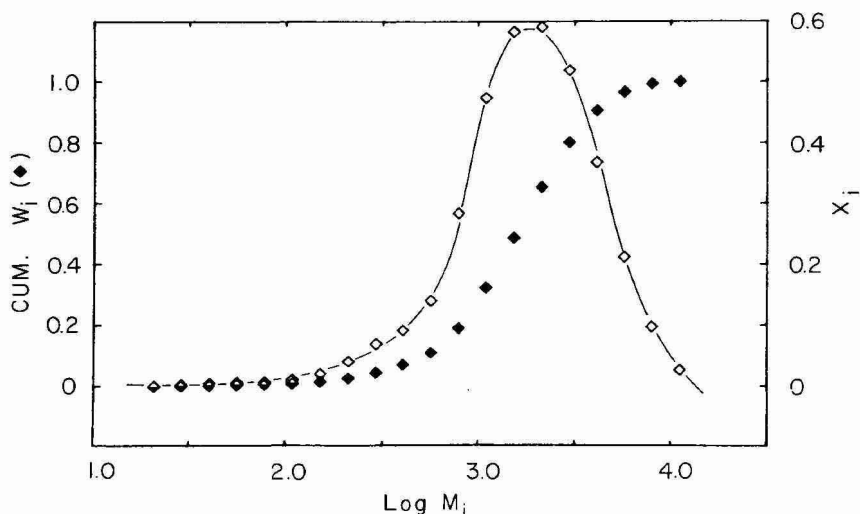
Fig. 5. The cumulative weight average (■) and molecular weight frequency distribution (□) of the EESEAL shown in Fig. 4.

APPARENT MOLECULAR WEIGHT (× 10⁻¹)
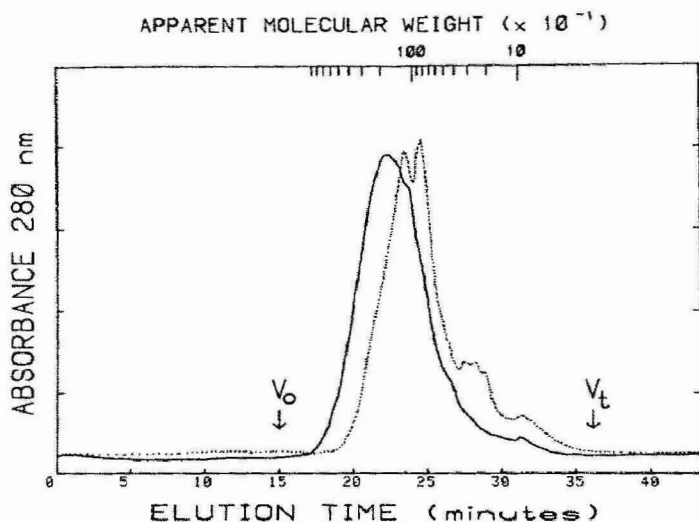
Fig. 6. The elution profile of fractionated EESEAL into acid-insoluble (——) and acid-soluble (····) lignin fractions, from methanolic solution at pH = 7.

from the plot of log M *vs.* K_d for the compounds in Table I. Fig. 5 shows the results from hand integration[41] of the lignin curve shown in Fig. 4. The weight-average molecular weight, $\bar{M}_w$, and the number-average molecular weight, $\bar{M}_n$, were found to be 1980 and 925, respectively. The ratio $\bar{M}_w/\bar{M}_n = 2.14$.

The chromatograms of the two fractions of EESEAL —the acid-insoluble and acid-soluble lignins, prepared as described in Materials, methods and theoretical, from methanolic solutions at pH = 7 and pH = 12 are shown in Figs. 6 and 7, respectively. The fractionation procedure employed separates the high-molecular weight acid-insoluble fraction from the low-molecular-weight fraction, which demonstrates paucidispersity. These components are seen to increase when the fractionation is carried out

APPARENT MOLECULAR WEIGHT (× 10⁻¹)
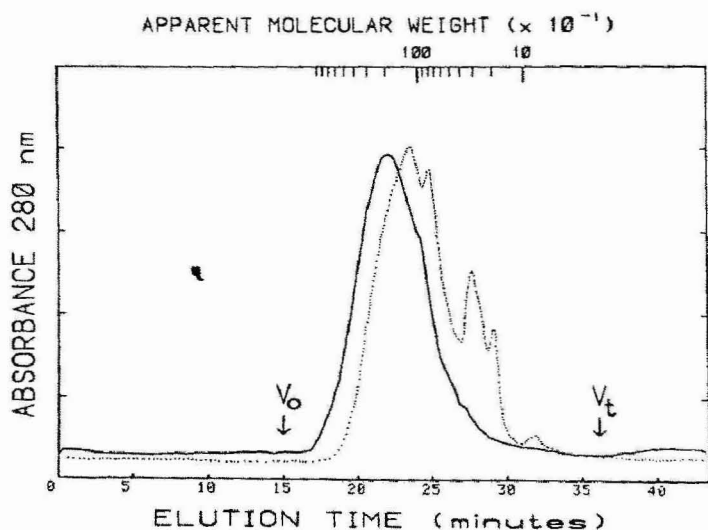
Fig. 7. The elution profile of fractionated EESEAL into acid-insoluble (——) and acid-soluble (····) lignin fractions, from methanolic solution at pH = 12.
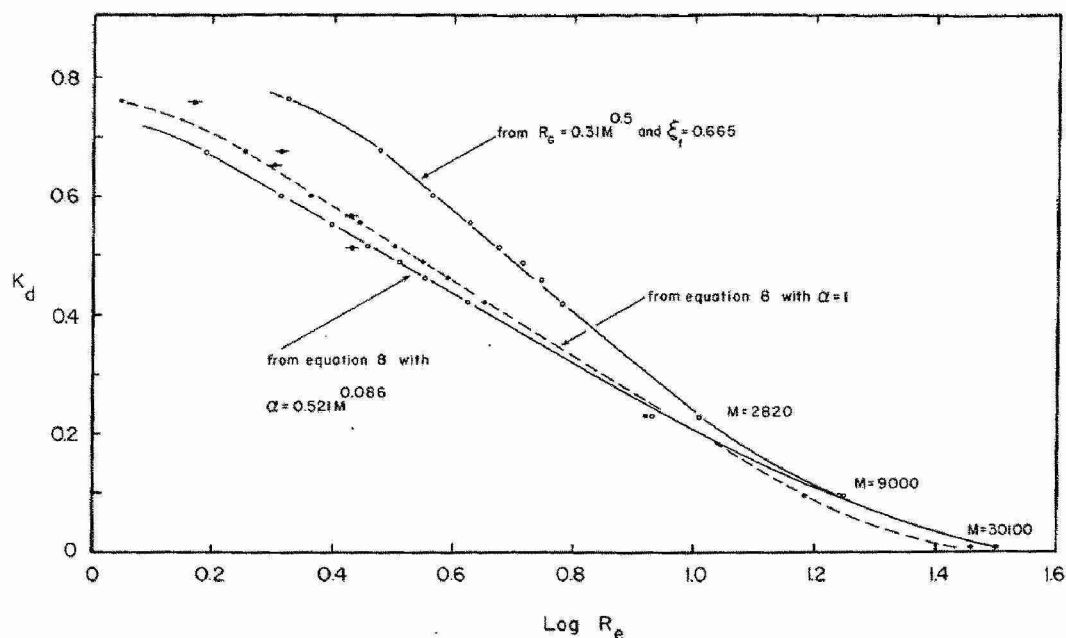
Fig. 8. Comparison of calculated values for R_e found statistically with elution volumes for the polystyrene standards (PS 1 to PS 288 from left to right). Several model compounds are also plotted with values found by direct volume measurement (solid circles with error bars for uncertainty in molecular volume). These model compounds, from left to right, are: PS 1, BMB, PS 2, BMME and BMHMP.

at pH = 12. Average molecular weight values found for the AI fraction are shown in Table III.

Although the lignins examined in this study were described in terms of apparent molecular weights found from direct polystyrene calibration, it is worthwhile to compare the elution of lignin model compounds when described in terms of the effective hydrodynamic radius, R_e. Fig. 8 shows the comparison of values of R_e calculated by several methods from the known molecular weights of polystyrenes of $\sigma < 600$ when plotted against the elution parameter, K_d. R_e values for styrene and distyrene, as well as BMB, BMME and BMHMP, are also plotted in Fig. 8 (as solid circles). For these compounds, values of R_e were found by volumetric approximation of R_G, followed by the definition of ξ_f in terms of several specific assumptions (see Theoretical). The agreement observed in Fig. 8 for these values of R_e is quite good.

DISCUSSION

The HPSEC of polystyrenes and lignin model compounds on μSpherogel columns in dioxane–chloroform (50:50, v/v) proved to be well-behaved and reproducible. The highest correlation coefficients from these data were obtained by plotting log M vs. K_d. The standard deviation from this function was $\pm 0.10M$. Acetovanillone was found to elute early (the solid squares in Fig. 2). Unlike the model compounds BMB, BMME and BMHMP which contain protected phenolic hydroxy groups and benzyl groups and appear to chromatograph predictably, acetovanillone behaves as if the solvent is a "poor" solvent. Because acetovanillone contains an unprotected phe-

TABLE III

COMPARISON OF HPSEC DATA ON STEAM-EXPLODED ASPEN (*POPULUS TREMULOIDES*) LIGNINS

THF = Tetrahydrofuran; DMF = dimethylformamide. N.A. = Not available.

Isolation of lignin (gun conditions)	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_w$	Mobile phase	Lignin concentration (%, w/v)	Column material	Detection technique	Ref.
Methanol–water extraction, repre-cipitation with water (234°C, 45 sec)	700	1700–1900	2.4–2.7	THF	0.5	TSKG4000H8 + G2000H8 or G2500H8 × 2 (Toyo-Soda)	Differential refractometry and light scattering	8
Ethanol–water* extraction; EESEAL (240°C, 55 sec)	925	1980	2.1	Dioxane–chloroform (50:50, v/v)	0.15–0.45	μSpherogel 100 × 2 + 500 Å	UV	This work
Al fraction from EESEAL (pH = 12)	1070	2130	2.0	Dioxane–chloroform (50:50, v/v)	0.15–0.45	μSpherogel 100 × 2 + 500 Å	UV	This work
NaOH extraction** (N.A.)	800–1200***	2600	3.25–2.2	DMF–0.1 *M* LiBr	N.A.	μSpherogel 500 + 1000 +	UV	10
NaOH extraction* (N.A.)	600	1900	3.5	DMF–0.1 *M* LiBr	N.A.	μSpherogel 500 + 1000 + 10,000 + 100,000 Å	UV	10

* Sample from Iotech Corp., 1981.
** Sample from Iotech Corp., 1978.
*** Obtained by osmometry.

nolic hydroxy group and an α-carbonyl group, the segment–segment contacts are preferred to solvent–segment contacts. This condition may lead to an increased effective molecular volume and early elution. However, we have also shown that at low molar concentrations these associative effects are largely minimized (see Fig. 3).

The steam-exploded aspen lignins examined in refs. 15–17 are different due to the different methods of isolation and purification, and also to the different explosion conditions employed. The results of the various molecular-weight distributions from those references are assembled in Table III, and are compared with our present data. The data of Marchessault and St.-Pierre[15] and ours agree reasonably well. The more severe explosion conditions in our case justify the higher $\bar{M}_w$ and $\bar{M}_n$ observed. As expected, the method of extraction of lignin greatly influences its polydispersity. Solvent extraction leads to materials with polydispersities of 2–2.5, whereas larger values (≈ 3) are found in the higher-yield extraction by sodium hydroxide.

It does seem from all of these data (Table III) that on the column materials employed and in the various mobile phases used, association is not a major effect. The good resolution of the various oligomers achieved in the chromatograms of the acid-soluble fractions in the present work is gratifying.

Although the empirical relationship log M vs. K_d describes reasonably well the elution behavior of intermediate-molecular-weight polystyrenes, i.e., S.D. $= \pm 10\%$ M, the comparison of polymers of different structures by molecular weight alone cannot always allow correct elution prediction. A more generic parameter for elution behavior, such as R_e, should be a better comparative measure. However, the calculation of values of R_e for polymers of model compounds of lignins or even polystyrenes of low σ is not straightforward. Fig. 8 demonstrates the results of one such comparison. From light scattering data, the relationship $R_G = 0.31 M^{0.50}$ was established for polystyrenes from $M = 40,000$ to 5×10^6 in a good solvent at $25°C$[42]. At high values of $\sigma, \xi_f = 0.665$. The upper solid line in Fig. 8 was then calculated from the values of M in Table I. A statistical approach for the calculation of R_G (eqn. 5) and ξ_f (eqn. 3) yields eqn. 8 (see Theoretical). The lowest solid curve in Fig. 8 was found using eqn. 8 with $\alpha = 0.521 M^{0.086}$. This value for α was determined from viscosity data for polystyrenes of $40,000 < M < 5 \times 10^6$ in benzene[25], which is considered a "good" solvent for the polystyrenes. The upper and lower solid curves in Fig. 8 superimpose at high values of M, e.g., 9000 and 30,100. This is very satisfactory as the upper curve is generated largely from experimental data and the lower from a statistical solution-conformation treatment. The dashed line in Fig. 8 indicates elution data plotted with R_e found from one limiting condition for R_e, where $\alpha = 1$. This condition reflects the maximum contraction of the polymer in an "ideal" solvent, and is observed in Fig. 8 at values of $M > 2000$. A crossover point of the curve from eqn. 8 with $\alpha = 1$ and the curve with $\alpha = AM^x$ occurs at approximately $\sigma = 36$. This point may reflect the limit in one or several assumptions in extending the high polymer theory to values of σ lower than 36. It is doubtful that l_{av} or β are in error here as these parameters are primarily molecular weight independent and only slightly temperature dependent. However, failure in these assumptions at values of $\sigma = 1$ or 2 probably leads to the deviation seen at low values of r_e, i.e., for styrene. The parameter α is highly dependent, however, on molecular weight and solvent. The assumption that $\alpha = 0.521 M^{0.086}$ at σ values less than 36 may prove incorrect. The solid circles with error bars in Fig. 8 represent the elution behavior of PS 1 and PS

2 as well as BMHMP, BMME and BMB. Here R_e was calculated from volumes found from geometrical models and with ξ_f calculated using eqn. 4. The error bars represent the level of confidence in estimating possible conformations obtainable in solution. A difficulty arises in the assignment of σ (or M_0) for BMHMP, BMME and BMB. Clearly, from Fig. 8, the fit of R_e found volumetrically for PS 1 and PS 2 (for which M_0 is known) is good. However, the spread of data here shows the probable compounding of small errors which render distinction between good or ideal solvent conditions impossible.

Although some observations have been made on the correlation of values of R_e to elution volume of intermediate polystyrenes, more primary data are needed to find values of R_e for the low-molecular-weight lignins. Light scattering measurements made in HPSEC solvents and values of intrinsic viscosity are needed to substantiate R_G and α, respectively. Alternatively, the lignin polymer may be fractionated by HPSEC, followed by the direct determination of molecular weight averages for these fractions by sedimentation equilibrium. We are presently fractionating these lignin polymers and plan to subject them to sedimentation equilibrium studies as well as careful characterization (analytical and spectroscopic). However, the absence of curvature in the data plotted with the empirical relationship log M vs. K_d indicates that this approach is a good first approximation to the true lignin molecular weights. Furthermore, good agreement was shown in values of R_e found for low DP polystyrenes and lignin model compounds by molecular volumetric estimation and statistical polymer theory. This indicates the validity of the application of Kirkwood–Riseman theory to polymer solution dynamics and HPSEC elution behavior. Eventually, this approach may lead to the determination of polymer molecular weights from elution behavior based on column calibration in terms of R_e. For now, the relationship of molecular weight to R_e, specific for each polymer–solvent system, would be determined by a direct method. Hopefully, future extensions of Kirkwood–Riseman treatment will allow prediction of polymer solution conformation from chemical composition alone.

ACKNOWLEDGEMENTS

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GRADIENT ELUTION IN SUPERCRITICAL FLUID CHROMATOGRAPHY

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SUMMARY

The applicability to supercritical fluid chromatography (SFC) of elution by gradients in eluent composition is demonstrated, using alkanes and diethyl ether as the primary eluent and alcohols, cyclohexane and dioxane as the secondary component. For the separation of styrene oligomers, the combination of an alkane with dioxane turned out to be the most efficient. The SFC apparatus used was a modified high-performance liquid chromatographic instrument.

INTRODUCTION

Solutions to chromatographic problems with respect to optimization of resolution and analysis time often require the application of gradients. Among the substrates requiring gradient techniques are oligomer and polymer systems. The need to work with non-linear gradients arises from the fact that within a homologous series the differences in physical properties of the molecules, *e.g.*, solubility, become smaller with increasing molecular weight.

In supercritical fluid chromatography (SFC), gradients in pressure, temperature and eluent composition can be used. Whereas the usefulness of pressure gradients has already been established for a longer period of time (*cf.* recent reviews[1-3]), temperature gradients have been shown to be of less importance[4,5]. Gradients in eluent composition have been applied to SFC only lately but have already shown encouraging results[6,7]. In this paper, we report some results on the application of eluent gradients to oligomers of styrene and 2-vinylpyridine using diethyl ether and alkanes as the primary eluents.

EXPERIMENTAL

The SFC apparatus used for the investigation was a modified high-performance liquid chromatographic (HPLC) instrument (Model 1084B, Hewlett-Packard). It closely resembles the SFC apparatus described previously[5] and is shown schematically in Fig. 1.

Eluents that boil below 50–70°C are pre-pressurized in steel storage tanks (1) by applying a helium pressure. Alternatively, pre-pressurization can be accomplished
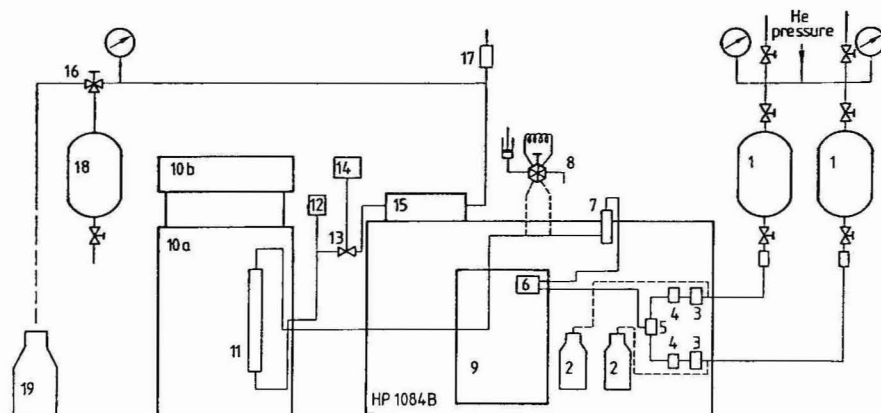
Fig. 1. Schematic diagram of SFC apparatus.

by increasing the temperature of the storage tank above ambient, using an external heating tape. If one of the components of the mobile phase possesses a sufficiently high boiling point, it can be fed without pre-pressurization from the glass bottles (2) provided with the HPLC instrument. The components are metered separately in the liquid state by the pumps of the Model 1084B instrument (3) and consecutively pass the damping system combined with a device for measuring the feed rate (4), the mixing chamber (5), a filter (6) and the variable-volume injection system (7). For work with low-boiling eluents, *e.g.,* propane and butane, as a primary component, a multiport-valve (8) is used for injection instead of the variable-volume injector. The stream then enters the oven of the Model 1084B instrument (9).

For SFC operation, the stream is usually led directly out of the unheated oven to a larger external oven (10a) with forced air circulation (W. C. Heraeus, Hanau, F.R.G.; UT-5042 EK). This external oven can be heated to higher temperatures (300°C) and also accommodates larger columns. In addition, the oven can be temperature programmed by a programmer (10b) (W. C. Heraeus, Kelvitron TPG 2) equipped with a Pt-100 temperature sensor. The stream of mobile phase which is in the supercritical state within the oven passes through the separation column (11) and is allowed to liquefy after having left the oven. The pressure at the column end is monitored by an electric pressure transducer (12) (Siemens, Messumformer Teleperm D, M 56 441), while the pressure upstream of the column is measured by the pressure transducer of the Model 1084B instrument itself. Control of the pressure at the column end is provided by a needle metering valve (13). For creating pressure gradients, the stem of this valve can either be connected to a motor which is controlled by a pressure feedback loop or more simply with the potentiometer axis of a 'cut-a-curve' electromechanical time programmer (14). The eluate is then returned to the Model 1084B instrument, specifically to its UV detector. An external metering valve (16) allows the pressure in the detector to be raised to a level that prevents the formation of gas in the mobile phase. To keep the pressure constant and to protect the detector, an adjustable spring-actuated safety valve is provided (17). The eluent is finally collected in a pressure-resistant metal container as a liquid under its own vapour pressure or, in the case of higher boiling eluents, in a glass bottle (19).

The mobile phases, except for butane and propane, were distilled from sodium and degassed. Diethyl ether and 1,4-dioxane were treated with iron (II) sulphate in addition in order to remove peroxides. Butane (99.5%) and propane (99.5%) (Linde, Höllriegelskreuth, F.R.G.) were used without pre-treatment. Pentane and diethyl ether were fed to the chromatograph with pre-pressures of 0.2–1 bar, and propane and butane of 12–15 bar.

The stainless-steel columns (25 cm × 4.6 mm I.D.) were packed with Li-Chrosorb Si 100 (Merck, Darmstadt, F.R.G.) by means of a slurry method. The oligostyrene sample used in this investigation was PS 800 (number-average molecular weight, $\bar{M}_n$ = 810 g/mol; ratio of weight-average molecular weight to number-average molecular weight, $\bar{M}_w/\bar{M}_n \leqslant 1.12$; from Pressure Chemical, Pittsburgh, PA, U.S.A.; the data are as given by the manufacturer). The oligo(2-vinylpyridine) sample was prepared by azobisisobutyronitrile-initiated radical oligomerization as described in detail elsewhere[8].

The baseline shifts in the chromatograms are due to different UV absorptions of the different eluent components; this effect is less pronounced in Figs. 4–6 according to higher detector attenuation.

Chromatograms were either run with the column end pressure kept constant, i.e. variable valve setting, or else keeping the valve setting constant, thereby incurring a change in column end pressure during the run.

RESULTS

The primary components diethyl ether and linear alkanes are known to be solvents for low-molecular-weight oligostyrenes. In particular, pentane has been shown to be a suitable eluent for the separation of oligostyrenes by SFC. The choice of the secondary eluents cyclohexane and dioxane was based on the increased solution power of these solvents for oligo- and polystyrenes compared with linear alkanes and ethers. Thus an increasing content of cyclohexane or dioxane in the eluent mixture is expected to reduce the retention times, especially for the higher members of the homologous oligostyrene series. Alcohols, on the other hand, are poorer solvents than diethyl ether and linear alkanes for oligostyrenes, and therefore a decrease in alcohol content should show a comparable effect. For oligo(2-vinylpyridine), however, the solubility behaviour in alkanes and alcohols is different to that with oligostyrenes: the solubility in alkanes is negligibly small and methanol is an excellent solvent. Therefore, chromatography of oligo(2-vinylpyridines) has to be carried out with an increasing methanol content. The results obtained for the two oligomer systems are described below for each component pair.

Diethyl ether–ethanol

As mentioned, a negative ethanol gradient had to be applied for oligostyrenes in order to increase the elution power of the eluent. A typical chromatogram is shown in Fig. 2a. This eluent pair turned out to be difficult. Although steep eluent composition gradients were applied, it was difficult to compensate for the broadening of the peaks with elution times longer than 10 min. Also, the pressure decrease due to a decreasing ethanol content with a given valve setting (valve 13 in Fig. 1) necessitated pressure regulating. Regulating the pressure, however, was difficult, as erratic pressure changes occurred. These pressure changes probably arise from particles of
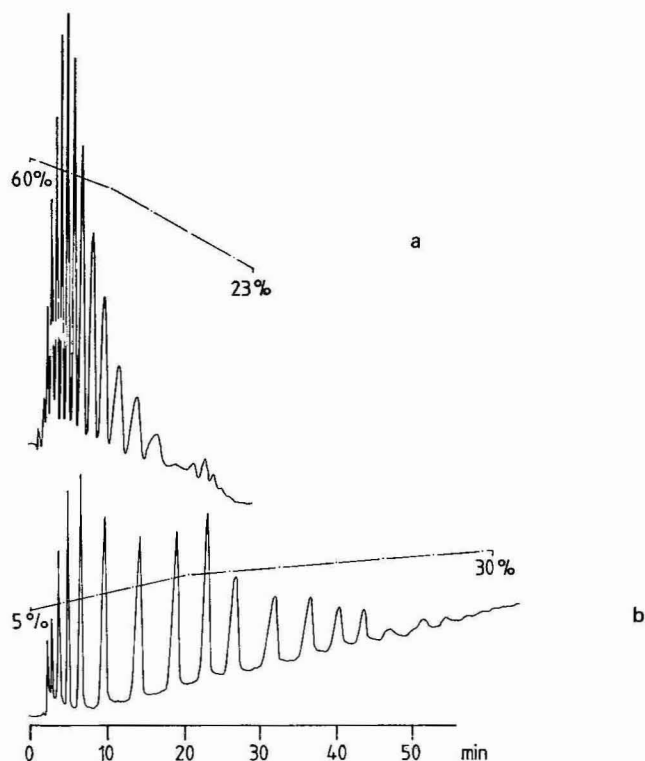
Fig. 2. Chromatograms of PS 800 with diethyl ether as the primary component. (a) Diethyl ether–ethanol; initial column end pressure 70 bar, increased after 20 min to 90 bar to shorten the analysis time. (b) Diethyl ether–dioxane; initial column end pressure 56 bar. ———, Detector signal; - · – · – · , content of ethanol and dioxane in the eluent mixture. Stationary phase: LiChrosorb Si 100, 10 μm. Detection: UV, 254 nm. Sample solvent: dioxane. Flow-rate: 1 ml/min. Oven temperature: 250°C.

silica gel that arise from a significant dissolution process involving the stationary phase in ethanol-containing supercritical mobile phase.

Diethyl ether–dioxane

This eluent pair was much more suitable for SFC experiments with oligostyrenes than the preceding one. The eluent composition gradients could be less steep and the pressure increased with increasing dioxane content at a given setting of the valve at the column end. Thus the effect of the eluent gradient was not counteracted as was the case with diethyl ether–ethanol. Also, no erratic pressure changes were observed. Fig. 2b shows a separation of the PS 800 sample. The applicability of this eluent pair was not restricted to low-molecular-weight oligostyrenes, and oligomers up to a degree of oligomerization of about 40 could be resolved fairly well.

Pentane–cyclohexane

The significant difference in the solubilities of oligostyrenes for the two components of this eluent pair led to gradients similar to those with diethyl ether–dioxane, as is demonstrated in Fig. 3. In order to eliminate any doubts about whether the
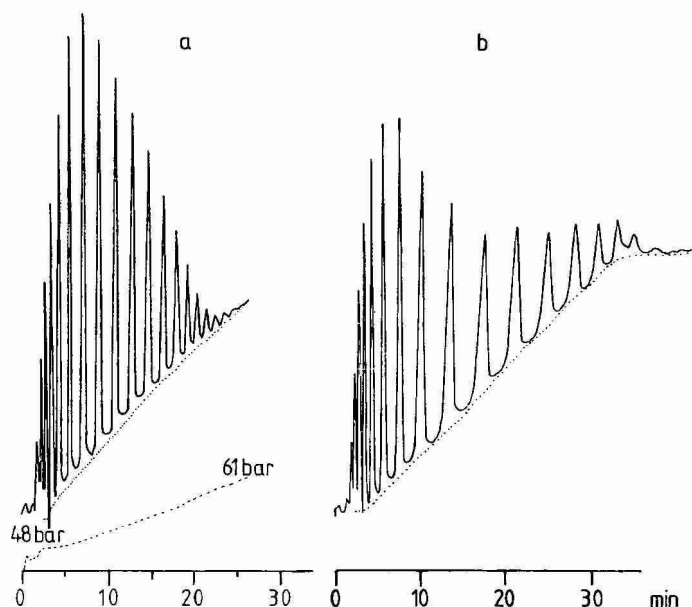
Fig. 3. Chromatograms of PS 800 with pentane–cyclohexane. Linear gradient: 5 to 30% cyclohexane within 30 min. (a) Run with pressure increase at a fixed valve setting; (b) run at constant pressure of 50 bar. ———, Detector signal; ......, baseline; --------, column end pressure. Stationary phase: LiChrosorb Si 100, 7 μm. Detection: UV, 262 nm. Sample solvent: hexane. Flow-rate: 1 ml/min. Oven temperature: 250°C.

chromatographic resolution obtained is due to the eluent gradient or to the pressure increase during the gradient, both causing better solubility of the sample, runs were carried out with the column end pressure maintained constant (Fig. 3b). It can be seen that the pressure increase is indeed responsible for part of the effect. The main influence, however, is due to the increasing amount of cyclohexane, whereby equidistant peaks may be obtained without a pressure increase by changing the gradient programme.

Alkanes–dioxane

This combination proved to be most suitable for oligostyrenes, as shown by the examples in Figs. 4–6. A comparison between the four alkanes is made in Figs. 4 and 5. It was found that the pressure and/or the dioxane content needed for baseline resolution within 80 min or less for PS 800 decreases on going from propane to hexane. The column temperature of 240°C was the same for all the chromatograms in Figs. 4 and 5, except for Fig. 4b, where the temperature had to be increased to 275°C on account of the relatively high critical temperature of hexane. The critical data of the eluent components are given in Table I.

Another interesting feature can be derived from Figs. 4 and 5: it appears to be easier to achieve good resolution for low-molecular-weight oligomers with low-boiling alkanes as primary eluents. On the other hand, elution of oligomers of high molecular weights with the higher alkanes requires lower working pressures. At the end of the chromatogram in Fig. 4a, the column end pressure reached about 140 bar,
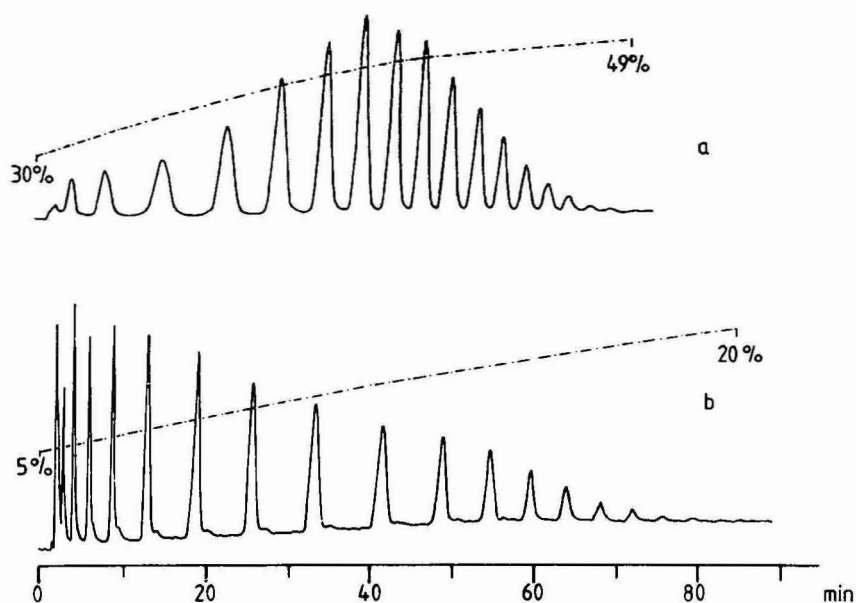
Fig. 4. Chromatograms of PS 800 with (a) propane–dioxane and (b) hexane–dioxane. ———, Detector signal; – · – · – · , content of dioxane in the eluent mixtures. Stationary phase: LiChrosorb Si 100, 10 μm. Detection: UV, 258 nm. Sample solvent: hexane. Flow-rate: 1 ml/min. Oven temperature: (a) 240°C; (b) 275°C. Initial column end pressure: (a) 80 bar; (b) 41 bar.
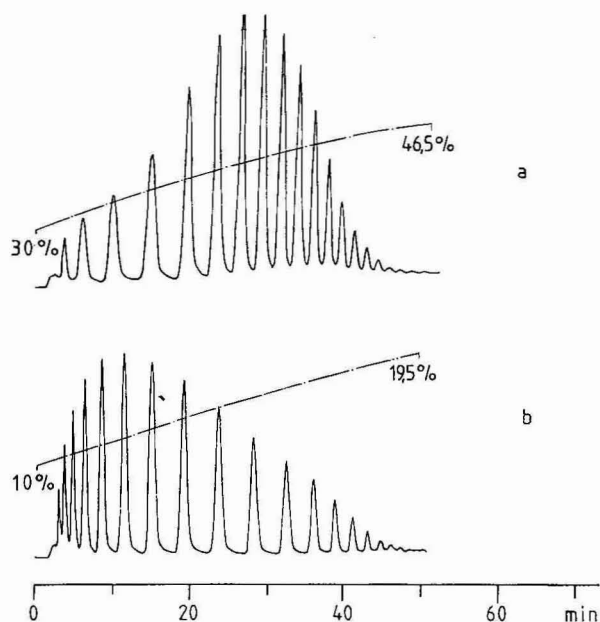
Fig. 5. Chromatograms of PS 800 with (a) butane–dioxane and (b) pentane–dioxane. ———: Detector signal; – · – · – · , content of dioxane in the eluent mixtures. Stationary phase: LiChrosorb Si 100, 10 μm. Detection: UV, 258 nm. Sample solvent: hexane. Flow-rate: 1 ml/min. Oven temperatures: 240°C. Initial column end pressure: (a) 69 bar; (b) 50 bar.

TABLE I

CRITICAL DATA OF THE COMPONENTS OF THE ELUENTS UNDER INVESTIGATION
(COMPILED FROM LITERATURE DATA[10,11])

Compound	T_c (°C)	P_c (bar)	ρ_c (g cm⁻³)
Propane	96.8	42.6	0.225
Butane	152	38.0	0.228
Pentane	196.6	33.7	0.232
Hexane	234.7	30.3	0.234
Diethyl ether	192.6	36.1	0.267
Cyclohexane	279.9	40.3	0.270
1,4-Dioxane	314.8	52.1	0.360
Methanol	240	79.5	0.275
Ethanol	243.1	63.9	0.280

whereas the value for the chromatogram in Fig. 4b was only about 60 bar. However, column temperatures for the lower alkanes can be decreased significantly while still staying above T_c, which in turn leads to decreased pressures for the same dissolution power of the mobile phase for a given alkane.

Fig. 6 demonstrates the influence of the dioxane programme on the separation of PS 800. Using otherwise identical conditions as for Fig. 5b, the higher initial dioxane content and the steeper gradient shorten the analysis time from 50 to 20 min with a still reasonable resolution.

Pentane–methanol

For separating oligo(2-vinylpyridines) a positive methanol gradient has to be applied. A chromatogram of a radically oligomerized sample is shown in Fig. 7. The sample is well separated with respect to the degree of oligomerization, *n*. The assignment of groups of peaks for an individual degree of oligomerization was carried out by means of oligomers having a defined degree of oligomerization and defined tacticity[8]. Within a group of peaks of given *n*, further differentiation, probably tactic isomers, can be observed, as Fig. 7 also shows.
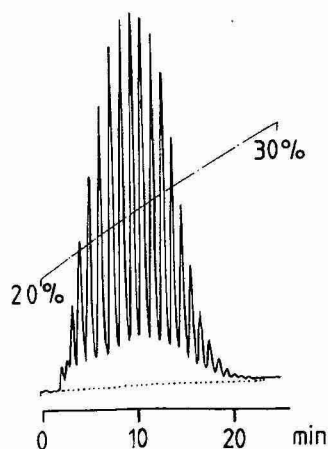
Fig. 6. Chromatogram of PS 800 with pentane-dioxane. ....., Baseline. Other conditions as in Fig. 5.
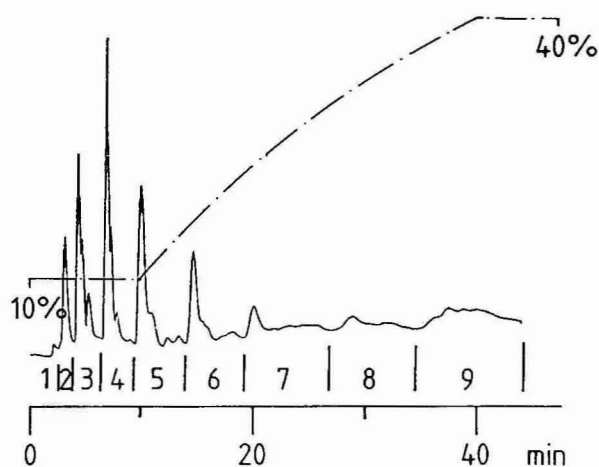
Fig. 7. Chromatogram of an oligo(2-vinylpyridine) sample with pentane–methanol. ————, Detector signal; – · – · – · , content of methanol in the eluent mixture. Stationary phase: LiChrosorb Si 100, 10 μm. Detection: UV, 262 nm. Sample solvent: methanol. Flow-rate: 1 ml/min. Oven temperature: 250°C. Initial column end pressure: 66 bar. The numbers below the detector signal trace correspond to the degree of oligomerization, n.

It should be pointed out, however, that the difficulties that arose with supercritical ethanol are even more pronounced with methanol. After two or three chromatographic runs, the supercritical phase has dissolved enough silica gel to make the loss in stationary phase visibly obvious. Moreover, pressure irregularities caused by particles in the stream passing the valve at the column exit become troublesome and dissolution of the stationary phase can be expected to result in a decreased resolution.

DISCUSSION

The results show that alkanes and ethers are well suited as eluents for SFC. Alcohols are less suitable because of their ability to dissolve the silica gel stationary phase, especially when used at higher concentrations. This may be observed with methanol and also to a lesser extent with ethanol. At lower alcohol concentrations (5–10%, v/v) the dissolution problems are not as severe and may even be disregarded. For chromatographing specifically oligostyrene, eluents composed of alkanes and dioxane yield particularly good resolutions.

In this study, the applicability of SFC has been extended to a greater number of mixed eluents for the technique of gradient elution[6]. Thus, two main gradient techniques are applicable to SFC: pressure gradients, where the mobile phase density is the parameter altered to change the analysis time and resolution, and eluent gradients, where the eluent composition is altered. If the column end pressure is not kept constant during a run, combined effects of the two gradient techniques are obtained. Additionally, it could be shown that the instrumental expenditure when working with SFC is not as large as might be believed. The development of SFC instruments does not need to be a development of completely novel instrumentation. Commercial HPLC instruments additionally equipped with an oven capable of work-

ing at temperatures up to 300°C and with a pressure-regulating device would satisfy many of the requirements for SFC, as has also been demonstrated recently by Gere et al.[9].

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC ELUTION CHARACTERISTICS OF SUBSTITUTED N-ETHYLBENZAMIDES

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SUMMARY

The reversed-phase liquid chromatographic retention of sixteen N-ethylbenzamides substituted with methyl, methoxy or phenyl groups at the 4-phenyl position and/or at the 2-ethyl position was investigated using two different octadecyl phase columns and a phenyl phase column with water–methanol solvent mixtures. For isomeric amides an increased retention was obtained for the isomer having the larger substituent at the 4-phenyl position. Satisfactory linear correlations were obtained by plotting log k' obtained on one column against log k' on a second column at the same or different eluent compositions. This suggests that quantitative structure–retention relationships can be transformed from one reversed-phase system to another. The molecular connectivity indices, χ, to third order were calculated for the amides, and a high degree of correlation was observed between them and the measured log k'.

INTRODUCTION

The effect of chemical structure on retention in liquid chromatography is a topic of much current research[1–9]. Wells and co-workers[1–3] have recently published a complete study on the retention of $C_1–C_5$ N-alkylbenzamides using different octadecyl bonded phase columns. The N-alkylbenzamides were formed by the reaction between benzoyl chloride and all possible primary aliphatic amines containing 1–5 carbon atoms. Wells and co-workers[3–5], like many others[6–9], have used molecular connectivity indices to correlate chromatographic parameters with molecular structure.

The molecular connectivity indices reflect the shape and atomic interactions of a molecule. Detailed discussions of this concept and of its calculation have been given by Kier and Hall[10] and Wells *et al.*[3]. When the nature of the atom is not taken into consideration the index is referred to as the connectivity level, χ, while if such characteristics are allowed for the index is referred to as the valence level, χ^v. Connectivity indices have been extended to include indices of different orders (the order being the number of bonds involved in the subgraph of the molecule and denoted by a left-side superscript), as well as subgraphs composed of paths, clusters, path/clusters and chains which are denoted by the subscripts p, c, pc and ch, respectively. Wells and co-workers[3–5] have used such extended indices.

This paper reports the reversed-phase chromatographic properties of sixteen N-ethylbenzamides on three different stationary phases. Molecular connectivity indices to the third order-were calculated and correlated with log capacity factor (k'). The effect of the stationary phase on retention is also discussed.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a 79875A variable wavelength spectrophotometer detector was used. Absorbance spectra were determined in the UV-region, 200–320 nm, on a Kontron Uvikon 820 spectrophotometer.

Reagents and chemicals

Ethylamine hydrochloride, propylamine hydrochloride, β-phenylethylamine, 4-toluoyl chloride and biphenyl-4-carbonyl chloride were obtained from Fluka (Buchs, Switzerland). 4-Methoxybenzoyl chloride, methylene chloride, benzene, acetone, sodium nitrate and sodium sulphate were obtained from E. Merck (Darmstadt, G.F.R.). 2-Methoxyethylamine was from EGA-Chemie (Steinheim, G.F.R.). Benzoyl chloride was from BDH (Poole, Great Britain). Light petroleum (b.p. 40–60°C) was from May & Baker (Dagenham, Great Britain) and was distilled before use. Freshly distilled diethyl ether was from Kemira Corporation (Vihtavuori, Finland). High-performance liquid chromatographic (HPLC) grade methanol was from Orion Corporation (Espoo, Finland) and water was distilled and deionized. Amines and benzoyl chlorides were pro analysis grade (except toluoyl chloride which was practical), and were used without further purification.

Preparation of derivatives

The appropriate amine or amine hydrochloride (3 mmol) was mixed with 100 ml of 2 N sodium hydroxide in a three-necked flask equipped with a magnetic stirrer, reflux condenser and a funnel. Benzoyl chloride or the appropriate 4-substituted benzoyl chloride (1.5 molar excess) was added dropwise from the funnel. The resulting mixture was stirred for 1 h at room temperature, then extracted twice with diethyl ether or methylene chloride. The combined organic layers were washed twice with water, dried with sodium sulphate and evaporated. The residue was purified by recrystallization from light petroleum–benzene (1:2, v/v).

Columns

A phenyl bonded phase and two different octadecyl bonded phase analytical HPLC columns were used: a 17 cm × 4.0 mm I.D. stainless-steel column packed with Spherisorb S5 ODS2 (5 μm; Phase Separations, Queensferry, Great Britain), a 20 cm × 4.0 mm I.D. stainless-steel column packed with μBondapak C_{18} (10 μm; Waters Assoc., Milford, MA, U.S.A.) and a 25 cm × 4.0 mm I.D. stainless-steel column packed with Nucleosil $7C_6H_5$ (7 μm; from Macherey, Nagel & Co., Düren, G.F.R.). The columns were packed by a slurry technique using acetone as the suspending medium for the octadecyl phase and water–ethanol (58:42) for the phenyl phase. Five grams of packing material were mixed with 50 ml of the appropriate solvent in a

TABLE I

ABSORPTION MAXIMA AND MOLAR ABSORPTION COEFFICIENTS FOR DERIVATIVES IN-VESTIGATED

R_1—⬡—$\overset{\overset{O}{\|}}{C}$—NH—$CH_2$—$CH_2$—$R_2$
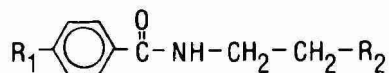
Compound No.	R_1	R_2	λ_{max} (nm)	$\varepsilon_{\lambda_{max}}$ (cm^{-1} M^{-1})
1	H	H	224	10,300
2	H	CH_3	224	10,300
3	H	OCH_3	225	10,200
4	H	C_6H_5	225	13,900
5	CH_3	H	234	13,700
6	CH_3	CH_3	235	12,700
7	CH_3	OCH_3	235	13,700
8	CH_3	C_6H_5	235	14,400
9	OCH_3	H	251	15,600
10	OCH_3	CH_3	251	15,300
11	OCH_3	OCH_3	251	15,900
12	OCH_3	C_6H_5	251	21,100
13	C_6H_5	H	266	24,800
14	C_6H_5	CH_3	266	25,100
15	C_6H_5	OCH_3	267	24,000
16	C_6H_5	C_6H_5	267	26,100

ultrasonic bath and the packing was performed with a Shandon HPLC packing pump using 200 ml methanol at 350 bar pressure. Both octadecyl phases were end-capped so that no free silanol groups were left.

Chromatographic procedures

The eluent was pumped isocratically at a flow-rate of 1.0 ml/min and the oven temperature was 40°C. The column dead volume was taken as the elution volume for a 2-μl injection of an aqueous solution containing sodium nitrate, with the UV detector operating at 254 nm and 0.01 a.u.f.s. Sufficiently dilute samples were prepared to give the smallest detectable peaks (< 20 % of scale). The UV detector was operated at the wavelength of maximum absorbance for each of the compounds (Table I). Capacity factors were calculated in the usual manner and at least two injections were made in each case.

The specific gravity was measured for each batch of HPLC solvent mixture. The methanol content of solvent mixtures was checked by a pycnometric method at 20 ± 0.02°C. Duplicate analysis were performed. The concentrations of water and methanol in the eluent are given as volume percentages.

RESULTS AND DISCUSSION

Table I lists the N-ethylbenzamide derivatives investigated together with their absorption maxima and molar absorption coefficients. All compounds are white solids except N-2-methoxyethyl-4-methoxybenzamide (11) which is oily.

TABLE II

PARAMETERS OF LOG k' VS. METHANOL CONTENT FOR SPHERISORB S5 ODS2 COLUMN

See Table I for compound identification.

Compound No.	Intercept	Slope	r	d*
1	1.276	−0.0220	−0.999	0.0094
2	1.737	−0.0270	−0.999	0.0094
3	1.322	−0.0235	−0.999	0.0137
4	2.900	−0.0400	−0.999	0.0197
5	1.868	−0.0288	−0.999	0.0138
6	2.279	−0.0325	−0.999	0.0143
7	1.847	−0.0288	−0.998	0.0206
8	3.428	−0.0452	−0.999	0.0214
9	1.498	−0.0252	−0.999	0.0089
10	1.956	−0.0299	−0.999	0.0128
11	1.490	−0.0256	−0.998	0.0216
12	3.109	−0.0426	−0.999	0.0255
13	3.241	−0.0432	−0.998	0.0290
14	3.684	−0.0476	−0.999	0.0249
15	3.262	−0.0439	−0.998	0.0286
16	4.728	−0.0584	−0.999	0.0208

★ Mean distance of data points from the line.

TABLE III

PARAMETERS OF LOG k' VS. METHANOL CONTENT FOR μBONDAPAK C_{18} COLUMN

Other details as in Table II.

Compound No.	Intercept	Slope	r	d
1	1.167	−0.0173	−0.992	0.0279
2	1.605	−0.0223	−0.994	0.0309
3	1.186	−0.0180	−0.993	0.0289
4	2.785	−0.0364	−0.997	0.0377
5	1.749	−0.0243	−0.995	0.0311
6	2.192	−0.0293	−0.996	0.0357
7	1.724	−0.0244	−0.992	0.0404
8	3.660	−0.0476	−0.990	0.0889
9	1.473	−0.0220	−0.996	0.0374
10	1.876	−0.0260	−0.993	0.0388
11	1.398	−0.0209	−0.989	0.0426
12	3.041	−0.0398	−0.996	0.0471
13	3.156	−0.0405	−0.996	0.0445
14	3.678	−0.0466	−0.995	0.0630
15	3.155	−0.0410	−0.996	0.0507
16	4.738	−0.0580	−0.998	0.0452

TABLE IV

PARAMETERS OF LOG k' *VS.* METHANOL CONTENT FOR NUCLEOSIL $7C_6H_5$ COLUMN

Other details as in Table II.

Compound No.	Intercept	Slope	r	d
1	0.975	−0.0138	−0.990	0.0253
2	1.250	−0.0173	−0.992	0.0289
3	1.011	−0.0144	−0.991	0.0259
4	2.272	−0.0300	−0.992	0.0501
5	1.334	−0.0185	−0.994	0.0272
6	1.623	−0.0220	−0.994	0.0325
7	1.359	−0.0188	−0.991	0.0328
8	2.705	−0.0354	−0.992	0.0595
9	1.215	−0.0170	−0.991	0.0297
10	1.510	−0.0207	−0.993	0.0320
11	1.255	−0.0175	−0.991	0.0306
12	2.492	−0.0325	−0.994	0.0457
13	2.607	−0.0341	−0.992	0.0564
14	2.894	−0.0375	−0.994	0.0535
15	2.611	−0.0341	−0.993	0.0534
16	3.828	−0.0480	−0.997	0.0447

Log k' was calculated for compounds 1–16 at 40, 50, 60 and 70 % methanol–water eluent mixtures and plotted against the methanol content of the eluent. The intercepts, slopes and correlation coefficients of the lines so obtained with each column are listed in Tables II–IV together with the mean distances of data points from the plots of log k' *vs.* methanol content.

Retention of isomeric derivatives

The lines of log k' *vs.* methanol content for the isomeric compounds (2 and 5, 3 and 9, 4 and 13, 7 and 10, 8 and 14, and 12 and 15, see Table I) are quite close to each other, revealing that the differences in retention are not large. A larger log k' value reflects greater retention, and in every case the line for the isomer having the larger group at the 4-phenyl position lies above the line of the 2-ethyl-substituted isomer. Hence, for monosubstituted compounds, 4-phenyl substitution results in greater retention than 2-ethyl substitution. Consistently, for disubstituted isomeric compounds, that having the larger group at the 4-phenyl position exhibits the greater retention. Of the compounds of general formula $R_1C_6H_4CONH(CH_2)_2R_2$, Nos. 10 ($R_1 = OCH_3$, $R_2 = CH_3$), 14 ($R_1 = C_6H_5$, $R_2 = CH_3$) and 15 ($R_1 = C_6H_5$, $R_2 = OCH_3$) have greater retention than, respectively, compounds 7 ($R_1 = CH_3$, $R_2 = OCH_3$), 8 ($R_1 = CH_3$, $R_2 = C_6H_5$) and 12 ($R_1 = OCH_3$, $R_2 = C_6H_5$).

Comparison of columns

The statistical analysis system (SAS) procedure RSQUARE was run to obtain correlations of log k' values on one column with log k' values on a second column at 40, 50, 60 and 70 % methanol–water, *i.e.*, log k_1' (40) *vs.* log k_2' (40), log k_1' (40) *vs.* log k_2' (50) ... log k_1' (70) *vs.* log k_2' (70), where the subscripts denote the different stationary phases and the percentage of methanol is given in parentheses. In this way

TABLE V

STATISTICAL DATA OBTAINED BY PLOTTING LOG k' VALUES ON ONE COLUMN AGAINST LOG k' VALUES ON A SECOND COLUMN AT 40, 50, 60 AND 70% METHANOL-WATER

Column pair	N	Correlation coefficient		
		Range	Average	Standard deviation
Spherisorb S5 ODS2–μBondapak C$_{18}$	16	0.9945–0.9996	0.9977	0.0014
Spherisorb S5 ODS2–Nucleosil 7C$_6$H$_5$	16	0.9824–0.9891	0.9891	0.0033
μBondapak C$_{18}$–Nucleosil 7C$_6$H$_5$	16	0.9820–0.9941	0.9899	0.0034

sixteen correlations were obtained for each column pair. The statistical data for these correlations are presented in Table V.

The correlations at 50% methanol–water are

$$\log k'_1 = 1.096 \pm 0.009 - (0.127 \pm 0.008) \log k'_2 \quad r = 0.9996 \tag{1}$$

$$\log k'_1 = 1.432 \pm 0.059 - (0.198 \pm 0.042) \log k'_3 \quad r = 0.9884 \tag{2}$$

$$\log k'_2 = 1.306 \pm 0.052 - (0.065 \pm 0.040) \log k'_3 \quad r = 0.9891 \tag{3}$$

where the subscripts 1, 2 and 3 denote the data obtained on the Spherisorb S5 ODS2, μBondapak C$_{18}$ and Nucleosil 7C$_6$H$_5$ columns, respectively; the uncertainties are the standard errors of the regression coefficients. The results suggest that it is possible, even at different eluent compositions, to transfer a set of log k' values from one chromatographic system to another, and thus facilitate the prediction of log k' values from structural parameters on different stationary phases.

Comparison of log k' values with molecular connectivity indices

The molecular connectivity indices to the third order, $^0\chi$, $^0\chi^v$, $^1\chi$, $^1\chi^v$, $^2\chi$, $^2\chi^v$, $^3\chi_p$, $^3\chi_c$, $^3\chi_p^v$ and $^3\chi_c^v$, were calculated for compounds 1–16. The SAS procedure RSQUARE was used to obtain regressions of log k' against all possible one- and two-variable combinations of the connectivity level, χ, and the valence level, χ^v, indices, their reciprocals, squares and reciprocal squares. When the best combinations had been selected, procedure SYSREG was used to evaluate the regression coefficients.

The best one-variable fit was that using the second order valence level, $^2\chi^v$, on the two octadecyl phase columns and the first order valence level, $^1\chi^v$, on the phenyl phase column. The range of the regression coefficients ($N = 12$) was from 0.9531 to 0.9847 with a mean of 0.9701 and standard error of 0.0107.

The first order valence level, $^1\chi^v$, and the first order connectivity level, $^1\chi$, were chosen as variables for the best two-variable combinations in all except one case, where the zero order connectivity level, $^0\chi$, and the first order valence level, $^1\chi^v$, were chosen. The range of the regression coefficients ($N = 12$) was from 0.9911 to 0.9944 with a mean of 0.9933 and standard error of 0.0011. The third order indices were not chosen at all. The selected indices are given in Table VI.

TABLE VI

SELECTED MOLECULAR CONNECTIVITY INDICES FOR THE N-ETHYLBENZAMIDES

See Table I for compound identification.

Compound No.	$^0\chi$	$^1\chi^v$	$^1\chi$	$^2\chi^v$
1	8.1044	3.6755	5.3426	2.2255
2	8.8115	4.1755	5.8425	2.6219
3	9.5187	4.1653	6.3425	2.6147
4	11.9244	5.7326	8.3602	3.8575
5	8.9747	4.0862	5.7364	2.7255
6	9.6818	4.5862	6.2364	3.1219
7	10.3889	4.5760	6.7364	3.1147
8	12.7947	6.1433	8.7540	4.3575
9	9.6818	4.1985	6.2744	2.5880
10	10.3889	4.6985	6.7743	2.9844
11	11.0960	4.6883	7.2744	2.9772
12	13.5018	6.2557	9.2920	4.2200
13	12.0876	5.7468	8.3089	3.8028
14	12.7947	6.2468	8.8089	4.1993
15	13.5018	6.2367	9.3089	4.1921
16	15.9076	7.8040	11.3265	5.4349

The regression equations at 50 % methanol–water with one- and two-variable combinations are:

Spherisorb S5 ODS2

$$\log k' = (0.544 \pm 0.039)\, ^2\chi^v - 1.132 \pm 0.139 \qquad r = 0.9645 \qquad (4)$$

$$\log k' = (1.207 \pm 0.097)\, ^1\chi^v - (0.550 \pm 0.066)\, ^1\chi - 1.406 \pm 0.072 \quad r = 0.9935 \qquad (5)$$

µBondapak C_{18}

$$\log k' = (0.489 \pm 0.035)\, ^2\chi^v - 0.920 \pm 0.122 \qquad r = 0.9668 \qquad (6)$$

$$\log k' = (1.074 \pm 0.086)\, ^1\chi^v - (0.482 \pm 0.059)\, ^1\chi - 1.173 \pm 0.064 \quad r = 0.9937 \qquad (7)$$

Nucleosil $7C_6H_5$

$$\log k' = (0.0260 \pm 0.001)\, (^1\chi^v)^2 - 0.098 \pm 0.040 \qquad r = 0.9828 \qquad (8)$$

$$\log k' = (0.051 \pm 0.008)\, (^1\chi^v)^2 - (0.012 \pm 0.004)\, (^1\chi^v)^2 - 0.094 \pm 0.032 \quad r = 0.9914 \qquad (9)$$

Choosing a connectivity level, χ, in a regression of this type implies that the nature of the atom itself is not important. If, however, a valence level, χ^v, is selected, the identity of the atom (carbon *vs.* oxygen) is important for the correlation.

CONCLUSIONS

A high degree of correlation was observed between the log k' on one column and the log k' on a second column, as well as between the molecular connectivity indices and the log k' at various eluent compositions on all three columns. The same connectivity parameters were chosen as the best descriptors of retention in these systems. These observations imply that in the range of alkylbenzamides examined the same structural features are important in the reversed-phase chromatographic process on these columns.

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RETENTION BEHAVIOUR OF DISUBSTITUTED BENZENE ISOMERS ON ACETYLATED CYCLODEXTRIN STATIONARY PHASES

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SUMMARY

Chemically bonded α- and β-cyclodextrin stationary phases were treated with acetic anhydride. The resulting acetylated stationary phases exhibit selectivity in the separation of disubstituted benzene isomers by liquid chromatography, as do the unmodified, parent cyclodextrin stationary phase. However, the acetylated β-cyclodextrin stationary phase is superior to the unmodified one and can completely separate the o-, m- and p-isomers of toluidine or dinitrobenzene, which cannot be done on the unmodified stationary phase. Acetylation of the α-cyclodextrin stationary phase does not necessarily bring about a similar improvement in the separation.

INTRODUCTION

The chemical modification of cyclodextrins has been investigated in an attempt to improve their complexing and catalytic abilities. Various functional groups have been introduced onto the rim of cyclodextrins[1–4], resulting in changes in the depth of the cyclodextrin cavity, in the hydrogen-bonding ability and various other physical properties, compared with those of the unmodified, parent cyclodextrins. It has been observed that the host–guest interaction in complexes of methylated α-cyclodextrin is quite different from that in those of unmodified α-cyclodextrin, in which the guest molecule (benzaldehyde or p-nitrophenol) is positioned upside down[5].

α- and β-cyclodextrins have been immobilized covalently on polyacrylamide[6] and silica[7,8] gels via the spacer arms. The resulting stationary phases yielded efficient, selective separations of various aromatic compounds in liquid chromatography. The retention behaviour of modified cyclodextrin stationary phases is of great interest because selectivity changes in solute retention are expected.

In this work, α- or β-cyclodextrin immobilized on silica was acetylated with acetic anhydride, and the retention behaviours were studied for several disubstituted benzene derivatives and compared with those on the cyclodextrin phases before acetylation.
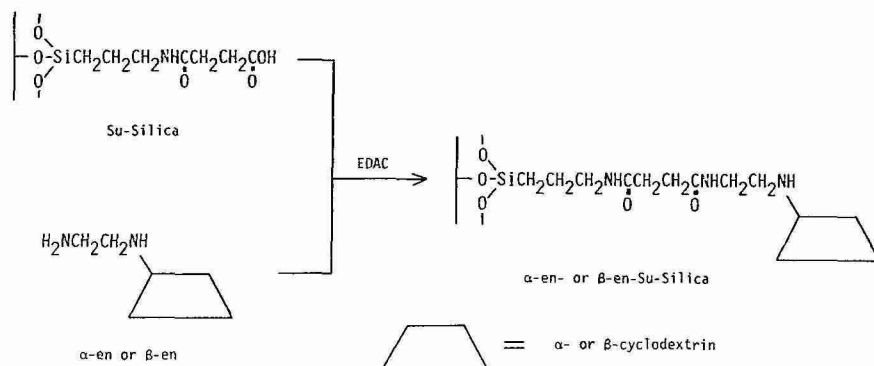
Fig. 1. Immobilization of cyclodextrin on silica gel. EDAC = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

EXPERIMENTAL

The reagents and apparatus used were as described previously[8].

Preparation of acetylated cyclodextrin stationary phases

Ethylenediamine-monosubstituted α- or β-cyclodextrin was immobilized on the carboxylated derivative of silica as described previously[8]. The stationary phase obtained was denoted by α-en-Su-Silica or β-en-Su-Silica, respectively (Fig. 1).

α-en- or β-en-Su-Silica (1.7 g) was suspended in dry pyridine (10 ml). Acetic anhydride (6 ml) was added to this suspension kept at 45°C. After 6 h the acetylated cyclodextrin phase was filtered off, thoroughly washed successively with methanol, water and methanol and dried *in vacuo* at 80°C for 12 h. The acetylated α- or β-cyclodextrin stationary phase thus obtained is denoted by Ac-α-en-Su-Silica or Ac-β-en-Su-Silica, respectively.

Chromatography

The acetylated cyclodextrin stationary phase was packed by a balanced density slurry method into a stainless-steel column (15 cm × 4 mm I.D.). The flow-rate of eluent (water or methanol–water) was 1.0 ml/min. The wavelength used for detection was 254 nm. The concentration of sample solution was 0.2 mM, and a volume of 20 μl was injected except where specified.

TABLE I

ANALYTICAL DATA FOR CYCLODEXTRIN STATIONARY PHASES

Phase	Amount of cyclodextrin immobilized ($\mu mol/g$)	Elemental analysis (%)		
		C	H	N
α-en-Su-Silica	31.5	8.68	1.52	1.15
Ac-α-en-Su-Silica	31.5	10.36	1.71	1.14
β-en-Su-Silica	21.7	8.32	1.63	1.06
Ac-β-en-Su-Silica	21.7	9.59	1.59	1.04
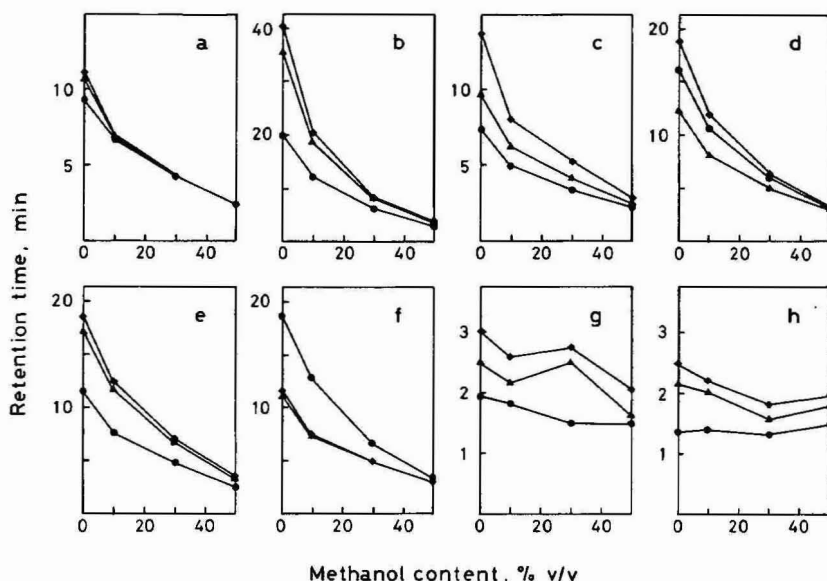
Fig. 2. Effect of methanol content in the eluent on retention times of disubstituted benzene isomers ($\bullet$, o-; $\blacktriangle$, m-; and $\blacksquare$, p-) on Ac-α-en-Su-Silica. Solutes: a = cresol; b = iodoaniline; c = toluidine; d = nitroaniline; e = nitrophenol; f = dinitrobenzene; g = aminobenzoic acid; h = nitrobenzoic acid.

RESULTS AND DISCUSSION

The amounts of cyclodextrins immobilized on α-en- and β-en-Su-Silica were evaluated spectrophotometrically by determining D-glucose formed after hydrolysis with H_2SO_4[8]. Table I gives the cyclodextrin capacities of the stationary phases together with the results of elemental analyses before and after acetylation. The increase of the carbon content after acetylation indicates complete modification of the cyclodextrin units in α-en- or β-en-Su-Silica.

Dependence of retention upon eluent composition

The retention times of disubstituted benzene derivatives on both Ac-α-en- and Ac-β-en-Su-Silica were measured by changing the methanol–water ratio in the eluent from 0:100 to 50:50. The results are shown in Figs. 2 and 3, respectively. In the case of aminobenzoic or nitrobenzoic acid, 5 μl instead of 20 μl of a 0.2 mM solution were injected because leading effects appeared when the larger sample volume was employed. A decrease in retention with increasing methanol content was found for the disubstituted benzenes studied on Ac-α-en- and Ac-β-en-Su-Silica, except for aminobenzoic and nitrobenzoic acids. For the isomers of the latter benzoic acids the dependence of retention on methanol content was more complicated. On the unmodified phase, α-en-Su-Silica, the elution order of the o- and m-isomers of cresol, toluidine, nitroaniline or nitrophenol in methanol–water (10:90) ($m < o < p$) was reversed compared with that in water ($o < m < p$)[8]. In the cases of the acetylated cyclodextrin phases, the retention order of the o-, m- and p-isomers of each solute was not dependent upon the methanol content in the eluent.
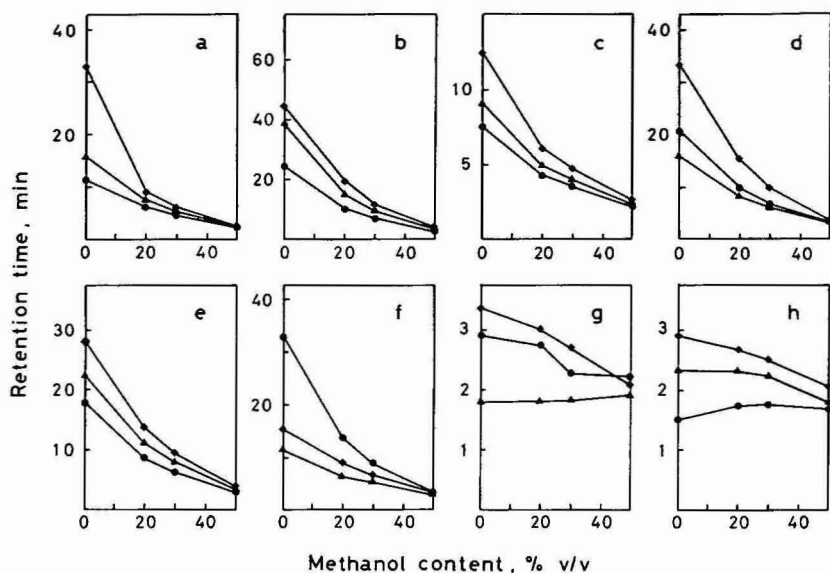
Fig. 3. Effect of methanol content in the eluent on retention times of disubstituted benzene isomers on Ac-β-en-Su-Silica. Solutes as in Fig. 2.

Considering both the separations of the three isomers and the total analysis times, the optimum eluent is pure water for Ac-α-en-Su-Silica. In this case, the isomers can be completely separated, with the exception of the *m*- and *p*-isomers of cresol, dinitrobenzene or nitrobenzoic acid. Similarly, the optimum eluent is methanol–water (20:80) for Ac-β-en-Su-Silica: except for the *o*- and *p*-isomers of aminobenzoic acid and the *m*- and *p*-isomers of nitrobenzoic acid, the isomers can be completely separated. *p*-Iodoaniline gives the longest retention time of 19.13 min. By using pure water as eluent, the three isomers of both aminobenzoic and nitrobenzoic acids can also be separated completely within 4 min on Ac-β-en-Su-Silica, as shown in Fig. 4.
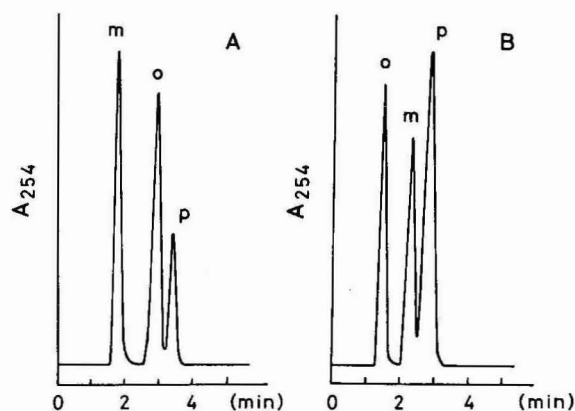
Fig. 4. Liquid chromatograms of aminobenzoic acid isomers (A) and nitrobenzoic acid isomers (B) on Ac-β-en-Su-Silica in water.
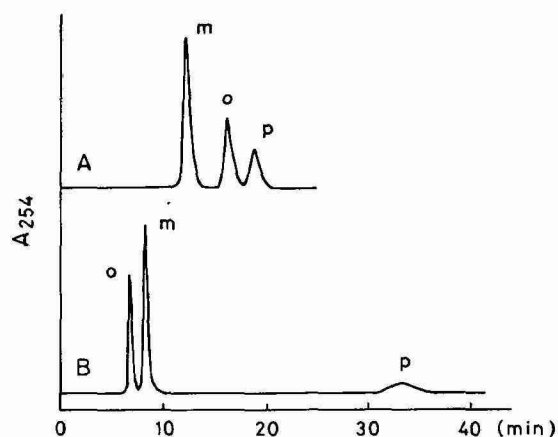
Fig. 5. Liquid chromatograms of nitroaniline isomers on Ac-α-en-Su-Silica (A) and α-en-Su-Silica (B).

Comparison of retention before and after acetylation

Fig. 5 shows typical liquid chromatograms of a mixture of o-, m- and p-isomers of nitroaniline on the α-cyclodextrin stationary phases before and after acetylation (α-en-Su-Silica and Ac-α-en-Su-Silica). A complete separation of the three isomers can be obtained earlier on Ac-α-en-Su-Silica than on α-en-Su-Silica.

Table II gives the retention times of the eight kinds of disubstituted benzene derivatives both on Ac-α-en-Su-Silica and α-en-Su-Silica in water. Both stationary phases must have the same α-cyclodextrin capacity. Therefore, it is reasonable to assume that the difference in the retention time of a solute on the different phases directly reflects the difference in the interaction between unmodified and acetylated α-cyclodextrin with the solute. The p-isomers of iodoaniline, nitroaniline and nitro-

TABLE II

RETENTION TIMES ON α-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: water.

Solute	Retention time (min)					
	Ac-α-en-Su-Silica			α-en-Su-Silica		
	o-	m-	p-	o-	m-	p-
Cresol	9.30	10.61	11.09	4.30	5.15	5.76
Iodoaniline	20.05	35.40	40.85	7.35	42.20	115.2
Toluidine	7.39	9.70	13.70	3.19	3.87	4.55
Nitroaniline	16.30	12.25	18.95	6.66	8.17	32.95
Nitrophenol	11.54	16.98	18.50	6.10	10.19	104.3
Dinitrobenzene	18.70	11.25	11.48	4.81	3.96	4.23
Aminobenzoic acid*	1.95	2.51	3.05	–	–	–
Nitrobenzoic acid*	1.35	2.15	2.46	–	–	–

* Not eluted within 60 min on α-en-Su-Silica.

TABLE III

RETENTION TIMES ON β-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: methanol–water (20:80).

Solute	Retention time (min)					
	Ac-β-en-Su-Silica			β-en-Su-Silica		
	o-	m-	p-	o-	m-	p-
Cresol	6.13	7.30	8.39	4.25	5.13	7.22
Iodoaniline	9.85	14.64	19.13	6.44	10.66	20.15
Toluidine	4.31	4.90	6.05	3.05	3.22	4.05
Nitroaniline	9.91	8.40	15.20	5.35	4.50	12.94
Nitrophenol	8.65	11.14	13.49	14.53	7.62	34.70
Dinitrobenzene	13.64	6.48	8.58	5.96	2.95	3.05
Aminobenzoic acid*	2.76	1.80	3.08	–	–	–
Nitrobenzoic acid*	1.75	2.32	2.65	–	–	–

* Not eluted within 60 min on β-en-Su-Silica.

henol and the m-isomer of iodoaniline strongly interact with unmodified α-cyclodextrin, which is apparent from the long retention times. However, their retention is considerably reduced by acetylation of the α-cyclodextrin units. This fact indicates a decrease in the complexing ability of acetylated α-cyclodextrin, compared with that of unmodified α-cyclodextrin. On the other hand, the retention times of the other solutes are longer on Ac-α-en-Su-Silica than on α-en-Su-Silica. This may be due to the in-
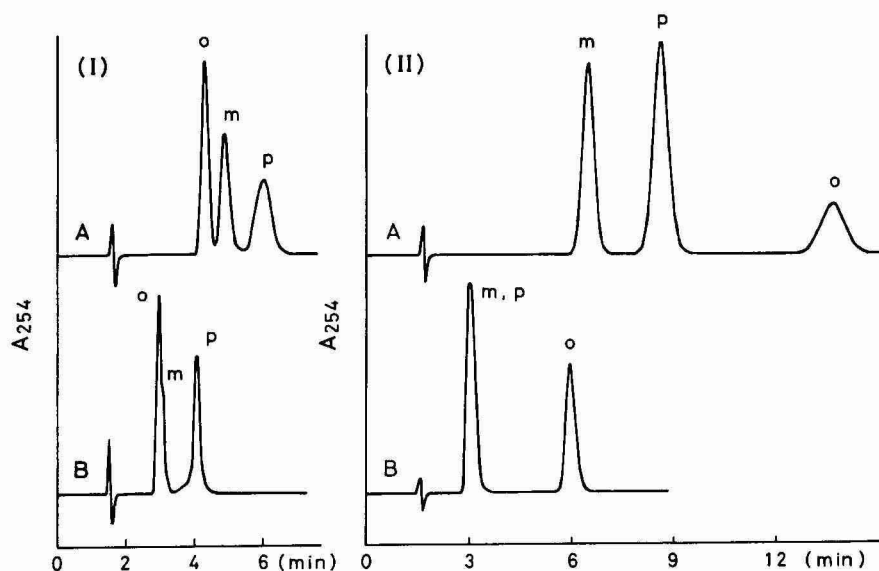
Fig. 6. Liquid chromatograms of toluidine isomers (I) and dinitrobenzene isomers (II) on Ac-β-en-Su-Silica (A) and on β-en-Su-Silica (B).

crease in the hydrophobicity of the phase upon conversion of the hydroxyl groups of α-cyclodextrin into acetoxy groups. The most remarkable change in retention behaviour after acetylation is that of aminobenzoic and nitrobenzoic acids which can be eluted with pure water within about 3 min; they cannot be eluted within 60 min on α-en-Su-Silica. Although the separation of the toluidine isomers is improved by the acetylation, α-en-Su-Silica is superior to Ac-α-en-Su-Silica in separating the other disubstituted benzene isomers.

The retention times of the disubstituted benzenes on both Ac-β-en-Su-Silica and β-en-Su-Silica in methanol–water (20:80) are given in Table III. Except for o- and p-nitrophenol and p-iodoaniline, the retention of the solutes tested increases upon acetylation of the β-cyclodextrin units. As mentioned above, the three isomers of all the solutes investigated can be completely separated on Ac-β-en-Su-Silica. The toluidine or dinitrobenzene isomers are not completely separated on β-en-Su-Silica as shown in Fig. 6.

The elution order of the three isomers on Ac-α-en- or Ac-β-en-Su-Silica is the same as that on the corresponding, unmodified cyclodextrin stationary phase with only two exceptions: $m < o < p$ on Ac-α-en-Su-Silica and $o < m < p$ on α-en-Su-Silica for nitroaniline and $o < m < p$ on Ac-β-en-Su-Silica and $m < o < p$ on β-en-Su-Silica for nitrophenol. Considering the elution order of the isomers on octadecylsilyl silica (reversed-phase mode) or carboxylated silica before coupling with cyclodextrins[8], this result strongly suggests that an inclusion process is operative in the cases of the acetylated cyclodextrin stationary phases.

Column efficiency

Fig. 7 shows the effect of the eluent flow-rate on the capacity factors, k', for the nitroaniline isomers on Ac-β-en-Su-Silica. The k' values for each isomer are independent of the flow-rate in the range 0.16–1.0 ml/min; the selectivity is nearly constant.

Fig. 8 shows a plot of the height equivalent to a theoretical plate (HETP) for the nitroaniline isomers on Ac-β-en-Su-Silica *versus* the flow-rate of methanol–water (20:80). The HETP values for the o-isomer are larger than those for m- and p-isomers.
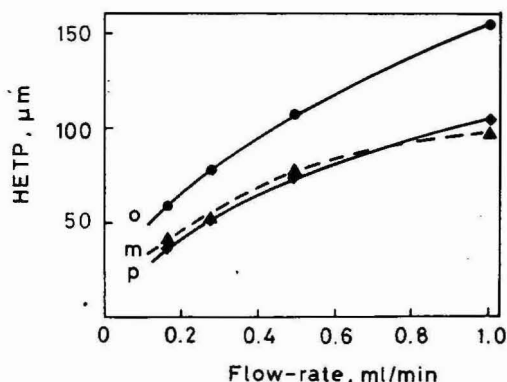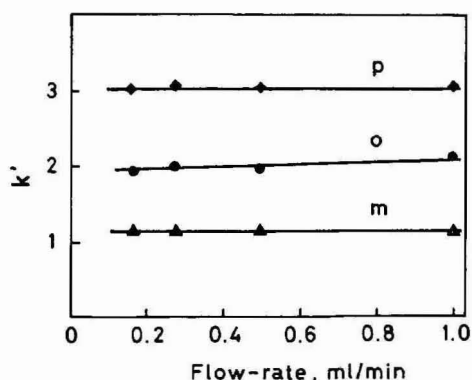
Fig. 7. Effect of eluent flow-rate on capacity factors, k', for nitroaniline isomers on Ac-β-en-Su-Silica.

Fig. 8. Effect of eluent flow-rate on height equivalent to a theoretical plate (HETP) for nitroaniline isomers on Ac-β-en-Su-Silica.

When calculated from the *m*- or *p*-isomer, the HETP value at a flow-rate of 1 ml/min is 100 μm, which corresponds to 1500 theoretical plates, and the HETP value at a flow-rate of 0.16 ml/min is 40 μm. These values are satisfactory for practical purposes.

In conclusion, stationary phases containing α- or β-cyclodextrin chemically bonded to silica have been modified by treatment with acetic anhydride. Compared with the unmodified phase, the acetylated β-cyclodextrin phase gives improved separations of the isomers of disubstituted benzenes.

ACKNOWLEDGEMENT

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SYNTHESIS AND STATIONARY PHASE PROPERTIES OF BROMO-PHENYL ETHERS

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SUMMARY

Electrophilic bromination is shown to be a useful reaction for the site-specific bromination of phenyl ethers containing from two to seven benzene rings. Compared to the phenyl ether stationary phases used in gas chromatography, bromination primarily raises the lower and upper operating temperatures, generally leads to a reduction in the efficiency of column packings and changes the selectivity and polarity of the phases only to a small extent. The three-ring bromophenyl ether, 1,3-dibromo-4,6-bis(4-bromophenoxy)benzene is shown to have the most useful stationary phase properties for the separation of organic compounds with a useable temperature range of 80–200°C.

INTRODUCTION

Several years ago work commenced in our laboratory aimed at the preparation of thermally stable polar reference phases for gas chromatography (GC). Such phases would be usable at high temperatures, contain polar/selective functional groups and have a well-defined non-polymeric structure reproducible via synthesis. After several trials, two possible solutions to the above problem have been developed: the substituted phenyl ethers[1–3] and the organic molten salts[4,5]. The phenyl ethers were selected for study because of their simple structure, chemical stability and remarkably low volatility for such low-molecular-weight compounds[6]. Commercial applications of the phenyl ethers take advantage of these properties, and phenyl ethers with different ring numbers are used as stationary phases in GC, lubricants for engines operated in extreme environments and as diffusion pump fluids in vacuum apparatus.

Mathews *et al.*[7] have prepared polysulfones by copolymerizing five or six ring phenyl ethers with diphenyl ether-4,4'-disulfonyl chloride. These polymers are useful high temperature polar phases which are commercially available. The approach we have taken is to explore several reactions for the site-specific introduction of reactive functional groups into the phenyl ether backbone[1]. These reactive centers provide points to which side chain groups containing any desired chromatographic selectivity can be attached. The most versatile reaction for providing site-specific reaction centers is thallium acetate catalyzed electrophilic bromination[2]. Under controlled con-
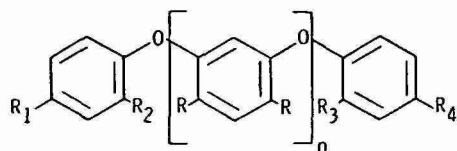
PPE-1 $R_1 = R_2 = R_3 = R_4 = R = H$, n = 3; 1,3-bis(3-phenoxyphenoxy)benzene

PPE-2 $R_1 = R_4 = R = Br$, $R_2 = R_3 = H$, n = 3; 1,3-dibromo-4,6-bis[2,4-dibromo-
         5(4-bromophenoxy)phenoxy]benzene

PPE-3 $R_1 = R_2 = R_3 = R_4 = R = Br$, n = 3; 1,3-dibromo-4,6-bis[2,4-dibromo)-
         5(2,4-dibromophenoxy)phenoxy]benzene

PPE-6 $R_1 = R_2 = R_3 = R_4 = R = H$, n = 1; 1,3-bis[phenoxy]benzene

PPE-7 $R_1 = R_4 = R = Br$, $R_2 = R_3 = H$, n = 1; 1,3-dibromo-4,6-bis[4-bromophenoxy]
         benzene

PPE-8 $R_1 = R_2 = R_3 = R_4 = R = H$, n = 5; 1,3-bis[3-phenoxyphenoxyphenoxy)benzene

PPE-9 $R_1 = R_2 = R_3 = R_4 = R = Br$, n = 5; 1,3-dibromo-4,6-bis[2,4-dibromo-5
         (2,4-dibromo-5((2,4-dibromophenoxy))phenoxy)phenoxy]benzene

Fig. 1. Structure and nomenclature of the phenyl and bromophenyl ethers used in this study.

ditions bromination occurs at positions *para* to the ether bond. More forcing con-
ditions result in *para* substitution in all rings as well as *ortho* substitution in the
terminal phenyl rings. Yields are often quantitative and minimal purification of the
reaction products is required. On account of the above properties, and the availability
of a wide range of bromophenyl intermediates, it was considered worthwhile to in-
vestigate their stationary phase properties. This might also yield useful information
concerning the affect of substitution on the physical, chemical and chromatographic
properties of the phenyl ethers.

The structures, abbreviations and systematics names for the phenyl and bromo-
phenyl ethers used in this study are given in Fig. 1. The compounds PPE-7 and PPE-
9 were prepared for the first time. The synthesis of PPE-6 is an improvement over
published procedures[8].

EXPERIMENTAL

Thallium(III) acetate sesquihydrate, copper powder, phenol and *m*-dibromo-
benzene were obtained from Aldrich (Milwaukee, WI, U.S.A.). PPE-1 and PPE-8
were obtained from Monsanto (St. Louis, MO, U.S.A.). The preparation of 4,4'-
dibromodiphenyl ether (DPE-2), 2,2',4,4'-tetrabromodiphenyl ether (DPE-3), PPE-
2 and PPE-3 has been described previously[2].

General methods for preparing bromophenyl ethers
    1,3-Bis(phenoxy)benzene (PPE-6). PPE-6 was prepared by a modification of
ref. 8. Phenol (0.2 mol), potassium hydroxide 86% (w/w) (0.1 mol) and copper
powder (1.0 g) were heated with stirring to 180°C under a nitrogen atmosphere. To
the mixture was added dropwise *m*-dibromobenzene (0.03 mol) while maintaining a

temperature of 180 $\pm$ 5°C during the exothermic reaction. The progress of the reaction was monitored by high-performance liquid chromatography (HPLC). Periodically small aliquots were removed by pipette, added to water and extracted with carbon tetrachloride. Three products were observed in the chromatogram of the organic phase corresponding to *m*-dibromobenzene, *m*-bromophenyl ether and PPE-6. Further quantities of phenol and copper powder were added as required to drive the reaction to completion. After 5-h reflux the reaction was essentially 95% complete. The reaction mixture was cooled to room temperature, poured into water (200 ml) and extracted with carbon tetrachloride. The organic layer was washed several times with water, dried over sodium sulfate and the solvent removed *in vacuo*. The light yellow oil was brominated without further purification. Distillation at reduced pressure (210–212°C at 10 mmHg) gave authentic samples of PPE-6 for stationary phase evaluation.

1,3-Dibromo-4,6-bis(4-bromophenoxy)benzene (PPE-7). To PPE-6 (0.02 mol) in carbon tetrachloride (100 ml) was added thallium acetate sesquihydrate (1.5 g) and the mixture stirred in the dark for 0.5 h. Bromine (0.08 mol) in carbon tetrachloride (25 ml) was added dropwise and the mixture stirred at room temperature for 0.5 h. The mixture was then heated to reflux for approximately 8 h. The reaction was monitored by HPLC and further quantities of catalyst added as required to give an essentially quantitative yield of PPE-7. The reaction mixture was cooled to room temperature, poured into sodium bicarbonate solution, the organic phase collected and washed with sodium bicarbonate solution and water, dried over sodium sulfate and the solvent removed on a Rotovapor. PPE-7 was obtained as a viscous light yellow oil giving a single peak on HPLC. Its structure was confirmed by NMR spectroscopy [7.90 (singlet), 7.50 (doublet), 6.90 (doublet) and 6.70 ppm (singlet)].

1,3-Dibromo-4,6-bis{2,4-dibromo-5-[2,4-dibromo-5-(2,4-dibromophenoxy)-phenoxy]phenoxy}benzene (PPE-9). To PPE-8 (6.1 g) in carbon tetrachloride (150 ml) was added thallium acetate sesquihydrate (1.5 g) and the mixture stirred in the dark for 0.5 h. A mixture of bromine (19 g) in carbon tetrachloride (50 ml) was added dropwise followed by stirring at room temperature for 0.5 h. The mixture was heated to reflux for a few hours, cooled and the clear supernatant transferred to a second flask leaving behind a muddy precipitate containing catalyst and impurities originally present in PPE-8. Refluxing was continued with a fresh addition of a small amount of catalyst and bromine (5 g). Progress of the reaction was monitored by HPLC and was complete after about 105 h. The reaction mixture was worked-up as described for PPE-7. Preparative HPLC was used for final purification to give PPE-9 as a viscous yellow oil in about 85% yield. The oil crystallized slowly on standing to give a solid, m.p. 93–95°C. Its structure was confirmed by NMR spectroscopy [8.06 (doublet), 7.90 (doublet), 7.70 (singlet), 7.05 (singlet) and 6.80 ppm (singlet)].

HPLC analysis

Reversed-phase HPLC was used to monitor the progress of the synthesis of the above compounds and to establish the final product purity. A Varian 5000 gradient-elution liquid chromatograph with a variable-wavelength UV–visible detector set to λ = 245 nm was used. The column was a 30 cm × 4.0 mm I.D. Micro-Pak CH-10 octadecylsilane type, particle diameter 10 μm, from Varian. For monitoring the syn-

thesis of PPE-6 and PEE-7 a linear gradient of methanol–water (1:1) to methanol over 15 min was used at a flow-rate of 1.5 ml min^{-1}. Retention times for *m*-bromophenyl ether, PPE-6 and PPE-7 were 8.25, 9.25 and 11.50 min respectively. To monitor the synthesis of PPE-9 a linear gradient of acetonitrile–water (3:1) to acetonitrile over 15 min with an isocratic hold of 5 min at 100 % acetonitrile and a flow-rate of 2.0 ml min^{-1} was used. The retention times of PPE-8 and PPE-9 were 10.1 and 17.05 min respectively.

Identification by NMR

NMR analysis was performed on a Varian T-60 ^{1}H NMR spectrometer in carbon tetrachloride or deuterochloroform solutions using tetramethylsilane as internal standard. Structural assignments were made by analogy to previously studied bromophenyl ethers[2].

GC evaluation

For GC a Varian 3700 gas chromatograph with flame-ionization detectors was used. Column packings were prepared by the rotory evaporator technique using acetone or methylene chloride as solvent for the bromophenyl ethers and Chomosorb P AW (100–120 mesh) as support. Glass columns of various lengths, 0.5–3.0 m, and
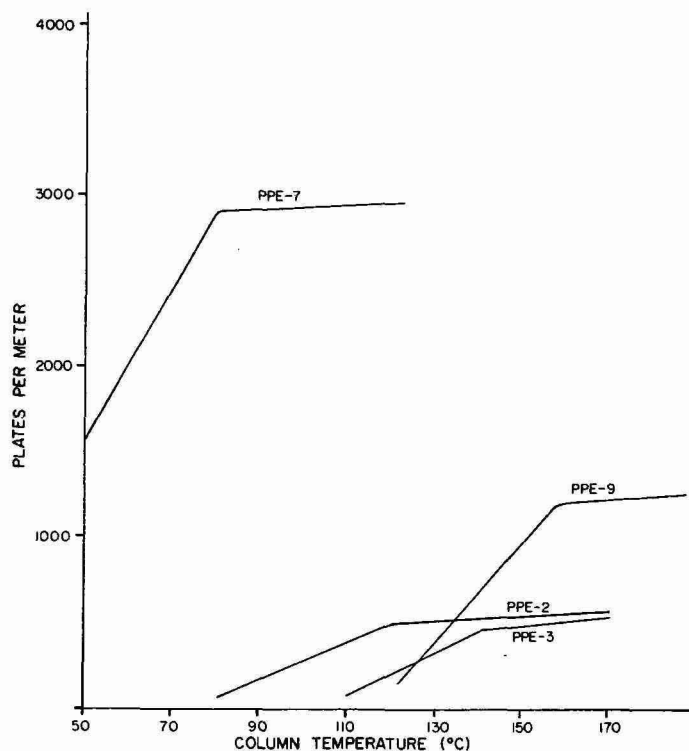
Fig. 2. Relationship between column efficiency and column temperature for the bromophenyl ethers.
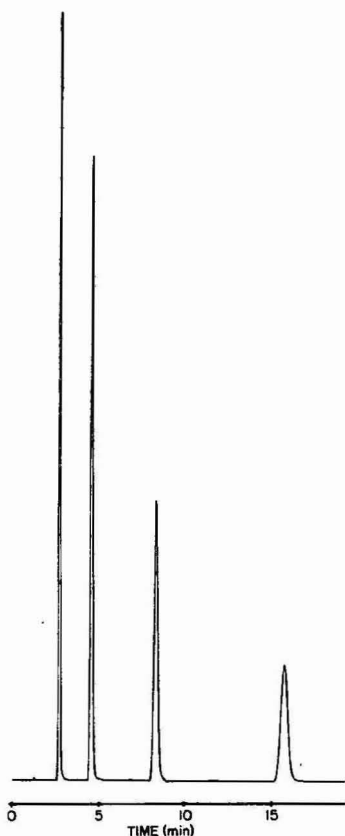
Fig. 3. Separation of C_8–C_{11} alkanes at 100°C on a 3 m × 2 mm I.D. column of 10% (w/w) PPE-7 on Chromosorb P (100–120 mesh) and nitrogen flow-rate 30 ml min^{-1}.

2.0 mm I.D. were vacuum packed with packings containing from 3 to 20% (w/w) of stationary phase. Separation conditions are given in the figure and table legends.

RESULTS AND DISCUSSION

The primary properties which characterize a stationary phase are its minimum and maximum operating temperature, the efficiency of the columns prepared using the phase compared to values normally obtained for other phases or predicted by theory and the polarity or selectivity of the phases determined by measuring McReynolds' constants. Minimum column operating temperatures were established from a plot of the column efficiency (measured as theoretical plates) against column temperature, Fig. 2. In all cases a sharp plateau exists in these plots which serves to define the minimum operating temperature. The maximum column operating temperature is established by evaporation or thermal degradation of the phase. This was measured by establishing the highest column temperature to which the column could be heated for 12 h without any change in the capacity factor, column efficiency or peak asymmetry values for the components of a test chromatogram measured at a

TABLE I

STATIONARY PHASE CHARACTERISTICS OF THE PHENYL ETHERS

Phenyl ether	Melting point (°C)	Operating temperature range (°C)		Average plate count, N (m^{-1})
		Minimum	Maximum	
DPE-2	52–53	–	180	< 50
DPE-3	78–80	–	–	< 50
PPE-1	Liquid	< 20	200	3200
PPE-2	80–81	120	325	500
PPE-3	95–96	145	325	500
PPE-6	Liquid	< 20	185	3500
PPE-7	Liquid	80	200	3000
PPE-8	Liquid	< 20	290	3500
PPE-9	93–95	155	325	1200

* Calculated for a stationary phase loading of 10% (w/w) on Chromosorb P (100–120 mesh).

lower temperature before and after the conditioning period. A typical test chromatogram for PPE-7 is shown in Fig. 3. The data for minimum and maximum operating temperature and average column efficiencies for all phases studied are summarized in Table I. For the phases which are solid at room temperature the minimum operating temperature is well-above the melting point and presumably corresponds to the clearing point temperature. The principal effect of introducing bromine into the phenyl ethers is to increase the temperature for minimum and maximum allowable column operation. An upper temperature limit of 325°C was found for PPE-2, PPE-3 and PPE-9. Above this temperature the bromophenyl ethers are thermally unstable. Thus

TABLE II

McREYNOLDS' CONSTANTS FOR PPE-6 AND PPE-7 MEASURED AT 100°C

I = Retention index value; ΔI = McReynolds' constants; Σ_1^5 = sum of first five McReynolds' constants; ΔI_6^7 = retention index difference between PPE-7 and PPE-6.

Test probe	Squalane	PPE-6		PPE-7		ΔI_6^7
		I	ΔI	I	ΔI	
Benzene	653	860	207	815	162	− 45
Butanol	590	900	310	842	252	− 58
2-Pentanone	627	819	192	830	203	11
1-Nitropropane	652	922	270	925	273	3
Pyridine	699	1012	3313	1006	307	− 6
1,4-Dioxane	654	1021	367	913	259	− 108
2-Methyl-2-pentanol	690	803	113	858	168	55
2-Octyne	841	958	117	950	109	− 8
1-Iodobutane	818	935	117	975	157	40
cis-Hydrindane	1006	1051	45	1090	84	39
Σ_1^5			1292		1197	

TABLE III

COMPARISON OF THE RETENTION OF McREYNOLDS TEST PROBES ON PPE-1, PPE-2, PPE-3 AND PPE-8 AT 150°C.

$\Delta I_A^B = I_B - I_A.$

Test probe	PPE-1 I	PPE-2		PPE-3		PPE-8 I
		I	ΔI_1^2	I	ΔI_1^3	
Benzene	822	848	26	854	32	822
Butanol	804	817	13	804	0	841
2-Pentanone	841	852	11	854	13	878
1-Nitropropane	961	952	−9	946	−15	916
Pyridine	981	1041	60	1041	60	1004
2-Methyl-2-pentanol	904	948	44	954	50	900
1,4-Dioxane	845	844	−1	850	5	984
1-Iodobutane	981	948	−33	946	−35	996
2-Octyne	988	1032	44	1036	48	965
cis-Hydrindane	1104	1152	48	1127	23	1120

thermal degradation and not phase volatility established the maximum operating temperature in these cases. The three ring bromophenyl ether, PPE-7, may be used up to 200°C before volatility losses become significant.

The phenyl ethers provide column packings with an average of 3200–3500 theoretical plates per meter. The bromophenyl ethers produce column packings of lower efficiency ranging from an acceptable value of 3000 plates per meter for PPE-7, through a usable value of 1200 plates per meter for PPE-9, to inadequate values of 500 plates per meter or less for DPE-2, DPE-3, PPE-2 and PPE-3. For the bromophenyl ethers there is no obvious correlation between melting point or structure and column efficiency. It must, therefore, depend on other properties such as viscosity or coating efficiency, although, visual inspection of the different packings did not provide evidence for uneven coating.

The McReynolds' constants for the phenyl ether PPE-6 and the bromophenyl ether PPE-7 are given in Table II. Surprisingly, bromine substitution causes fairly small changes in selectivity of the phase and actually leads to a reduction in its polarity, measuring the latter as Σ_1^5 value for the first five McReynolds' probes. The decrease in polarity is accounted for primarily by the reduced retention of benzene and butanol. The most marked change in selectivity is observed with dioxane. Thus the results in Table II would suggest that bromination of the phenyl ether primarily caused a reduction in the charge transfer and proton donor/acceptor properties of PPE-7 compared to PPE-6.

Squalane was not sufficiently stable at 150°C to permit direct measurement of the McReynolds' constants of PPE-2, PPE-3 and PPE-9. For the purpose of comparison the retention index values of the McReynolds test probes were determined on PPE-1, PPE-2 and PPE-3. The results are summarized in Table III. Although differences in selectivity can be seen between PPE-1 and PPE-2 or PPE-3, these differences are really quite small compared to the extensive structural changes made by the introduction of bromine. A comparison between PPE-8 and PPE-9 could not be made due to the low capacity factor values for the test probes on PPE-9.
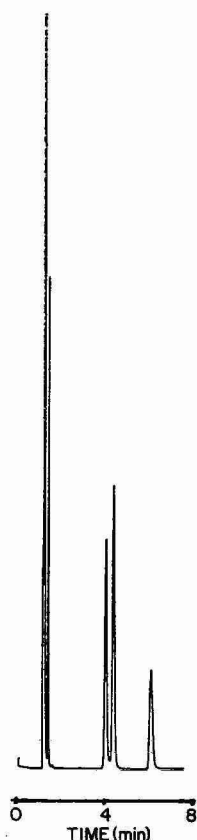
Fig. 4. Separation of decane, decene and decyne at 135°C. The conditions are the same as for Fig. 3.

Among the bromophenyl ethers investigated, PPE-7 is the most promising in terms of its chromatographic properties. It provides efficient column packings with a usable temperature range of 80–200°C. Fig. 3, discussed previously, shows a good separation of a mixture of alkanes. The phase has useful selectivity for the separation of alkanes, alkenes and alkynes. This is illustrated in Fig. 4 for the separation of decane (b.p. 174°C), decene (b.p. 181°C) and decyne (b.p. 174°C). Fig. 5 is an example of a separation of compounds covering a wide polarity range. The substituted benzenes are separated without obvious signs of peak tailing further indicating the useful properties of PPE-7 for the separation of different compound types. Its McReynolds' constants are similar, if not identical, to the five ring phenyl ether (PPE-1), which is commercially available.

The bromophenyl ethers are easily prepared by thallium acetate catalysed electrophilic bromination of the two to seven ring phenyl ethers. Bromination does not always impart useful chromatographic properties to the phenyl ethers. It lowers their volatility leading to higher available operating temperatures but generally results in packings having lower chromatographic efficiency. The presence of bromine in the phenyl ethers does not produce large changes in the selectivity of the phases. It is unlikely that the bromophenyl ethers will replace common phases already in use,
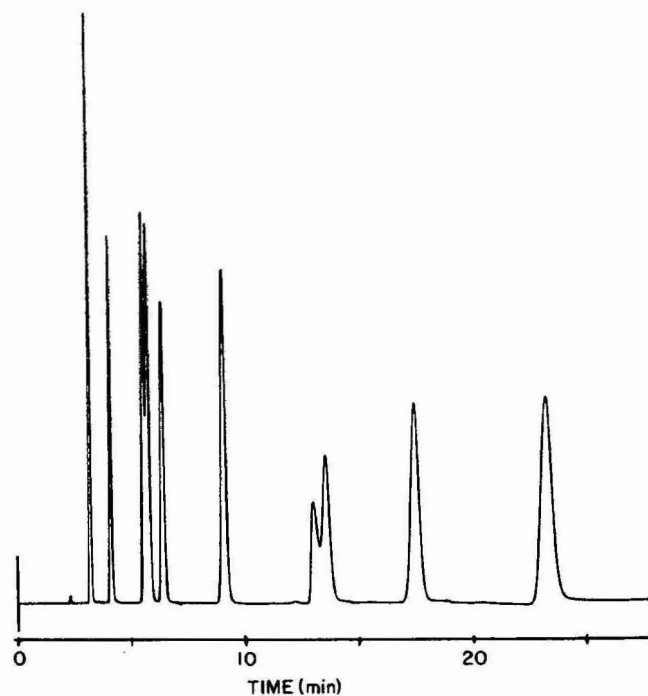
Fig. 5. Separation of substituted benzenes at 170°C. Other conditions the same as for Fig. 3. Order of elution: benzene, toluene, *m*-xylene, chlorobenzene, *o*-xylene, *p*-dichlorobenzene, benzaldehyde, *o*-dichlorobenzene, iodobenzene and nitrobenzene.

particularly as their properties are somewhat similar to the parent phenyl ethers. Their main use remains that of intermediates from which other reference stationary phases can be prepared by substitution reactions.

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COMPOSITIONAL ANALYSIS OF AN H-COAL LIQUID BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY–COMPUTER TECHNIQUE*

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SUMMARY

The H-Coal atmospheric still overhead (ASO) liquid was subjected to preparative high-performance liquid chromatography on Porasil followed by gas chromatographic and gas chromatographic–mass spectrometric analysis of the collected fractions, except for the forerun and column residue. Among the intermediate 5 fractions, there were 29 single compounds and 12 compound types identified. This analysis involved interpretation of 60 mass spectra aided by the PBM and STIRS computer search of the Cornell mass spectral data base. The identified compounds represent about half of the H-Coal liquid.

INTRODUCTION

The commercial feasibility of the H-Coal process (developed by Hydrocarbon Research, Inc.) to liquefy coal to produce refinery feedstock has been tested in a large scale demonstration plant[1]. A thorough knowledge of the composition of the liquid distillates will facilitate the assessment of process efficiency and quality assurance. The gas chromatography–mass spectrometry (GC–MS) method is particularly applicable to the volatile components as has been shown in many analyses of coal-derived samples[2–4]. Such an analysis generates a high volume of ion detection data, the interpretation of which is suited to a computer search technique using the "Probability Based Matching" (PBM) system and the interpretive algorithm "Self-Training Interpretive and Retrieval System" (STIRS) developed by McLafferty and co-workers[5,6]. In our continuing studies of the physicochemical properties of H-Coal liquids, we have determined the distributions of molecular weights by gel permeation chromatography[7,8] and of aliphatic and aromatic carbons by carbon-13 Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR)[9,10]. We wish to report herein a compositional analysis of the H-Coal atmospheric still overhead (ASO). This is achieved by fractionating the ASO liquid by normal-phase high-performance liquid chromatography (HPLC) and subjecting the intermediate fractions, except for the forerun (fraction 1, baseline material according to an UV-absorbance detector) and

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* Part of a series of reports on the physicochemical properties of H-Coal liquids.

the polar residue (fraction 7, methanol backflush from the column), to GC–MS–computer analysis. By virtue of this combined separation and analytical technique, we have made assignments by compound structure or compound type for ≈50 wt. % of the ASO liquid.

EXPERIMENTAL

Materials

The liquified coal sample under investigation was received from the University of Kentucky Institute for Mining and Minerals Research (IMMR). The liquefaction was performed by Hydrocarbon Research Inc., Trenton, NJ, U.S.A., by the H-Coal process in the "Syncrude" mode with reactor temperature at 454°C, exit reactor partial pressure of hydrogen at 2245 p.s.i.g. The sample designated atmospheric still overhead (ASO) has a nominal boiling range of C_4-200°C.

TABLE I

GAS CHROMATOGRAPHY ANALYSIS OF FRACTION 2

Temperature ramp 50°C (1 min) and 5°/min increase to 290°C. Retention times are relative to tetralin.

Sample			Standards	
Peak No.	% of peak areas	RRT	Compound	RRT
1	1.8	0.21		
2	0.9	0.24		
3	7.5	0.28	*n*-Propylcyclo-hexane	0.28
4	4.5	0.36		
5	1.0	0.40	Camphane	0.39
6	6.5	0.44	Hexahydro-indane	0.44
7	3.5	0.47		
8	3.8	0.53		
9	17.6	0.57	*trans*-Decalin	0.58
10	5.7	0.68	Adamantane	0.68
11	5.9	0.72		
12	6.9	0.81		
13	1.0	0.89		
14	2.8	0.94		
15	1.3	0.98		
16	3.4	1.03		
17	2.0	1.07		
18	7.9	1.15	Bicyclohexyl	1.15
19	1.1	1.19	Cyclododecane	1.20
20	3.5	1.25		
21	3.4	1.29		
22	2.8	1.32		
23	3.0	1.39		
24	1.1	1.42		
25	1.1	1.55		

High-performance liquid chromatography

The instrument for chromatography consisted of a U6K injector, M6000A pump and Model 440 absorbance detector operating at 254 nm, purchased from Waters Assoc. (Milford, MA, U.S.A.). The packing material, Porasil A, also obtained from Waters, was dry packed into two 610 × 7.8 mm stainless-steel columns, each containing 14.1 g. The two columns were connected in series and eluted with *n*-hexane at 4.0 ml/min. In preparative runs, injections of 100 μl of the coal liquid sample (143 mg/ml) were made. Collected fractions were distilled to remove hexane, the residues weighed and submitted to GC–MS analysis as methanolic solutions. Fraction 7, the polar material, was collected by backflush of the column with methanol.

GC–MS analysis

GC separation of a fraction collected above was carried out on a 6 ft. × 2 mm glass column packed with 3 % OV-17 (methyl phenyl silicones, 50 % each of methyl and phenyl) on Supelcoport (100–120 mesh), with helium carrier at 30 ml/min, using a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector. The temperature ramps were as specified below: fractions 2–4, 50–290°C, 5°/min; fractions 5 and 6, 100–290°C, 6°/min. For GC–MS analysis, the Finnigan Model 3300 gas chromatograph–mass spectrometer was used. The spectrometer was operated in the electron impact mode at 70 eV. The PBM/STIRS analysis[5,6] of each mass spectrum was executed on the IBM VM 370/165 computer of Cornell University via terminal, through the TYMNET time sharing system.

RESULTS AND DISCUSSION

The ASO fraction was subjected to preparative HPLC on Porasil A eluted with *n*-hexane. The separation is expected to be by chemical class, yielding in the order of alkanes, benzenes, naphthalenes and polyaromatics as that observed on μPorasil by Dark *et al.*[11]. The forerun, fraction 1, contained UV-inactive aliphatics (12 %, w/w), and the methanol backflush, fraction 7, the polar aromatic residue (11 %, w/w). The five intermediate fractions 2–6 accounted for 14, 17, 20, 15 and 11 % (w/w) respectively, of the coal liquid. The HPLC was followed by a temperature-programmed GC analysis of the latter five fractions. The relative retention times (RRTs) of the GC peaks as well as those of reference compounds, chromatographed under the same conditions, are shown for fractions 2–6 in Tables I–V, respectively. The standard compound is listed by the unknown peak only when comparison of the relative retention times is within 0.01 deviation. For fraction 2, 7 of the 25 peaks, accounting for 47 % of total peak area, correlate with cycloalkane standards. For fraction 3, 14 of 26 peaks, representing 65 % of total peak area, correlate with standards of the alkylbenzenes. For fraction 4, 92 % of the twelve peak areas is contributed by eight peaks which find correlation with standard naphthalenes, whereas four of the nine peaks in the fraction 5 chromatogram, with 24 % of the peak area, are correlatable with biphenyl types. Only 18 % of the fifteen peak area for fraction 6 is identifiable with four tri- and tetraaromatic compounds.

Mass spectral analysis of these GC peaks was undertaken. From a total of 87 peaks in these five fractions, there were 60 mass spectra obtained from the Finnigan

TABLE II

GAS CHROMATOGRAPHY ANALYSIS OF FRACTION 3

Temperature ramp 50°C (1 min) and 5°/min increase to 290°C.

Sample			Standards	
Peak No.	% of peak areas	RRT	Compound	RRT
1	0.3	0.45	n-Propylbenzene	0.45
2	2.8	0.47	1,3,5-Trimethyl-benzene	0.48
3	0.2	0.52	tert.-Butylbenzene	0.53
4	1.5	0.54	1,2,4-Trimethyl-benzene	0.54
5	8.1	0.68	1-Phenylbutane	0.67
			Indane	0.69
6	6.1	0.78	2-Phenylpentane	0.77
7	0.4	0.86		
8	5.9	0.92	1-Phenylpentane	0.91
9	2.8	0.96		
10	15.0	1.00	Tetralin	1.00
11	2.4	1.05	1,2,3-Trimethyl-benzene	1.06
12	6.9	1.10		
13	3.6	1.14	1-Phenylhexane	1.13
14	12.4	1.23		
15	2.5	1.25		
16	7.2	1.33	Phenylcyclohexane	1.33
17	1.8	1.35	1-Phenylheptane	1.36
18	1.0	1.40		
19	2.8	1.44		
20	0.8	1.49		
21	5.2	1.51	Tetrahydroace-naphthene	1.50
22	1.7	1.55		
23	4.4	1.61	Hexamethylbenzene	1.61
24	5.2	1.72		
25	0.6	1.78		
26	2.8	1.84		

GC–MS system. In Tables VI–X are tabulated the mass spectra and computer analysis. For Each GC peak, the mass spectrum, if obtained, is described in terms of five major mass ions, the total number of significant peaks and the molecular ion. Assignment of a tentative structure to each mass spectrum was made by comparison with the EIHC (Electron Impact on Hydrocarbons) file of coal-related organic compounds maintained at the mass spectral library of IMMR and the GC correlations shown above. As confirmation and further elaboration of the structural assignments, the MS data were matched by a computer search method using the Cornell University Mass Spectral Identification Systems[5,6]. This involves the retrieval system known as Probability Based Matching (PBM) which employs weighting of mass and abundance data and reverse searching, a system which has been shown to be superior for un-

TABLE III

GAS CHROMATOGRAPHY ANALYSIS OF FRACTION 4

Temperature ramp 50°C (1 min) and 5°/min increase to 290°C. Retention times are relative to tetralin.

Sample			Standards	
Peak No.	% of peak areas	RRT	Compound	RRT
1	30.2	1.11	Naphthalene	1.12
2	3.1	1.23		
3	9.4	1.31		
4	14.9	1.36	2-Methylnaph-thalene	1.36
5	4.2	1.41	1-Methylnaph-thalene	1.41
6	16.5	1.50	Tetrahydro-acenaphthene	1.50
7	4.9	1.59	2,6-Dimethyl-naphthalene	1.58
8	2.9	1.64	1,3-Dimethyl-naphthalene	1.63
9	5.9	1.82	Acenaphthene	1.81
10	3.2	1.94		
11	2.4	2.01		
12	2.4	2.17	9,10-Dihydro-phenanthrene	2.17

known spectra of mixtures. For further interpretation of the spectra, the MS data were treated to the only generally available interpretive algorithm "Self-Training Imperative and Retrieval System" (STIRS) which is applicable to most classes of organic compounds. The matching of the unknown data against the library files led to the identification of substructures or compound types. The structural candidates

TABLE IV

GAS CHROMATOGRAPHY ANALYSIS OF FRACTION 5

Temperature ramp 100°C (1 min) and 6°/min increase to 290°C. Retention times are relative to tetralin.

Sample			Standards	
Peak No.	% of peak areas	RRT	Compound	RRT
1	54.9	2.16		
2	1.7	2.41	Diphenylmethane	2.42
3	2.2	2.50		
4	13.6	2.66	4-Methylbiphenyl	2.65
5	5.1	shoulder		
6	12.1	2.88		
7	0.8	3.08	3,3'-Dimethylbiphenyl	3.09
8	8.0	3.29	9-Methylfluorene	3.29
9	1.6	3.78		

TABLE V

GAS CHROMATOGRAPHY ANALYSIS OF FRACTION 6

Temperature ramp 100°C (1 min) and 6°/min increase to 290°C.

Sample			Standards	
Peak No.	% of peak areas	RRT	Compound	RRT
1	0.7	2.45		
2	0.9	2.68		
3	2.5	2.72		
4	1.2	2.92		
5	2.1	3.16		
6	49.2	3.25		
7	4.9	3.80	9-Methylfluorene	3.29
8	22.1	3.81		
9	10.8	4.28	Phenanthrene	4.27
10	0.8	4.73		
11	0.6	4.88		
12	0.9	4.99		
13	0.6	5.51	Fluoranthene	5.50
14	0.9	5.75		
15	1.8	6.33	1-Methylpyrene	6.33

thus obtained are entered under the respective headings of PBM and STIRS in Tables VI–X.

Regarding fraction 2, 20 mass spectra out of 25 GC peaks were generated.' From the EIHC file, peaks 3, 6, 9 are assigned single structures, 5, 7, 20 structure types and the remainder, except two, with molecular formula information. The PBM search is not particularly useful for this fraction because of high background level. Nevertheless, it has allowed assignment of structures to 6 and 10, and the assignment of structural types to 2 and 5. The STIRS has confirmed structures for 6 and 10, assigned a structure to 23 and showed the substituents for 2 and 5. These results are shown in Table VI.

From fraction 3 there were obtained 25 mass spectra out of 26 GC peaks. Of these, the EIHC file has produced tentative assignments of specific structures for thirteen peaks (1, 2, 4, 5, 10, 12, 13, 14, 15, 18, 19, 21, 25), structural types for four (6, 11, 15, 26), molecular formulas for three, but no information on five (7, 9, 22, 23, 24). The computer search for this and subsequent fractions was quite fruitful. PBM has supplied fourteen specific structures, six structural types and one molecular formula, while STIRS substantiated nine structures and nine structural types. Only two (5, 20) of the tentative assignments were not further identified, and peaks 7, 23 and 24 remain unknowns. These results are shown in Table VII.

Of the twelve GC peaks in fraction 4, mass spectra are available for eight, all of which have received tentative assignments. They are analyzed by PBM/STIRS to yield identifications of six specific compounds and two compound types. These results are shown in Table VIII.

TABLE VI

IDENTIFICATION OF COMPONENTS IN FRACTION 2 BY MS-COMPUTER ANALYSIS

Italicized values denote base peaks as 100% intensity.

GC peak no.	MS data		Tentative assignment		Computer search	
	No. of significant peaks	Major m/e	M$_r^+$ obs.	Compound or type	PBM	STIRS
1	20	40, 41, 53, *54*, 97	126	Cyclo C$_9$H$_{18}$		
2	12	41, 53, 54, *96*, 97	126	C$_9$H$_{18}$	An alkylcyclo-hexane	Me
3	20	53, *54*, 81, 82, 83	126	Propylcyclo-hexane		
4	40	40, 53, 54, *96*, 97	140	C$_{10}$H$_{20}$		
5	13	40, 41, *54*, 55, 97		an alkylcyclo-hexane	An alkylcyclo-hexane	Me, Et, Pr
6	23	66, 67, 81, 82, 97	124	Hexahydroindane	cis-Hexahydro-indane	cis-Hexahydro-indane
7	17	53, 54, *81*, 82, 83	140	A butylcyclo-hexane		
8	21	66, 67, 80, 81, 96	138, 154	Bicyclo C$_{10}$ + cyclo C$_{11}$		
9	20	66, 67, 68, *81*, 95	138	Decalin		
10	65	66, 67, 81, 95, 152	152	Bicyclo C$_{11}$	2-Methyl-trans-decalin	2-Methyl-trans-decalin
12	26	40, 41, *54*, 55, 56	166			
14	64	41, 53, 54, *82*, *83*	166	Bicyclo C$_{12}$		
15	35	53, 54, *62*, *81*, 82	164			
16	68	40, 41, *54*, 55, 56	164	C$_{12}$H$_{20}$		
18	24	53, *54*, 66, 67, 82	166	C$_{12}$H$_{22}$		
19	29	41, 53, 54, 67, 97	164	C$_{12}$H$_{20}$		
20	59	53, 54, *67*, 82, 83	166	An ethylbicyclo-decane		
23	55	66, 67, *81*, 95, 137	152	A methylbicyclo-decane		2-n-Butyl-decalin
24	30	54, 67, 81, 96, 97	164	C$_{12}$H$_{20}$		
25	64	66, 67, *81*, 135, 192	150, 164	C$_{11}$H$_{18}$ + C$_{12}$H$_{20}$		

TABLE VII

IDENTIFICATION OF COMPONENTS IN FRACTION 3 BY MS-COMPUTER ANALYSIS

Italicized values denote base peaks as 100% intensity.

GC peak no.	MS data Major m/e	No. of significant peaks	Tentative assignment M+ obs.	Compound or type	Computer search PBM	STIRS
1	64, 65, 90, *91*, 120	16	120	n-Propylbenzene	A substituted benzene	Phenyl
2	77, 79, 91, *105*, 120	28	120	1,3,5-Trimethylbenzene	1,3,5-Trimethylbenzene	
3	77, 79, 91, *105*, 120	14	120	C$_9$H$_{12}$		A C$_3$H$_7$-benzene
4	77, 91, 103, *105*, 120	24	120	1,2,4-Trimethylbenzene	1,2,4-Trimethylbenzene	
5	91, 115, *117*, 118, 119	37	118	1-Phenylbutane + indane		
6	91, 115, *117*, 132, 133	39	132	A methylindane	1-Methylindane	1-Methylindane
7	77, 91, *119*, 120, 131	30				
8	77, 91, *105*, 106, 148	30	148	C$_{11}$H$_{16}$	A substituted benzene	p-Isobutyltoluene or a dimethylethyl-benzene
9	91, 115, *117*, 118, 132	36	132		Monomethylindanes	Monomethylindanes
10	78, 91, *104*, 115, 132	39	132	Tetralin	Tetralin	
11	91, 115, *117*, *131*, 132	22	146	A methyltetralin	5-Methyltetralin	1- or 2-methyltetralin
12	91, *104*, 115, 131, 146	26	146	2-Methyltetralin	2-Methyltetralin	2-Methyltetralin
13	91, 115, 117, *131*, 132	38	146	5-Methyltetralin	5-Methyltetralin	5-Methyltetralin
14	117, *118*, 131, 132, 146	48	146	6-Methyltetralin	6-Methyltetralin	6-Methyltetralin
15	91, 115, *117*, 118, 131	70	160	A propylindane	A substituted indane	An alkylindane
16	91, 104, 117, *118*, 160	67	146, 160	Phenylcyclohexane and a 2,x-dimethyl-tetralin	Phenylcyclopentane and 2,7-dimethyl-tetralin	Phenylcyclopentane and 2,7-dimethyl-tetralin
18	115, 117, *118*, 129, 145	48	146, 160	A dimethyltetralin	1,1-Dimethyltetralin	1,1-, 1,5-, 2,6-, 2,7-dimethyltetralin
19	91, 115, 117, *131*, 132	57	148	5-Ethyltetralin	An ethyltetralin	An ethyltetralin
20	91, 92, *105*, 129, 148	20	148	C$_{11}$H$_{16}$		
21	115, 128, 129, *130*, 131	46	158	Tetrahydroacenaphthene	2a,3,4,5-Tetrahydro-acenaphthene	2a,3,4,5-Tetrahydro-acenaphthene
22	91, 117, 118, *131*, 132	37			C$_{10}$H$_{12}$	C$_{10}$H$_{12}$
23	115, *129*, 130, 131, 145	27				
24	91, 117, 129, *159*, 160	40				
25	91, *104*, 105, 131, 132	22	188	2-Butyltetralin	A tetralin	2-tert.-Butyltetralin
26	115, 117, 131, *145*, 146	30	188	5- or 6-butyltetralin	6-Butyltetralin	5- or 6-butyltetralin

TABLE VIII

IDENTIFICATION OF COMPONENTS IN FRACTION 4 BY MS–COMPUTER ANALYSIS

Italicized values denote base peaks as 100% intensity.

GC peak no.	MS data		Tentative assignment		Computer search	
	Major m/e	No. of significant peaks	M⁺ obs.	Compound or type	PBM	STIRS
1	51, 63, 102, 127, *128*	32	128	Naphthalene	Naphthalene	
3	73, 117, 118, 131, 148	39	146	A methyltetralin	5- or 6-methyltetralin	5- or 6-methyltetralin
4	63, 115, 139, 141, *142*	53	142	2-Methylnaphthalene	2-Methylnaphthalene	
5	63, 115, 139, 141, *142*	40	142	1-Methylnaphthalene	1-Methylnaphthalene	
6	115, 128, 129, *130*, 145	52	158	Tetrahydroace-naphthene	Tetrahydroace-naphthene	
7	115, 128, 129, *141*, 156	38	156	An ethylnaphthalene	A naphthalene	2-Ethylnaphthalene
9	129, 144, 152, *153*, 154	22	154	Acenaphthene		Acenaphthene
11	115, 128, 129, *141*, 142	27	154	A naphthalene	A naphthalene	A naphthalene

TABLE IX

IDENTIFICATION OF COMPONENTS IN FRACTION 5 BY MS–COMPUTER ANALYSIS

Italicized values denote base peaks as 100% intensity.

GC peak	MS data		Tentative assignment		Computer search	
	Major m/e	No. of significant peaks	M⁺ obs.	Compound or type	PBM	STIRS
1	51, 76, 152, *153*, *154*	60	154	Biphenyl	Biphenyl	Biphenyl
4	51, 63, 115, 152, 168	30	168	A methylbiphenyl		4-Methyl-biphenyl
6	52, 53, 64, *139*, 168	27	168	Dibenzofuran	Dibenzofuran	
8	45, 82, 83, *152*, 153	45		9-Methylfluorene		A diaromatic

TABLE X

IDENTIFICATION OF COMPONENTS IN FRACTION 6 BY MASS SPECTRA–COMPUTER ANALYSIS

Italicized values denote base peaks as 100% intensity.

GC peak	MS data		Tentativ; assignment		Computer search	
	Major m/e	No. of significant peaks	M^+ obs.	Compound or type	PBM	STIRS
6	63, 82, 139, *165*, 166	31	166	Fluorene	Fluorene	Fluorene
8	40, 74, 75, 76, 89	25	180	1-Methyl-fluorene	1-Methyl-fluorene	1-Methyl-fluorene
9	63, 75, 76, 89, 178	17	178	Phenanthrene	Phenanthrene	Phenanthrene

TABLE XI

COMPOSITION SUMMARY OF FRACTIONS 2–6 OF H-COAL ASO LIQUID

Fraction 1 (12%) contains UV-inactive aliphatic hydrocarbons and fraction 7 (11%) the polar or polynuclear aromatics.

Fraction	Components identified (% of GC trace)	% of fraction by GC peak area	% of ASO*	Av. mol. wt.	Av. molecular formula
2	Alkylcyclohexanes (5.4), hexahydroindane (6.5), methyldecalin (5.7)	17.6	2.4	129	C_9H_{17}
3	Trimethylbenzenes (4.3), methylindanes (8.9), tetralin (15.0), alkyl-tetralins (32.3), tetra-hydroacenaphthene (5.2)	65.7	11.4	143	$C_{11}H_{14}$
4	Tetrahydroacenaphthene (16.5), methyltetralins (9.4), naphthalene (30.2), alkylnaphthalenes (24.0)	80.1	16.1	141	$C_{11}H_{11}$
5	Biphenyl (54.9), dibenzofuran (12.1)	67.0	10.1	157	$C_{12}H_{10}$
6	Fluorene (49.2), methyl-fluorene (22.1), phenanthrene (10.8)	82.1	8.9	172	$C_{13}H_{11}$

* Calculated from % of fraction × % (w/w) of fraction.

There are nine GC peaks for fraction 5, of which four, representing 89 % of the total peak area, have yielded mass spectra. The same analysis routines have led to determination of three compounds and one tentative assignment. These results are shown in Table IX.

Although only three mass spectra were derived from fifteen of the GC peaks of fraction 6, the spectra are indicative of 83 % of the material as represented by GC peak area. Three single compounds were unanimously picked by the three MS analysis routines as shown in Table X.

CONCLUSIONS

The HPLC fractions 2–6 have yielded 60 mass spectra from 87 GC peaks. Interpretation of the MS data by making use of the EIHC file of caol-related compounds and the huge Cornell mass spectra library have generated structures for 29 single compounds and 12 compound types. It appears that the HPLC fractionation of the H-Coal ASO liquid on Porasil A is by chemical class. Fraction 1 is comprised of UV-inactive aliphatic hydrocarbons. Fraction 2 contains cycloalkanes from al-

kylcyclohexanes to alkyldecalins. Fraction 3 is made up of monoaromatics from a trimethylbenzene to an alkyltetralin. The fraction 4 materials are naphthalenes, fraction 5 the biphenyl types and fraction 6 the fluorenes and phenanthrenes. The larger polynuclear aromatics are probably contained in the polar fraction 7. In Table XI are summarized the above compositional analyses. The components identified as single compounds or compound types, their distributions in each HPLC fraction and in the whole liquid are shown. The average molecular weights and molecular formulas are calculated for each fraction on the basis of the components identified. It is noted that $\approx 49\%$ by weight of ASO is accounted for by components containing about eleven carbons.

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TRACE ANALYSIS OF AMINES AND ISOCYANATES USING GLASS CAPILLARY GAS CHROMATOGRAPHY AND SELECTIVE DETECTION

I. DETERMINATION OF AROMATIC AMINES AS PERFLUORO FATTY ACID AMIDES USING ELECTRON-CAPTURE DETECTION

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SUMMARY

A method for the trace analysis of aromatic amines is presented. It involved conversion of the amines into the corresponding amides by reaction with a perfluoro fatty acid anhydride. The amides were separated by glass capillary gas chromatography and quantitation was achieved using on-column injection and electron-capture detection.

The method was applied to amines of interest from work environment health aspects. Detection limits were in the low picogram range. Columns suitable for this application and their preparation are discussed together with detector behaviour and the mass spectra of investigated perfluoro fatty acid amides.

INTRODUCTION

Recently a gas chromatographic (GC) method for the trace analysis of isocyanates in air, developed at this laboratory, was presented[1]. It involved absorption of the isocyanates in acidic solution, where they were hydrolysed to the corresponding amines. After addition of excess base, the amines were extracted with toluene and reacted with a perfluoro fatty acid anhydride to yield the corresponding amides. These were separated by packed column GC and monitored with an electron-capture detector. The method was applied to a small number of aromatic isocyanates.

In this continuation work, our attention was focused on the assay of aromatic amines that are encountered in industrial atmospheres, thus being of interest from work environmental health aspects. In order to increase selectivity and versatility, capillary instead of packed column GC was applied to the separation of the amides.

The amines in toluene solution are converted into the corresponding amides by treatment with a perfluoro fatty acid anhydride, preferably pentafluoropropionic acid anhydride (PFPAA), according to the reaction
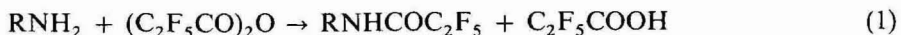
$$RNH_2 + (C_2F_5CO)_2O \rightarrow RNHCOC_2F_5 + C_2F_5COOH \qquad (1)$$

TABLE I

AMINES INVESTIGATED

No.	Amine	Abbreviation	Abbreviation of corresponding isocyanate*
1	Aniline	—	PhI
2	2,6-Toluenediamine	2,6-TDA	2,6-TDI
3	2,4-Toluenediamine	2,4-TDA	2,4-TDI
4	2,4-Diaminoanisole	2,4-DAA	—
5	α-Naphthylamine	—	—
6	β-Naphthylamine	—	—
7	4-Aminobiphenyl	—	—
8	1,5-Naphthalenediamine	NDA	NDI
9	4,4'-Diaminobiphenyl (benzidine)	—	—
10	4,4'-Methylenedianiline	MDA	MDI
11	o-Tolidine (4,4'-diamino-3,3'-dimethyl-biphenyl)	TODA	TODI
12	4,4'-Methylenebis-(o-chloroaniline)	MOCA	—
13	4,4'-Diamino-3,3'-dimeth-oxybiphenyl (o-dianisidine)	DADA	DADI

* The isocyanate formula is obtained by exchange of NH_2 groups in the amine for NCO groups.

Excess reagent and pentafluoropropionic acid formed in the reaction are extracted with a pH 7.0 phosphate buffer and the remaining toluene solution of amides is analysed by glass capillary GC using on-column injection and an electron-capture detector.

The method is also applicable, as will be described in a forthcoming paper, to the determination of aromatic isocyanates after absorption in acidic solution and extraction of the amines formed. Table I lists the investigated amines and corresponding isocyanates currently encountered in industrial atmospheres. Amines without counterparts among the isocyanates are carcinogenic substances, being entered on the A and B lists of the Swedish Work Environment Board.

EXPERIMENTAL

Apparatus

Chromatograph. A Carlo Erba Fractovap Model 4160 chromatograph with on-column injection system was employed.

Detector. A ^{63}Ni (10 mCi) Carlo Erba Model HT-25 electron-capture detector with a control module 251 was used in the constant-current mode (voltage 50 V, pulse width 0.1 μsec, standing current 2.0 nA).

Glass capillaries. Glass capillaries were drawn from Pyrex or Duran 50 borosilicate glass tubes on a Carlo Erba Model 60 Glass Capillary Drawing Machine.

Column. A capillary Pyrex glass column (30 m × 0.3 mm I.D.) with 1.0 μm OV-73 stationary phase was employed, together with several other capillary columns,

all prepared in this laboratory as described below.

Recorder. A Servogor Model 310 recorder was used.

Integrator. A Hewlett-Packard Model 3390A Reporting Integrator was employed for peak evaluation.

Gases. The carrier gas used was helium and the make-up gas was argon–methane (95:5). Both were dried over activated molecular sieve 5A and deoxygenated using an Indicating Oxytrap (Chrompack, Middelburg, The Netherlands). The gas pressure regulators used were equipped with steel membranes (l'Air Liquide; provided by Alfax, Stockholm, Sweden).

Mass spectrometer. Mass spectrometric data were obtained on a Finnigan Model 4021 mass spectrometer operated in the electron impact mode with positive ion monitoring or in the chemical ionization mode using methane as ionization agent and negative ion monitoring.

Materials

Chemicals. Aniline, 2,4- and 2,6-toluenediamine (2,4- and 2,6-TDA), 2,4-diaminoanisole (DAA), 4,4'-diaminodiphenylmethane (MDA), benzidine and α- and β-naphthylamine were obtained from E. Merck (Darmstadt, F.R.G.). 4-Aminobiphenyl (BPA), 3,3-dimethoxybenzidine (DADA), 3,3'-dimethylbenzidine (TODA) and 1,5-diaminonaphthalene (NDA) were obtained from Fluka (Buchs, Switzerland) and 4,4'-diamino-3,3'-dichlorodiphenylmethane (MOCA) from Aldrich-Europe (Beerse, Belgium). Heptafluorobutyric (HFBAA), pentafluoropropionic (PFPAA) and trifluoroacetic (TFAAA) anhydrides were obtained from Pierce (Rockford, IL, U.S.A.) and, depending on batch quality, were distilled over phosphorus pentoxide (E. Merck). Hydrochloric acid, min. 37% (w/w), potassium dihydrogen phosphate and p.a. grade pentane were purchased from E. Merck and sodium hydroxide (p.a.) pellets from EKA (Bohus, Sweden).

Silylating agents for column deactivation, hexamethyldisilazane (HMDS), diphenyltetramethyldisilazane (DPTMDS), tetraphenyldimethyldisilazane (TPDMDS) and triphenylsilylamine (TPSA) were all obtained from Fluka. All stationary phases used were purchased from Chrompack.

Solvents and solutions. All water used was doubly distilled. Toluene was of glass-distilled grade (Rathburn Chemicals, Walkerburn, U.K.). Pentane, diethyl ether and methanol, all of p.a. grade, were from E. Merck. Saturated sodium hydroxide solution was obtained by addition of 150 g of sodium hydroxide to 100 ml of water. Phosphate buffer was prepared from potassium dihydrogen phosphate (136 g, 1 mol) and 1000 ml of water. The pH was adjusted to 7.0 with saturated sodium hydroxide solution.

Procedure

Standard solutions. Standard solutions of the amines were prepared by dissolving accurately weighed amounts (*ca.* 50 mg) in 100 ml of acetonitrile; 100-μl aliquots of this solution were then diluted to 100 ml with toluene to produce a solution with an amine concentration of *ca.* 500 pg/μl. This solution was further diluted with toluene to desired concentrations.

Derivative preparation. A 1-ml aliquot of the sample solution was transferred into a ground-glass stoppered test-tube, followed by the addition of 20 μl of the

appropriate perfluoro fatty acid anhydride. The contents of the test-tube were shaken for 1 min and then allowed to stand for 5 min. The excess reagent and the acid formed were removed by shaking with 1 ml of phosphate buffer (pH 7.0). The toluene layer containing the amide was then transferred into another test-tube, ready for injection into the gas chromatograph.

Column preparation. Columns with apolar stationary phases (OV-73, OV-1, SE-52 and SE-54) were prepared by the persilylation procedures described by Grob *et al.*[2]. Coiled Pyrex or Duran 50 glass columns with pre-straightened ends were rinsed and filled to 92 % with 20 % hydrochloric acid, flame-sealed and heated at 180°C for 16–18 h. After rinsing with water and drying under vacuum at 250°C the columns were treated by dynamic coating with a mixture of HMDS and DPTMDS (1 part of each in 2 parts of pentane), subjected to vacuum, sealed and heated at 400°C overnight. After rinsing with toluene, methanol and diethyl ether, the columns were coated by the static coating procedure at room temperature, using pentane solutions (1.3 %, w/v, to produce a *ca.* 1.0 μm stationary phase thickness) employing a sealing technique developed at this laboratory[3]. A similar procedure was applied to fused silica columns (obtained from Hewlett-Packard, Avondale, PA, U.S.A.), differing only in that the leaching procedure was replaced by merely rinsing with 20 % hydrochloric acid.

In the on-column injection method, the stationary phase is gradually flushed away from the first part of the column, and involatile substances will also be deposited at the inlet end of the column, thus impairing its performance. On that account, the inlet end has to be renewed from time to time. This is achieved in the following way: *ca.* 1 m of the column is cut off and stationary phase is removed from the first 7 cm of the remaining part of the column by the slow injection of 5 ml of solvent (pentane for apolar phases) against a nitrogen gas stream by means of a 5-ml syringe equipped with an on-column injection needle inserted as far as possible into the column. The column end is then straightened at a minimum temperature using an alcohol flame, as suggested by Grob *et al.*[4] or using a glass capillary straightener (Carlo Erba GESM Model 102-20). Occasionally the same procedure has to be applied to the outlet end of the column, where the column tends to deteriorate because of the high detector temperature (300°C).

Gas chromatography. Samples were analysed by on-column injection at a starting temperature near the solvent's boiling point (115°C for toluene). After elution of the solvent (*ca.* 2 min), the column temperature was programmed at 10°C/min to 300°C, where it was held for 5 min.

Quantitative analysis. The quantitative analysis was based on peak-height measurement. The linear response range for each derivative was established by plotting peak heights against concentration (Fig. 2) for injected standard solutions. In the applied mode, the response of the electron-capture detector is linear up to *ca.* 50 % of its dynamic range (see discussion below).

RESULTS AND DISCUSSION

Procedure

Formation of amides. Three perfluoroacylating agents, HFBAA, PFPAA and TFAAA, were investigated with regard to yield, separation properties and sensitivity.

The TFAA derivatives were discarded at an early stage of the investigation owing to their low sensitivities, being 10–20 times lower than for the HFBA and PFPA derivatives. This is also evident from recent work by Ebell et al.[5], who employed TFAAA for the assay of 2,4-TDA, 2,6-TDA and MOCA.

The sensitivity difference between the PFPA and HFBA derivatives is surprisingly small considering the difference in the number of electron-withdrawing fluorine atoms in these derivaties. However, the increased distance between the trifluoromethyl group and the electron-capturing carbonyl group in the HFBA derivative should diminish its electron-attracting effect compared with that in the PFPA derivative, as has been discussed by Clarke et al.[6].

The same derivatization procedure could be used for the two kinds of derivatives and the amines were fully converted into the amides in less than 1 min at room temperature. It was shown by GC–mass spectrometry (MS) that only one hydrogen atom at each amino group was replaced by a pentafluoropropionyl (PFP) or a heptafluorobutyryl (HFB) group (see Fig. 4).

Chromatograms of HFBA and PFBA derivatives of the investigated amines are shown in Fig. 1. The main difference between the chromatograms is the lower elution temperatures for the PFPA derivatives and the better separation between the PFPA derivatives of the two TDA isomers. It is therefore considered that PFPAA is the best derivatization reagent for the present application, in spite of the higher sensitivity of the HFBA derivatives. The fact that PFPAA can be obtained in a higher purity is an added advantage.

Extraction of excess reagent and liberated acid. Excess reagent and liberated acid are removed by extraction with a pH 7.0 phosphate buffer. Higher pH values will result in losses of derivatives, as demonstrated in earlier work in this laboratory[1], and lower pH values will give incomplete removal.

Chromatographic system

Capillary column. Trace analysis of the perfluoracylated amines requires glass capillary columns with high inertness and good temperature stability. On that account, polar stationary phases are less suitable because of more active surfaces and lower maximum temperatures. This is specially the case for the high-boiling derivatives of benzidine, MDA, TODA, MOCA and DADA, as shown by tests with OV-225 and Carbowax 20M stationary phases. Apolar columns prepared by the persilylation procedures described above, on the other hand, although requiring the same high elution temperatures as the polar columns, have shown remarkably good inertness and long-term stability.

Several silylating agents were tested in order to achieve optimal column performance. The agents finally chosen were a mixture of HMDS and DPTMDS as suggested by Grob et al.[2]. Attempts to use HMDS alone, as recently proposed by Godefroot et al.[8], gave columns with a durability of only a few days of high-temperature use. Other silylating agents suggested by Grob[7], namely TPDMDS and TPSA, did not give even initially acceptable columns.

Fused-silica columns were also prepared using the HMDS–DPTMDS mixture for deactivation, but although the initial inertness was acceptable, these columns rapidly deteriorated. This is probably due to the fact that persilylation temperatures above 330°C cannot be used, owing to the temperature limit set by the outer coatings
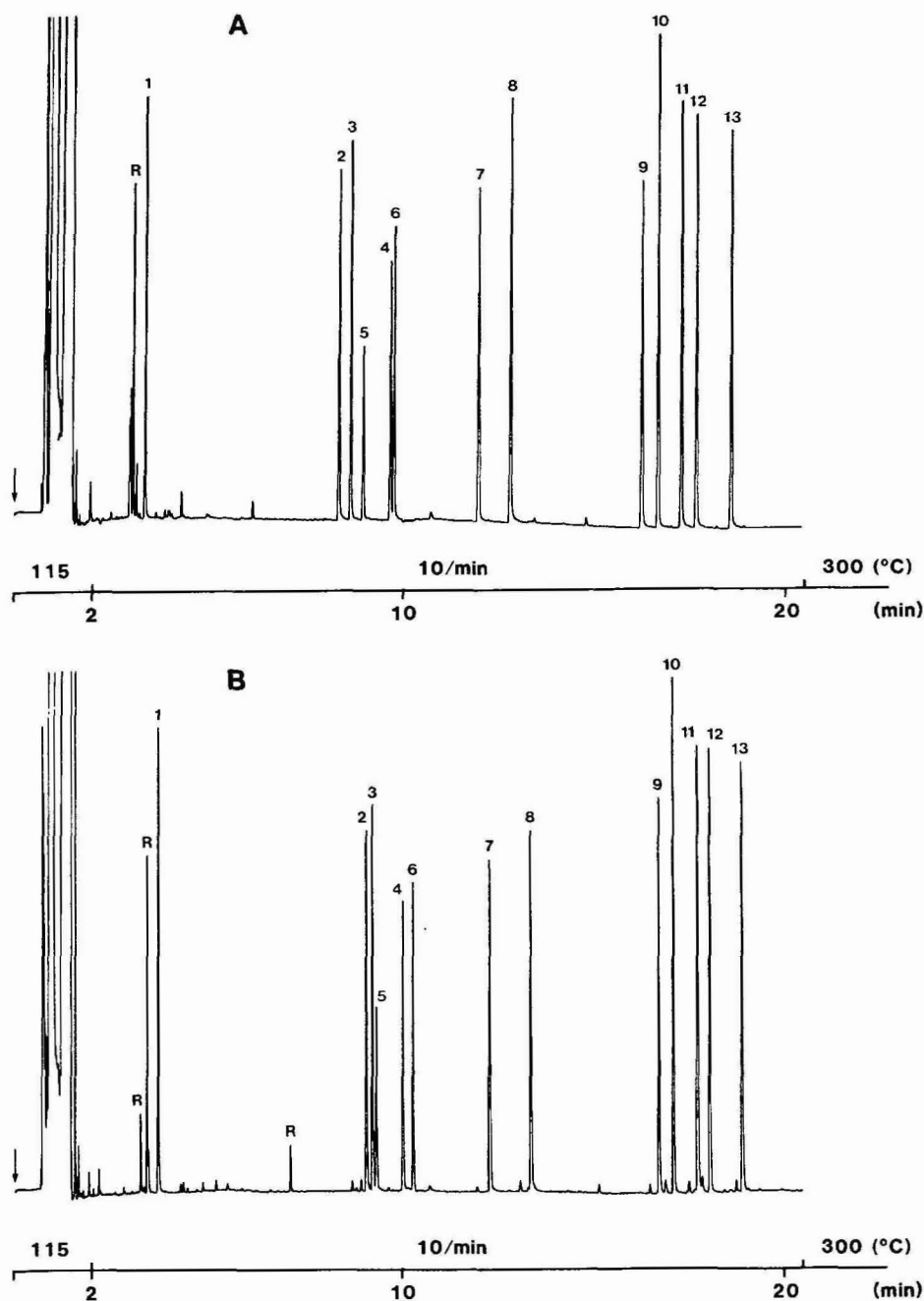
Fig. 1. Chromatograms of amine derivatives. (A) PFPA derivatives; (B) HFBA derivatives. Column, 25 m × 0.32 mm I.D. Pyrex glass capillary with OV-73 stationary phase, film thickness 1.0 μm. On-column injection of 2 μl of toluene solution at 115°C; after 2 min the temperature was programmed at 10°C/min to 300°C, where it was held for 5 min. Carrier gas, helium at 0.8 kg/cm². Electron-capture detector, constant-current mode; standing current, 2.0 nA; voltage, 50 V; pulse width, 0.1 μsec; temperature, 300°C; make-up gas, argon–methane (95:5); flow-rate, 55 ml/min. Peak identities according to Table I, each peak corresponding to 20–80 pg of amine. Peaks denoted R refer to reagent and solvent impurities.

of these columns. Initial work with polysiloxane deactivated columns, employing octamethylcyclotetrasiloxane (D4)[9] as deactivating agent, indicates that these columns may be well suited for the present application.

It should be emphasized that almost all of the *ca.* 50 columns prepared for this investigation were of acceptable quality when tested at nanogram levels with amide standard solutions or a "Grob test" mixture[10] and a flame-ionization detector, but only when tested at picogram levels with an electron-capture detector was their true suitability for the trace analysis of perfluoro fatty acid amides revealed.

One important factor with regard to the high inertness of the column finally chosen is the relatively high stationary phase film thickness (1 μm) used. Of the stationary phases tested (OV-73, OV-1, SE-52 and SE-54), OV-73 was selected as having very good long-term stability (one column was in constant use for 6 months) and separation efficiency, although differences between the tested phases in the latter respect were small. All stationary phases used were gum phases, being preferable in conjunction with on-column injection because of low solubility[11], and electron-capture detection, because of the low bleed rate[12].

The columns were fitted into the chromatograph using Vespel graphite ferrules (obtained from Carlo Erba, Milan, Italy). The use of Teflon or Viton ferrules was found to give ghost peaks, even when used at the cool injector end.

Carrier gas. Although hydrogen is generally the most suitable carrier gas in capillary GC, helium was employed because of the higher response and better linearity in conjunction with the electron-capture detector[13], although this meant about 20–30°C higher elution temperatures. Use of the make-up gas (argon–methane) as the carrier gas was not considered, as this would result in even higher elution temperatures, although the detector performance might be improved.

On-column injection. The on-column injection technique is the most reliable injection technique for quantitative analysis by capillary GC. The reproducibility of our chromatographic system was found to be acceptable, in that repeated injections of 2-μl aliquots of a 25 pg/μl standard solution of all PFPA derivatives at 115°C gave peak height standard deviations of 0.3% for the aniline derivative and 0.8–1.7% for the other derivatives, eluted at temperatures of up to 300°C.

Problems with removal of the stationary phase by solvent flushing were dealt with as described above. When renewing the capillary inlet end, both removal of the stationary phase prior to and the use of a protective atmosphere (nitrogen or helium) during straightening are essential, as increased column adsorption of samples will otherwise occur, as demonstrated by Grob *et al.*[4].

It has been demonstrated that non-extractable stationary phases can be prepared *in situ* from, *e.g.*, OV-73 by using dicumyl or dibenzoyl peroxide and other cross-linking agents[9,14–16]. For such stationary phases, the need to renew the inlet end of the column generally does not arise, as the phase is not flushed from the column during injection and involatile deposits can be removed with a suitable solvent. Deterioration of column ends due to high temperatures can not be prevented, however, and as renewal of the column end is not possible, the column life is considerably shortened. In addition, these stationary phases have a greater tendency to adsorb samples. On that account, non-extractable stationary phases are at present not recommended for the trace analysis of the amides in question.
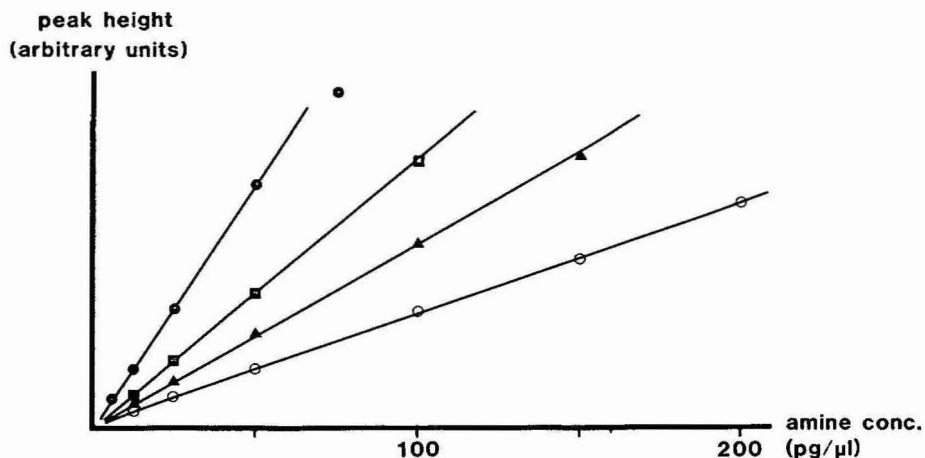
peak height
(arbitrary units)

Fig. 2. Calibration graphs for some amine PFPA derivatives in toluene. ● = 2,4 TDA; ■ = MDA; ▲ = benzidine; ○ = DADA.

Quantitative analysis

Linear range and detection limits. Fig. 2 demonstrates the linear range for some of the PFPA derivatives investigated. By the use of a high make-up gas flow-rate (60 ml/min), compared with the 10–20 ml/min recommended[17], the linear range was improved. By modification of the detector flow geometry, whereby the inner diameter of the detector jet and the area of the collector electrode were reduced, the linearity was further improved (Fig. 3).

The detection limits for the compounds investigated, calculated as the amount of amine giving a signal-to-noise ratio of 2:1, are in the region of 0.1–0.4 pg, but adsorption effects restrict practical detection limits to *ca.* 1 pg. Peak evaluation could be based on either peak height or peak area measurements, giving equivalent results.

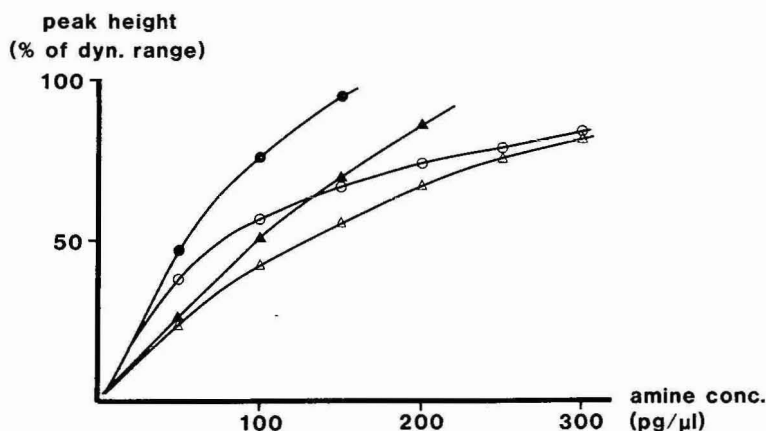**peak height**
(% of dyn. range)

Fig. 3. Effect of make-up gas flow-rate and detector modifications (see text) on response for PFPA derivative of 2,4-TDA. ○ = Original detector and ● = modified detector, flow-rate 20 ml/min; △ = original detector and ▲ = modified detector, flow-rate 55 ml/min.

Mass spectrometry

The structures of all PFPA derivatives were confirmed by GC–MS spectrometry using a Finnigan Model 4021 mass spectrometer in the electron impact mode. It was shown that one hydrogen was substituted at each amino group. Mass numbers (*m/z*) *versus* relative abundance of fragments of interest for the interpretation of the spectra are given in Table II.

As can be seen, a molecular ion is obtained in all instances, varying in relative abundance from 15 to 100%. CF_3^+ and $C_2F_5^+$ ions are also always split off, although the relative abundance of the latter is low in some instances. The structure of a major part of the fragments formed, $(M - X)^+$, can be formally interpreted as that of residual ions appearing when C_2F_5, C_2F_5CO and C_2F_5CONH alone, or in various combinations for difunctional derivatives, are split off from the molecular ion. In the case of 2,4-DAA, TODA and DADA, methyl groups are in addition lost. There is also an indication that hydrogen is eliminated from the methyl group in 2,4- and 2,6-TDA, a reaction that is known to occur with toluene (ref. 18, pp. 76 and 86).

The ions Y^+ in Table II are considered to be formed by rearrangement of the basic aromatic structure. Thus the PFPA derivatives of aniline, α- and β-naphthylamine, 1,5-NDA and 4-aminobiphenyl can give rise to cyclopentadienyl cations by expulsion of HCN from a residual ion (ref. 18, p. 323). For PFPA-DADA, the same kind of ion can, by analogy with anisole, be formed by abstraction of a methyl group followed by expulsion of carbon monoxide (ref. 18, p. 237). The appearance of tropylium ions in the mass spectra of diphenylmethanes is well known (ref. 18, pp. 87–89). On that account, the structural assignments given in Table II for the Y^+ ions of the PFPA derivatives of MDA and MOCA are considered to be well founded.

In most of the spectra, clusters of ions are found in the region of *m/e* 51–52, 63–65, 77–78, 89–92 and 101–107, which are known to arise from the decomposition of aromatic structures[18]. The base peak, *i.e.*, the highest peak in the spectrum, is formed in one instance by the molecular ion (M^+) and in four instances each by the $C_2F_5^+$ ion, an $(M - X)^+$ ion or a Y^+ ion.
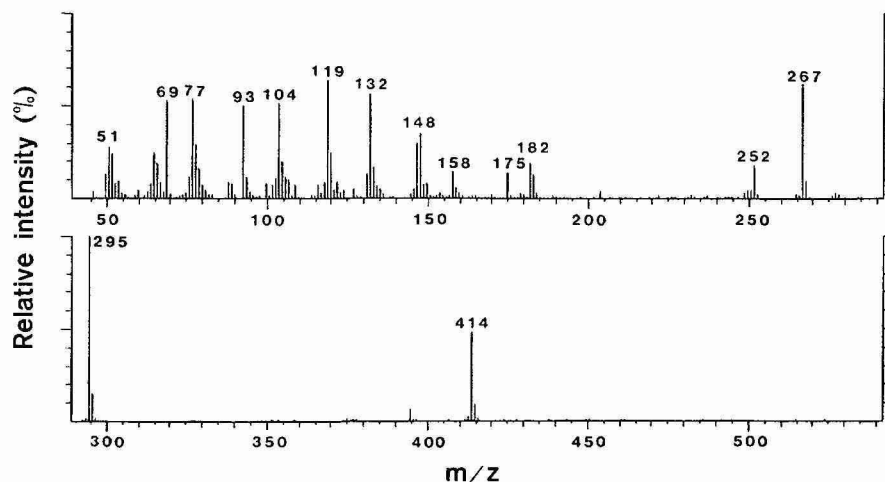

Fig. 4. Mass spectrum of PFPA derivative of 2,4-TDA, obtained by electron impact ionization and positive ion monitoring. For interpretation, see text and Table II.

TABLE II

MASS NUMBER (m/z)/RELATIVE ABUNDANCE (%) OF CERTAIN DIAGNOSTIC IONS FORMED ON ELECTRON IMPACT MASS SPECTROMETRY OF PFPA DERIVATIVES OF AROMATIC AMINES

One PFA group is substituted at each amino group.

PFPA derivative of	Base peak	M^+	CF_3^+	$C_2F_5^+$	$(M - C_2F_5)^+$	$(M - C_2F_5CO)^+$	$(M - X)^{+\star}$ Y^+	Tentative assignment for Y^+
Aniline	$(M - X)^+$	239/45	69/20	119/20	120/50	92/45	77/100[1] 65/65	Cyclopentadienyl ion $(C_5H_5)^+$
2,4-TDA	$(M - C_2F_5)^+$	414/45	69/45	119/60	295/100	267/35	252/20[1], 148/20[2], 132/25[3], 104/45[4] —	
2,6-TDA	$(M - C_2F_5)^+$	414/50	69/50	119/60	295/100	267/60	252/20[1], 148/40[2], 132/60[3], 104/50[4] —	
2,4-DAA	$C_2F_5^+$	430/30	69/95	119/100	311/5	283/20	415/12[5], 296/50[6], 149/70[7], 106/40[8] —	
α-Naphthylamine	Y^+	289/25	69/20	119/4	170/15	142/40	127/30[1] 115/100	Cyclopentadienyl ion $(C_9H_7)^+$
β-Naphthylamine	Y^+	289/35	69/23	119/4	170/8	142/55	127/50[1] 115/100	Cyclopentadienyl ion $(C_9H_7)^+$

1,5-NDA	M+	450/100	69/40	119/40	331/20	303/40	184/50[2] 156/50[9]	114/30	Cyclopentadienyl ion (C$_9$H$_6$)+
4-Aminobiphenyl	(M − C$_2$F$_5$CO)+	315/50	69/20	119/8	196/8	168/100	153/20[1]	141/50	Cyclopentadienyl ion C$_6$H$_5$(C$_5$H$_4$)+
Benzidine	C$_2$F$_5$+	476/45	69/60	119/100	357/3	329/80	182/25[9] 167/20[10]	—	
TODA	C$_2$F$_5$+	504/70	69/50	119/100	385/10	357/60	342/4[1] 210/25[9] 195/30[10] 180/30[8] 165/30[11]	—	
DADA	C$_2$F$_5$+	536/95	69/70	119/100	417/trace	389/8	402/20[6] 268/40[12] 242/8[9] 227/25[10] 212/25[8] 197/34[11]	169/20	Cyclopentadienyl ion CH$_3$OC$_6$H$_3$(C$_5$H$_3$)+
MDA	Y+	490/30	69/16	119/trace	371/trace	343/12	328/10[1]	165/20 132/100	Tropylium ion C$_6$H$_4$(C$_7$H$_5$)+ Tropylium ion (C$_7$H$_5$)+ NHCO
MOCA	Y+	558/15	69/45	119/50	439/trace	411/trace	523/20[13] 250/75[14]	166/100	Tropylium ion Cl(C$_7$H$_4$)+ NHCO**

* [1]X = C$_2$F$_5$CONH. [2]X = C$_2$F$_5$CO. [3]X = C$_2$F$_5$CONH + C$_2$F$_5$ + H. [4]X = C$_2$F$_5$CONH + C$_2$F$_5$CO + H. [5]X = CH$_3$. [6]X = C$_2$F$_5$ + CH$_3$. [7]X = C$_2$F$_5$ + C$_2$F$_5$CO + CH$_3$. [8]X = 2C$_2$F$_5$CONH. [9]X = 2C$_2$F$_5$CONH. [10]X = C$_2$F$_5$CO + C$_2$F$_5$CONH + CH$_3$. [11]X = 2C$_2$F$_5$CONH + CH$_3$. [12]X = 2C$_2$F$_5$ + 2CH$_3$. [13]X = 2C$_2$F$_5$CONH + CH$_3$. [14]X = 2C$_2$F$_5$ + 2Cl.

** The presence of one chlorine atom is shown by the simultaneous appearance of an ion 168/34.

For comparison, the mass spectra of the corresponding HFBA derivatives were also run. It appears that qualitatively they differ only slightly from those of the PFPA derivatives. However, in addition to CF_3^+ and $C_2F_5^+$ ions, $C_3F_7^+$ ions, of course, were formed. It is of interest that the abundance of CF_3^+ is considerably greater than for the PFPA derivatives. Thus, CF_3^+ is the base peak ion in seven cases, compared with none for the PFPA derivatives. Among the $(M - X)^+$ ions, no ions with $X = CF_3$ or $X = C_2F_5$ were found, only those with $X = C_3F_7$ being formed. Their abundance was generally lower than that for the corresponding $(M - C_2F_5)^+$ ions in the spectra of PFPA derivatives. Other $(M - X)^+$ ions present in the spectra of the HFBA derivatives were the same or corresponded to those given in Table II.

Selected ion monitoring (SIM), focusing the instrument on an ion in a mass spectrum, is a valuable technique for the quantitative and qualitative assay of complex mixtures of organic compounds, affording increased sensitivity and selectivity. The highest sensitivity for a given compound is achieved by focusing on the base ion, while monitoring on the basis of the molecular ion gives a high selectivity. Pairs of isomers such as derivatives of 2,4- and 2,6-TDA and α- and β-naphthylamine cannot be separately monitored using SIM technique, as their mass spectra are essentially identical. Focusing on common ions such as CF_3^+ and $C_2F_5^+$ could be suitable for recording chromatograms of PFPA derivatives of amines after separation by GC.

The data referred to above were all obtained by electron impact ionization and positive ion monitoring. For the SIM technique, preliminary work suggests that chemical ionization by methane and negative ion monitoring will give superior sensitivity and selectivity for the compounds investigated. Fig. 5 demonstrates a spectrum obtained in this mode.

CONCLUSION

The proposed trace analysis method for aromatic amines permits the simultaneous assay of many amines of special interest from work environmental health aspects. The high selectivity and sensitivity of the method makes it potentially useful for the analysis of complex industrial atmospheres.

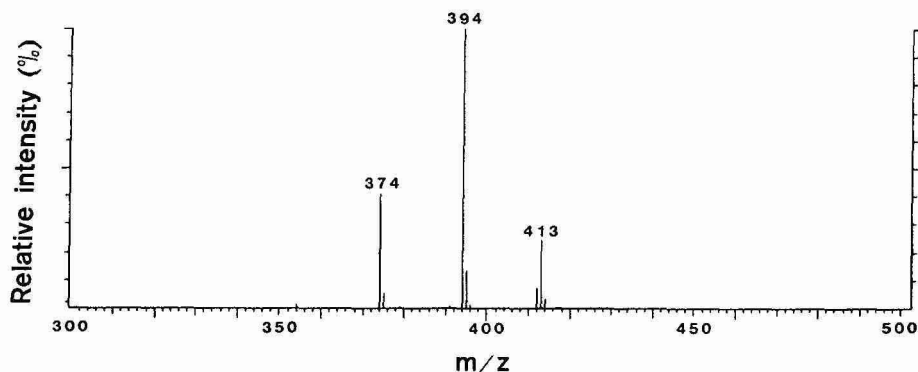The method is also applicable to the determination of aromatic isocyanates

Fig. 5. Mass spectrum of PFPA derivative of 2,4-TDA, obtained by methane chemical ionization and negative ion monitoring.

after sampling in acidic solutions, where they are converted into the corresponding amines. This problem will be discussed in a forthcoming paper.

Further applications of the method will include determination of aliphatic amines and isocyanates in working atmospheres and analytical studies in connection with moulding, welding etc. of polyurethane-based materials.

ACKNOWLEDGEMENTS

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ON-LINE DIODE ARRAY UV–VISIBLE SPECTROMETRY IN SCREENING FOR DRUGS AND DRUG METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The direct coupling of a multi-channel diode array UV–visible spectrophotometer to a powerful reversed-phase HPLC separation system is considered, especially for use in qualitative analysis, *e.g.*, screening/identification of drugs and drug metabolites. The approach is illustrated by the screening for metabolites of butoprozine and ticlopidine directly in human and rat bile.

INTRODUCTION

Recently a new generation of UV–visible spectrophotometers has been introduced in which the absorption spectrum of a compound is "pictured" within 1 sec by a multi-channel diode array system covering the range 200–800 nm. These detectors are capable of recording a series of spectra in the time and storing the data in their own memory and/or in external devices, such as magnetic tapes or disks, for later data handling and plotting. The potential of such an instrument as spectrophotometer has been discussed by James and Willis[1].

Being frequently involved in screening for unknown drugs and drug metabolites in biological materials, we felt that this diode array spectrophotometer (DAD) may also have great potential as an on-line detector for high-performance liquid chromatographic (HPLC) analysis (LC–DAD). In comparison with conventional single-wavelength detection it is evident that LC–DAD provides both multi-wavelength and spectral information in a single chromatographic run with the following advantages: saving of time, sample and eluent; smaller column leading; improved identification possibilities; rapid selection of optimal conditions for single-wavelength detection (*e.g.*, for quantitative purposes); possibilities for checking the purity of chromatographic peaks; rapid adaptation of the separation conditions to obtain better resolution.

In previous papers[2–5] we reported some preliminary experiences with LC–DAD in purity analysis and the detection of drug metabolites. In this paper we give a

detailed account of the general potentials of LC–DAD in screening for unknown drugs and drug metabolites with special relevance to biological materials.

EXPERIMENTAL

Drugs and chemicals

Butoprozine and ticlopidine were gifts from Sanofi S.A. The other drugs were present in our Department for general reference purposes and were donated by various manufacturers (Bayer, Astra, Homburg, Interpharm, Hoffman-La Roche, Wyeth, Ciba-Geigy). All drugs were 99% chromatographically pure by HPLC and thin-layer chromatography (TLC), or had been purified previously to a chromatographically pure state. Individual solutions and a mixture of the drugs were prepared in methanol with concentrations of 100–300 μg/ml per substance.

For the chromatographic analyses, water was obtained fresh from a Milli-Q water purifier (Millipore, Bedford, MA, U.S.A.), methanol was of HPLC grade from Baker (Deventer, The Netherlands) and ammonia, as a basic modifier, was of pro analysi grade from Merck (Darmstadt, G.F.R.). All other chemicals were also obtained from Merck and were of pro analysi grade.

Bile

Human bile samples were obtained from a male patient, 57 years old and 65 kg in weight, who was having a biliary drain. Butoprozine was administered intravenously in a dose of 15 mg diluted in a Baxter glucose solution during 15 min.

Rat bile samples were obtained from adult Wistar rats (body weight *ca.* 300 g). Ticlopidine was given by i.v. injection in a dose of 25 mg/kg.

Bile was collected in fractions of 1 h in ice-cooled tubes in the dark via a bile cannule. After collection, the bile was deep-frozen ($-18°C$) until taken for analysis.

HPLC

The same separation–detection set-up as described earlier[4] was used. The detection unit is shown in more detail in Fig. 1. The equipment consisted of an SF-770 variable single-wavelength detector (Schoeffel, Westwood, NJ, U.S.A.) (A); an HP-8450A multi-channel diode array UV–visible spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) (B) equipped with an M-178.32 QS flow-through quartz cell with a cell volume of 8 μl (Hellma, Mühlheim Baden, G.F.R.) (C); an HP-9875A tape cartridge using a dual tape drive (Hewlett-Packard) (D); an HP-9872A graphics plotter (Hewlett-Packard) (E); an Apple II plus (64K) computer (Apple Computers, Cupertine, CA, U.S.A.) with a video display (F and G); a stainless-steel column (15 cm × 4.6 mm I.D.) (H) packed with LiChrosorb RP-8 (5 μm) (Merck) or Nucleosil C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.); a WISP-710B autoinjector (Waters Assoc., Milford, MA, U.S.A.); two M-45 pumps (Waters Assoc.); and an M-720 solvent programmer (Waters Assoc.).

The columns were packed by means of a balanced density slurry method specially developed for the ammonia elution system[6]. Gradient elution was performed with water (0.005 *M* ammonia) and methanol as eluent components, using linear and/or stepwise gradient programmes. Elution usually started with water (0.005 *M* ammonia) to which methanol was added, according to the desired programme. The

Fig. 1. UV–visible detection set-up used in the HPLC experiments.

final elution was usually effected with 100 % methanol. Flow-rates were 1 ml/min. The eluent components water and methanol were saturated with helium before chromatographic use. During a run, a helium atmosphere was maintained in the eluent reservoirs.

RESULTS AND DISCUSSION

The spectrophotometer used as an HPLC detector was originally developed for UV–visible spectroscopic analysis only. By replacing the sample cell with a flow cell with a volume of 8 μl we could use it also as an HPLC detection system. When the spectra are stored in the memory of the instrument, consecutive spectra can be recorded with a time interval of 1 sec. However, because of the limited storage capacity, only 35 spectra can be stored in this way, which is usually not sufficient for a chromatographic run on a biological sample. Thus, for the latter purpose, the data must be transferred to an external storage device such as a tape or a disk, which takes at least 3 sec for one spectrum. Recording spectra with a minimum time interval of 3 sec was found to be more than adequate for our HPLC work. It will also be clear that,

because of the large amount of information generated by the detector, the data have to written into a memory device, as direct data plotting during analysis is not feasible under these conditions. During a run, a built-in monitor in the detector can provide either the full spectra that are being recorded or a portion thereof, or the chromatographic trace at a single, pre-selected wavelength. Comprehensive access to the stored data and plotting thereof, *e.g.*, in three-dimensional spectrochromatograms or in reconstructed, single-wavelength chromatograms is possible after the run.

In our experiments the diode array spectrophotometer was connected in series with a conventional single-wavelength detector as shown in Fig. 1, in order to compare the potentials of the two detectors[2,4]. It also had the advantage that we could now, during a run, display the absorption spectra of the eluting components via the monitor of the diode array detector and record the chromatogram at a single wavelength via the conventional detector. This set-up proved to be extremely useful when dealing with complex materials in relatively long runs. In this way the conventional chromatogram, recorded at a wavelength at which we anticipated that most of the compounds of interest would be detectable, served as a lead to recall relevant data from the memory after the run for further examination, manipulation and plotting.

In the following we describe three practical applications of LC–DAD in qualitative analyses.

Screening for butoprozine metabolites

Butoprozine is an indolizine derivative with anti-anginal properties[7,8]. As with all new drugs, the elucidation of its metabolic degradation pattern is important for a better understanding of its pharmacological and toxicological properties. The structure and UV spectrum of butoprozine are given in Fig. 2. In previous papers we described systematic approaches to the investigation of the three phases in drug metabolic profiling mainly based on conventional single-wavelength detection: (a) screening for unknown metabolites in the biological fluid of choice[9-12], (b) isolation
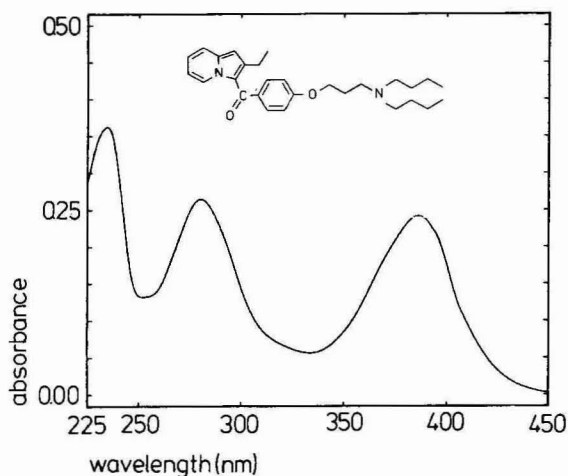

Fig. 2. Structure and UV spectrum of butoprozine. From ref. 4.

of the metabolites from the biological matrix[5,11] and (c) identification (structure elucidation) of the metabolites[11]. Separations in the screening and isolation phases were based on reversed-phase HPLC in both the isocratic and the gradient mode at alkaline pH established with amines or ammonia. Samples of excretory fluids such as bile and urine from different species were directly brought on to the column without extraction. In cases where a radioactive drug could be given the radioactivity of the eluent (measured on-line or off-line) provided a useful lead to the recognition of metabolites. However, in addition, UV detection at various wavelengths had to be carried out in order to obtain an insight in the UV-absorbing background of a metabolite peak and to develop screening methods for experiments in which only non-radioactive drug can be given. In a recent paper[11] we described an analytical approach to the recognition, isolation and identification of metabolites in human bile after administration of a non-radioactive drug. In these studies UV detection could only be carried out with conventional single-wavelength detection. With the DAD now being available we repeated the human bile investigations in order to compare the two UV approaches.

Fig. 3 shows the screening results represented in three spectrochromatograms taken with the DAD. Spectra were recorded with a time interval of 12 sec which resulted in 500 spectra of 400 absorption data points each in a complete run from water (0.005 M ammonia) to methanol (no ammonia) in 100 min, in a linear gradient. For reasons of time and simplicity only the relevant wavelength region 225–500 nm has been plotted (plotting of one complete spectrum from 200 to 800 nm takes about 50 sec); the region 500–800 nm was not of interest as none of the eluting compounds showed absorption there.

On the right is a spectrochromatogram of blank bile; the middle trace shows the bile situation during 0–6 h after administration of butoprozine. We call these chromatograms straight chromatograms because the sequence of the consecutive spectra corresponds to the time sequence. Comparison of these two runs shows readily (a) the presence of three main endogenous components (E_1, E_2 and E_3) in the blank and in treated bile, their concentrations changing during the time of the experiment, and (b) the presence of one main metabolite (M) in treated bile. This is in agreement with the single-wavelength experiments in which also one human main metabolite was found[11]. However, even though the plotting of the spectrochromatograms takes relatively long, LC–DAD provides a dramatic gain in time, by as much as 90 % compared with the classical UV approach. Peak recognitions are based on retention time comparison (provided by the high reproducibility of the separation system) and spectra comparison (provided by the highly informative detection system). Especially the latter gives additional evidence about the character of the peaks in comparison with conventional detection. Also, the spectrochromatograms can give direct structural information, as is demonstrated in the UV spectrum of M. This metabolite has a similar spectrum to the parent drug but shows very different chromatographic behaviour (eluting with about 60 % methanol instead of about 90 % methanol for the parent drug). This indicates the introduction of a polar group(s) in an otherwise intact aromatic system in M. Structure elucidation has revealed the introduction of one hydroxy group at the 1-position of the butoprozine molecule[11].

With all the absorbance data available on a tape or disk, plotting of the data points as well as connecting them can be done in various ways, provided that the
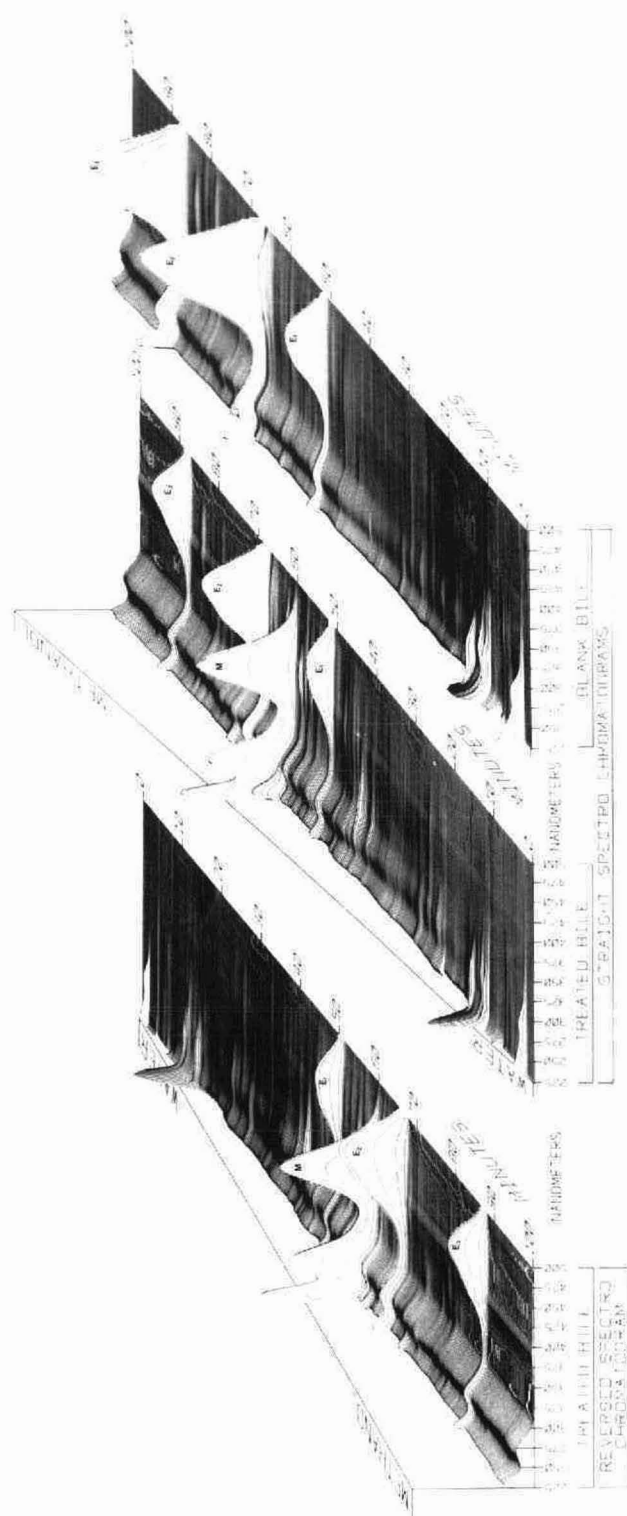
Fig. 3. Spectrochromatograms of blank human bile (−1 to 0 h before butoprozine administration) and treated human bile (0–6 h after butoprozine administration) for differentiation between metabolites and endogenous components. Conditions: column, 15 cm × 4.6 mm I.D.; packing, Nucleosil C_{18} (5 μm); eluent, 100% water (0.005 M ammonia) to 100% methanol (no ammonia) in 100 min; flow-rate, 1 ml/min; injection, 50 μl; absorbance, 0.0–1.0.

required computer programs are written for each purpose. Time, wavelength and absorbance axes can be varied in magnitude, direction and angle. A first demonstration is given in the left-hand spectrochromatogram in Fig. 3, where the time axis has been reversed so that we are "going back in time". This reversed spectrochromatogram allows us to see what is present behind a peak. A detailed study of the two treated chromatograms with respect to the blank chromatogram now shows the possible presence of other minor butoprozine metabolites just before and just after the main metabolite M. In order to obtain a better insight, this particular part of the run was plotted at a much higher sensitivity and with a greater distance between the spectra, as shown in Fig. 4. Now the presence of the minor metabolites a, b, c and d can be better observed. However, because we instructed the computer not to plot in the shadow of a peak, Figs. 3 and 4 still do not reveal what is present between M and E_2. To observe this, the spectra 41–50 were plotted (Fig. 5). Now the minor metabolite d can be observed better. These figures demonstrate that with the DAD a more detailed insight into the metabolic profiles can be obtained. In the earlier single-wavelength studies only the main metabolite could be recognized.

In the gradient runs also an increasing background in the wavelength region 225–310 nm can be observed when going from water to methanol. This is further illustrated in the eluent run shown in Fig. 6. It is possible to subtract such an eluent background from the blank and treated chromatograms by recording an eluent run before the analysis of the biological samples. However, it requires extra memory space and good run-to-run reproducibility of the chromatographic system. In our case, subtraction of the background in Figs. 4 and 5 confirmed the metabolic character of peaks a–d.
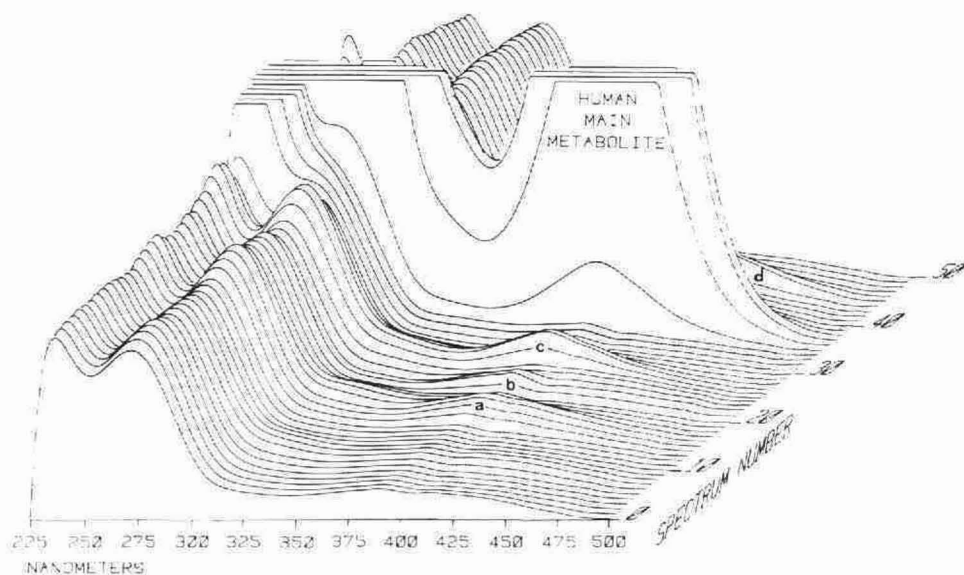

Fig. 4. Part of the treated straight spectrochromatogram in Fig. 3 for recognizing the minor metabolites (a, b, c and d). Absorbance, −0.01 to 0.2.
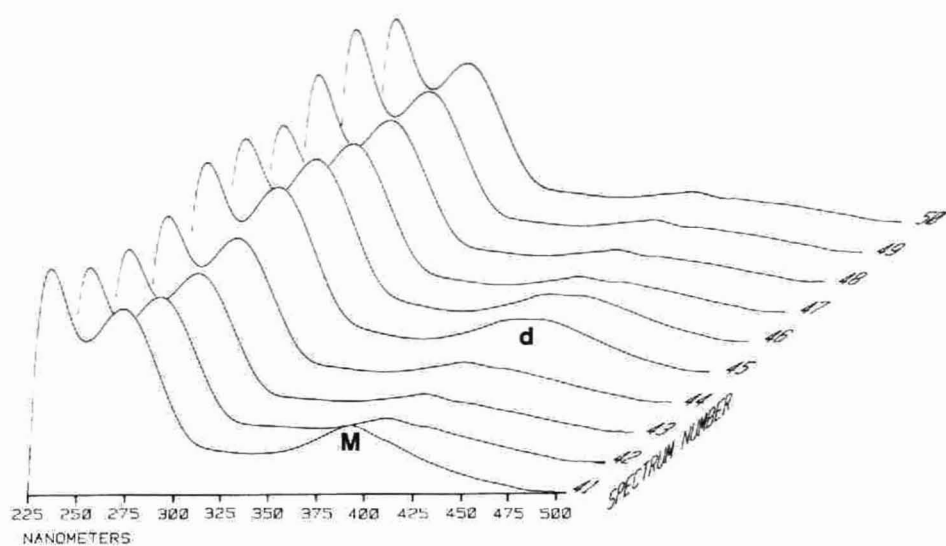
Fig. 5. Plots of spectra 41–50 in Fig. 4 just behind the human main metabolite for a complete view of minor metabolite d. Absorbance, −0.01 to 0.2.

Another way of manipulating the spectral data is demonstrated in Fig. 7, where conventional single-wavelength chromatograms have been reconstructed with an interval of 10 nm. In essence, it means connection of absorbance data points of the same wavelength and plotting in the conventional way at a sensitivity of choice. As
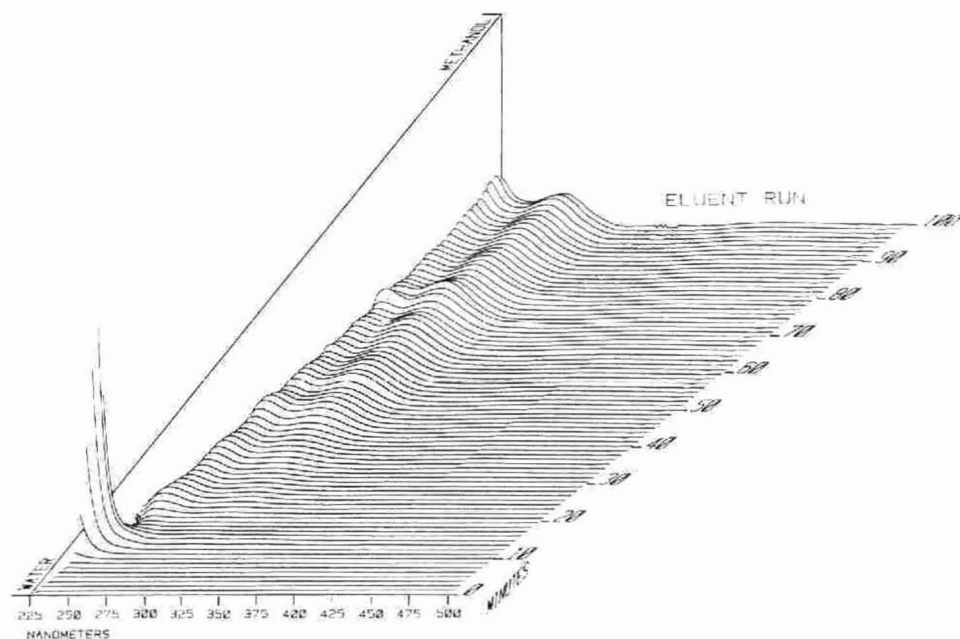

Fig. 6. Chromatographic run of water (0.005 M ammonia) to methanol to obtain more insight into the background. Conditions as in Fig. 3.
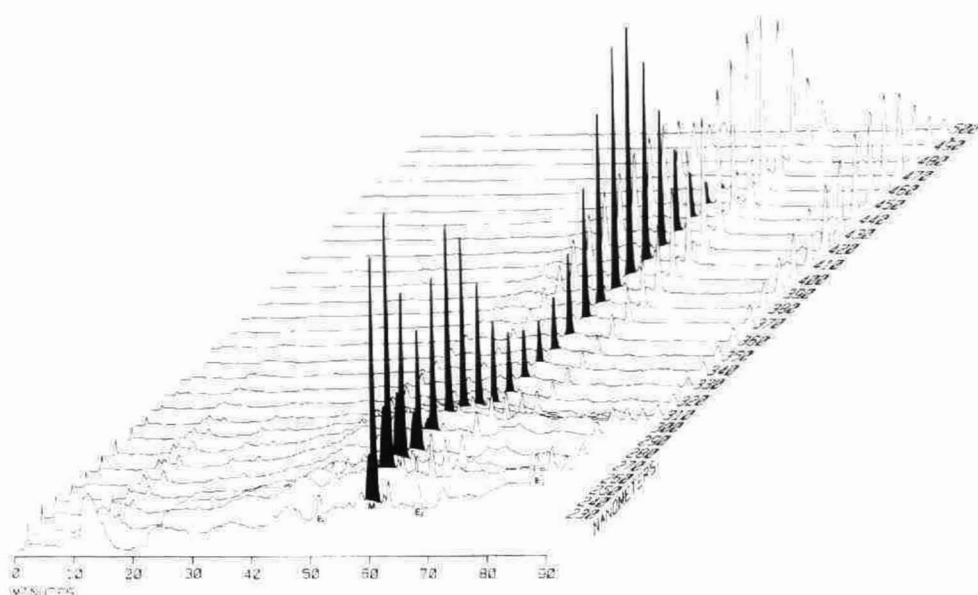
Fig. 7. Reconstructions of single-wavelength chromatograms from the treated bile data in Fig. 3. Attenuation, 1.0 a.u.f.s.

can be seen, the spectra of the main metabolite and the endogenous components are now represented in another way and at a different angle. This kind of plot especially facilitates the selection of a suitable wavelength for quantitation. The increase in the baseline from 225 to 310 nm in a gradient run can also be observed as in Fig. 3, but now in a more conventional way. From 300 to 500 nm M looks pure, but there clearly is a shoulder present, eluting just before M and absorbing between 230 and 290 nm. This shoulder can also be observed in the treated bile picture in Fig. 3 and may represent another metabolite as there is no peak indication in blank bile in this area. It should further be noted that this shoulder should be avoided when trying to isolate M for structure elucidation.

In Fig. 7 the sensitivity for plotting was 1.0 a.u.f.s. adapted to the main peaks. The same blank and treated runs were also followed by the conventional detector working at 400 nm at 0.2 a.u.f.s. The latter chromatograms are given in Fig. 8B and C, demonstrating the limitations of a conventional detector without a computer system. The wavelength and the attenuation are selected before the chromatographic run in the hope that a suitable result is obtained. Especially in screening analysis in which both qualitative and quantitative information about the compounds is not available, it is difficult to pre-select the optimal conditions, with the result that in most instances more runs must be carried out. With the data stored on tapes or disks the desired sensitivity can be chosen after the run. Fig. 8A shows a reconstruction of a chromatogram from the data recorded with the DAD and plotted on the same scale and with the same sensitivity as the other pictures in Fig. 8. A comparison between the pictures indicates that peak broadening in the detector set-up is negligible. Further, Fig. 8 demonstrates that the major human metabolite can be easily detected by
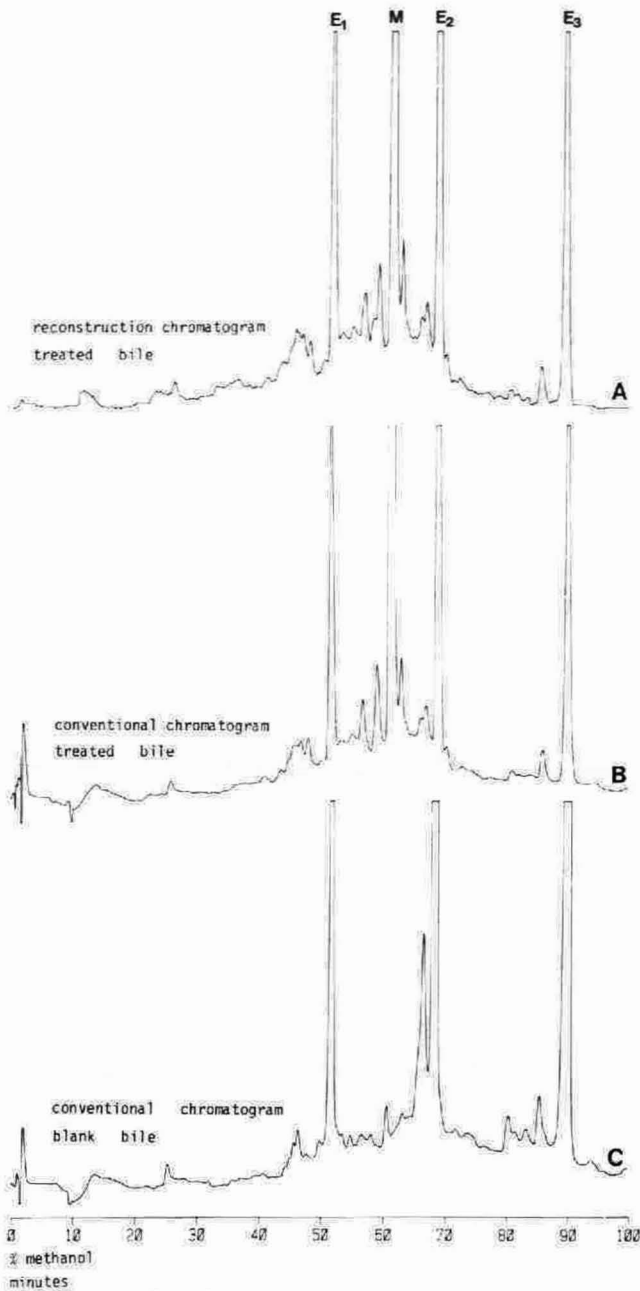
Fig. 8. Comparison of chromatograms obtained with the diode array detector (A) and with the single-wavelength detector (B) and C) recorded on-line. Conditions as in Fig. 3, except wavelength 400 nm and attenuation 0.2 a.u.f.s.

conventional detection but that it is virtually impossible to recognize the minor metabolites a–d in this way, even though the detector is being used at high sensitivity.

In the above application extensive use was made of spectrochromatograms.

This requires relatively long plotting times and time and effort are also lost on areas in the run where no compounds are eluted. Therefore, in the second application, we used the conventional detector to prepare a lead chromatogram at a suitable wavelength at which we can expect to recognize most if not all eluting compounds of interest. In addition, the drug used in the next application showed UV absorption only between 200 and 280 nm and was thus devoided of a characteristic absorption around 400 nm as was the case with butoprozine.

Screening for ticlopidine metabolites

Ticlopidine is a drug that reduces platelet aggregation. Its structure, UV spectrum and chromatographic behaviour in the general gradient with respect to butoprozine are shown in Fig. 9. From the UV spectrum of ticlopidine, 235 nm was chosen as initial wavelength for the conventional detector. At this wavelength we may expect to detect most metabolites with sufficient absorption and present in sufficient amount. The drug metabolic study was started on rat level after intravenous administration of ^{14}C-labelled ticlopidine. Radioactive measurements showed an excretion of 75 % in the bile and 25 % in the urine. In bile most radioactivity was found back in the first 2 h after drug administration. Therefore, blank bile (− 1 to 0 h) and treated bile (fractions 0–1 h and 1–2 h after drug administration) were taken for a first analytical study. In the chromatographic analysis of radioactive bile, on-line radioactivity detection was chosen as specific detection mode for detecting the separated metabolites containing the label. Also conventional UV detection was performed to gain more insight into the background, to detect possible metabolites without the label and to obtain a lead chromatogram for further DAD application.

In Fig. 10, three chromatograms at 235 nm are shown with the purpose of differentiating between ticlopidine metabolite peaks and endogenous bile peaks. Although it was possible to separate butoprozine metabolites without too much
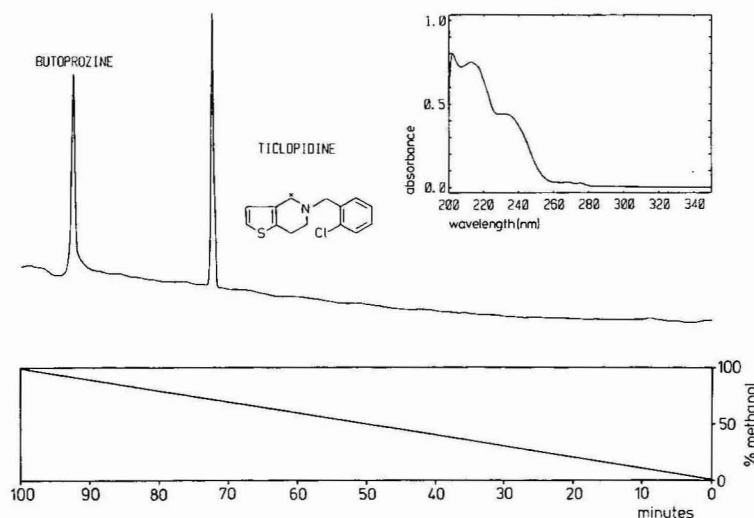

Fig. 9. Structure and UV spectrum of ticlopidine. Separation of a butoprozine–ticlopidine mixture in the general gradient. Conditions as in Fig. 3, except wavelength 235 nm and attenuation 0.5 a.u.f.s.
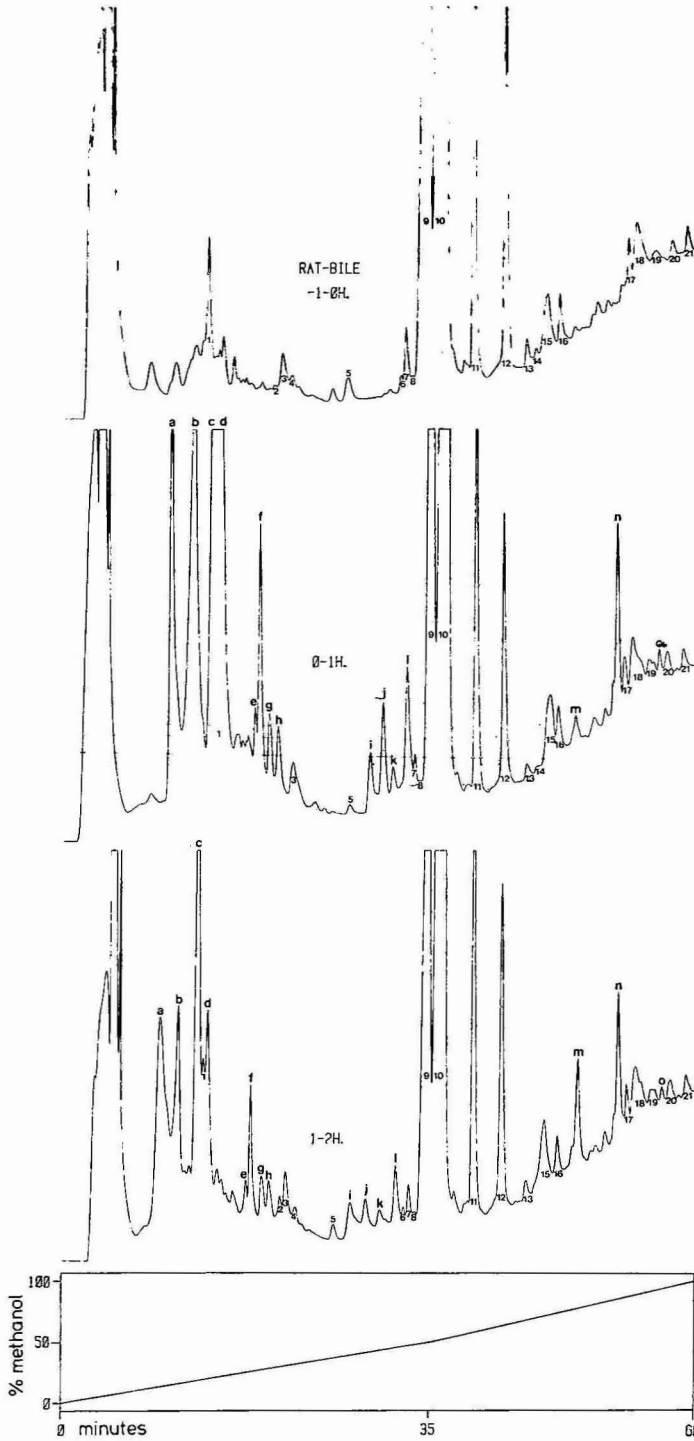
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Fig. 10. Comparison of chromatograms recorded with the single-wavelength detector of rat bile, the hour before and the first and second hours after ticlopidine administration. Simultaneous recording of UV spectra took place with the diode array detector. Conditions as in Fig. 9, except the gradient profile.

interference from endogenous components in the general continuous gradient of 100 min, with ticlopidine most metabolites were much more polar, eluting in the first part of this gradient at lower methanol concentrations, concomitant with various endogenous compounds. Hence, the gradient profile was adapted to these separation demands as shown in Fig. 10. In order to recognize the metabolites against the background based on retention time comparison only, a high reproducibility between the blank and treated runs is required. In daily routine HPLC, maintenance of the reproducibility for longer periods of time is not always possible. In Fig. 10, the runs were recorded on different days with the result that an absolute reliable retention time comparison becomes difficult, also owing to the large number of peaks. For more evidence about character (metabolite or endogenous) and the correspondence of metabolites in different fractions, additional characterization is thus required and for that purpose we evaluated the LC–DAD system.

During the runs shown in Fig. 10, every 4 sec a spectrum was recorded, monitored and stored on tape. The spectra were numbered 0, 4, 8, 12, etc., thus corresponding to the time axis of the conventional chromatograms. After the run we screened the tapes for compounds absorbing at 235 nm and plotted their UV spectra by taking it at the top of the elution profile (or at a shoulder) with subtraction of the background absorbance. We then scanned the tapes for peaks not absorbing at 235 nm that seemed to be metabolite related. After such a spectral screening, peaks with similar retentions and UV properties were given the same symbol, 1, 2, 3, etc., for endogenous compounds and a, b, c, etc., for potential metabolites.

Four spectra of peaks obtained in this way are shown in Fig. 11. Spectra 1 and 2 were derived from peak 3 in blank bile and the first treated bile fraction, respectively. After retention time and spectra comparisons we may assume we are dealing with the same endogenous component. Spectrum 1 is given in its real form as recorded in the gradient run and also after a smoothing technique[13] carried out with the computer. Spectra 3 and 4 were recorded of peaks a and n, respectively, which are present only in treated bile, so that we may assume that they are metabolites. This was checked with radioactivity measurement of the respective peaks. Peak n contained radioactivity but peak a did not, indicating a metabolite without the label or a possible endogenous component formed during the course of animal experiments. In such a case, labeling of another part of the molecule is necessary. In this way most metabolites were localized and checked in the bile.

Although a UV spectrum may be compound-characteristic in terms of its maxima and overall shape, it provides little structural information. Nevertheless, in our work with butoprozine it could be concluded from the DAD approach that most metabolites in various species still had an intact indolizine moiety[4,11,12]. However, with ticlopidine we encountered numerous metabolites with a UV spectrum completely different from that of the parent compound. For elucidation of the structures of these metabolites, additional spectroscopic analysis is necessary with more powerful techniques such as mass and NMR spectroscopy.

Compared with the first application, the DAD approach used here may not look so spectacular. However, the gain in information from each run on using DAD was very large and also saved considerable time.
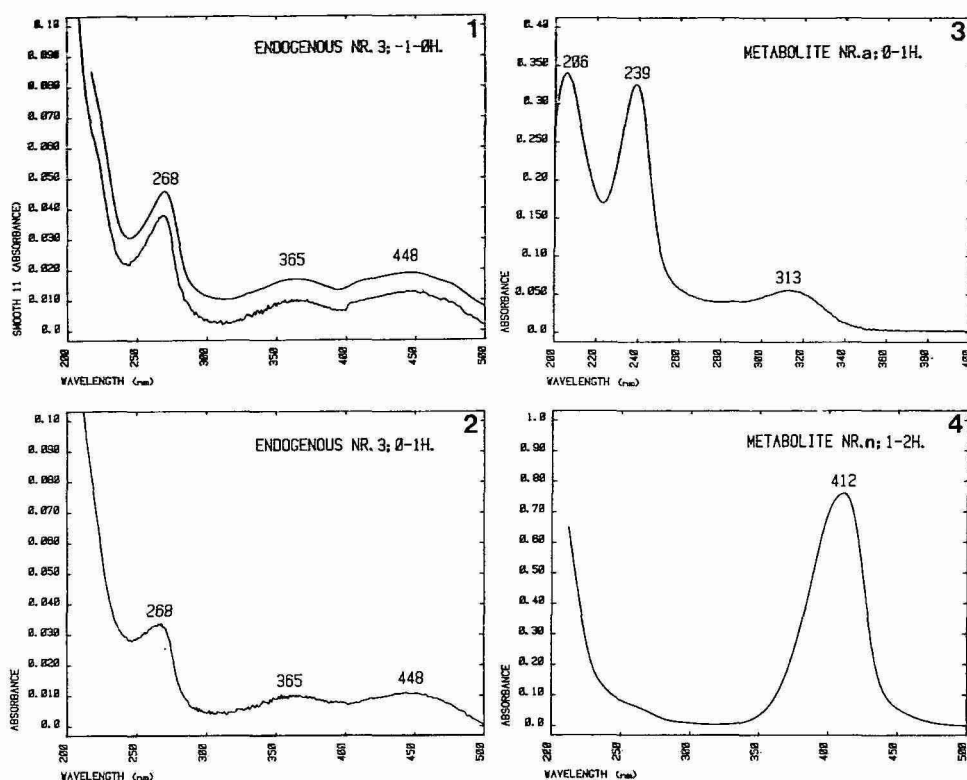
Fig. 11. Some UV spectra derived from the peaks in Fig. 10 by peak-top spectrum minus background.

Screening for drugs

There is a basic difference between screening/identification for drug metabolites and drugs. In drug metabolic profiling the metabolites are new biologically synthesized compounds. The only reference points are the structure and the properties of the parent drug from which they are being derived. Therefore, the screening is initially directed towards finding out how many metabolites have been formed, to see for how far the parent drug has been converted and if possible to obtain a relative quantitative picture about the drug and/or metabolite(s). Because many metabolic routes from the parent drug can be derived it is difficult to make exact predictions about the structure of the metabolites formed. To identify the metabolites, the screening must be followed by isolation and structural elucidation with spectroscopic techniques.

When starting a screening study on biological or other relevant materials for the presence of drugs, *e.g.*, in clinical or forensic toxicology, the number and identities of the drugs are also unknown. Contrary to the situation in metabolic profiling, however, the unknowns are not new compounds. Generally, reference compounds are available, from which we can compile a bank of reference data. When using a chromatographic system, the retention behaviour of the unknown compound can be checked against the data of the reference compounds in the data bank, thus present-
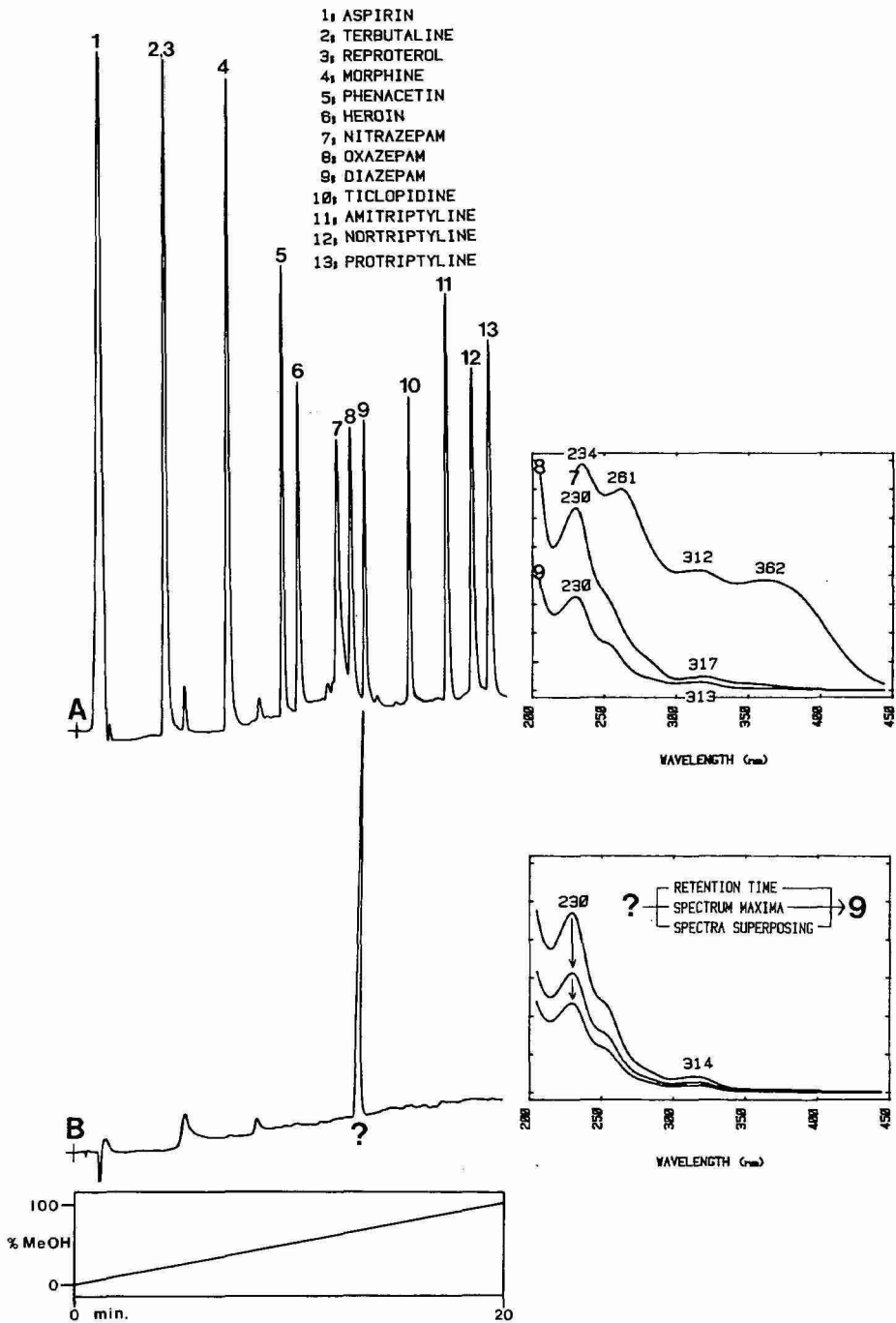
Fig. 12. Approach to the identification of drugs in biological fluids by comparisons of retention times and UV spectra. Conditions as in Fig. 9, except the gradient profile and packing, LiChrosorb RP-8 (5 μm). For further details see text.

ing a first possibility for identification. However, in view of the large number of drugs that have to be taken into account and the occurrence of intra- and inter-laboratory variations in the determination of the retention behaviour, a single retention time or R_F value usually is not sufficient to identify the compound under investigation. By using LC–DAD, the UV spectrum can serve as a second identification parameter which can be combined with the retention time parameter.

A preliminary application of this approach is depicted in Fig. 12. Here we used the HPLC system for rapid screening purposes, capable of dealing with a large variety of drugs, differing in character and polarity. More details about the separation system, such as kind of stationary phase, column packing procedures, gradient *versus* isocratic elution, kind of gradient profile and the basic modifier used and its concentration, will be given in a later paper[6].

Fig. 12A shows the separation of thirteen drugs in a linear gradient of 20 min. In this reference run, in addition to the determination of the retention time, recording of the UV spectrum also took place with the DAD. It should be noted that the spectra of the reference compounds in the data bank should be recorded at the eluent composition at which they elute in the chromatographic run. For three benzodiazepines (7, 8 and 9) such UV spectra are given in the upper insert after the chromatographic run A. Fig. 12B gives a chromatogram of a plasma sample after subtracting the endogenous background, showing a major unknown peak. Retention time comparison with the reference data allows the exclusion of drugs 1–6 and 10–13. Taking into account a certain spread in reproducibility, only compounds 7, 8 and 9 (and other drugs eluting in this region) are to be considered. For further identification, the UV spectrum was recorded in run B and plotted at different sensitivities after run B. Comparing the maxima with the reference maxima, nitrazepam (7) can be excluded. Differentiation between 8 and 9 is more difficult because they have very similar maxima. Now the overall shape of the spectra can be compared. Because this depends on concentration, the recorded spectrum of the unknown compound should be superimposed with reference spectra. With the DAD this is easy to perform. Now, it appeared that the unknown compound had the same spectrum as diazepam. Additional possibilities for confirming the identification are the generation of second or higher order derivative spectra with the DAD[14]. Of course, other chromatographic systems and/or mass spectrometry can also be considered.

Although this application represents a relatively simple case, it again illustrates the advantages of a high separation efficiency combined with highly informative detection.

CONCLUSIONS

This paper clearly shows the potential of LC–DAD especially in the area of qualitative analysis. Considering the detector unit of the HPLC equipment, the advantages with respect to conventional UV detection are evident. However, some limitations of the DAD used can be noted: first, the limited memory capacity for storing the spectral data of the instrument itself; second, the plotter technique, which is rather time consuming; and third, the limited possibilities for displaying the data during chromatographic analysis. With regard to the last aspect it is desirable that at least individual spectra plus the chromatographic trace at a pre-selected, fixed wave-

length can be displayed during the run. The ideal situation would be reached if a three-dimensional spectrochromatogram could be displayed.

It has also been shown that the information that can be obtained from a sample mixture is greatly enhanced when the spectrometer is coupled to a powerful HPLC separation unit. The system developed, based on reversed-phase HPLC at relative high pH established with ammonia, has proved to be both universal in chromatographing individual compounds with widely different structures and selective in the separation of complex mixtures, both a need in qualitative analysis.

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INFLUENCE OF MUTAROTATION CATALYSTS ON THE LIQUID CHROMATOGRAPHY OF MALTO-OLIGOSACCHARIDES ON A CATION-EXCHANGE RESIN

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SUMMARY

To avoid double peaks due to α- and β-anomers in complicated chromatograms of carbohydrate mixtures, mutarotation catalysts may be applied.

The effects of the addition of alkaline mutarotation catalysts to the aqueous mobile phase on the separation of malto-oligosaccharides at different separation temperatures are described. Addition of the catalyst results in a symmetrical and narrower peak for each malto-oligosaccharide tested. However, although the peak shape was improved, separation of a malto-oligosaccharide mixture at room temperature was not satisfactory, due to the peaks still being too broad. At elevated temperatures formation of isomerization products occur in the alkaline mobile phase during the separation.

The best chromatograms of malto-oligosaccharides mixtures were obtained with a cation-exchange resin in the calcium form using a neutral aqueous mobile phase and a column temperature of 90°C.

INTRODUCTION

Efficient liquid chromatographic separations of carbohydrates are often performed on a cation-exchange resin in the calcium form at elevated temperatures[1–4]. The separation mechanism is based on complex formation between the hydroxyl groups of the carbohydrates with the immobilized Ca^{2+} ions on the resin[2,5,6] and on size exclusion effects[7].

At room temperature the mutarotation of many reducing sugars is low. Consequently, the chromatographic separation of a mixture of carbohydrates at room temperature on a cation-exchange resin will result in a needlessly complicated chromatogram with double peaks and/or peaks with shoulders due to the (incomplete) separation between the α- and β-anomers of the respective sugars. Increasing the temperature increases the mutarotation by a factor of about 2.5 for every 10°C temperature rise[8]. As a result of the fast mutarotation, at elevated temperatures the α- and β-anomers of the respective carbohydrates elute together in one peak[1–4,7].

Recently, Verhaar and Kuster[9] and Verhaar[10] reported the application of a mutarotation catalyst in the chromatographic separation of carbohydrates. Addition of 0.001 M triethylamine (TEA) to the mobile phase catalyses the mutarotation of the reducing sugars at room temperature during the separation on the cation-exchange column. No splitting in the α- and β-anomer peaks occurred and useful sugar chromatograms could be obtained at room temperature.

Verhaar and Kuster restricted themselves to the separation of monosaccharides such as glucose, fructose, mannose and galactose[9]. In the starch industry, separations between malto-oligosaccharides are more important. For this reason, we studied the effect of several catalysing additives to the mobile phase on the efficiency of the separation of malto-oligosaccharides on a cation-exchange resin in the Ca^{2+} form.

It is well known that the mutarotation of reducing sugars is catalysed by both acids and bases[8,11-13]. In this study, no acid catalysts were tested because acid removes the Ca^{2+} ions from the cation-exchange resin and thus deteriorates the chromatographic separation. As discussed by Verhaar and Kuster[9], the TEA-containing mobile phase also displaced gradually the Ca^{2+} ions from the column, resulting in a continuous decrease in separation efficiency. Therefore, a column regeneration procedure was required after every 2 l of eluent pumped through the column. To avoid these time-consuming column regeneration procedures when using TEA, we also used a dilute calcium hydroxide solution. In order to maintain the previously reported benefits of a calcium ethylenediaminetetraacetate (CaEDTA)-containing mobile phase[8], solutions of CaEDTA in dilute calcium hydroxide were also applied.

EXPERIMENTAL

All separations were performed on a Hewlett-Packard 1084B liquid chromatograph equipped with an automatic sample introduction system (sample volume 50 μl), column oven and Hupe Busch 1033 differential refractometer detector (maintained at 37°C). A 25 cm × 9.0 mm I.D. column was packed with the cation-exchange resin AG 50W-X4 (Bio-Rad Labs., particle size 20–30 μm) in the calcium form[7].

The following solutions were used as mobile phases: pure water, an 50 ppm aqueous solution of CaEDTA (pH 6.2), a 250 ppm aqueous solution of CaEDTA (pH 6.4), a dilute aqueous solution of calcium hydroxide (pH 10.6), a 50 ppm solution of CaEDTA in diluted calcium hydroxide solution (pH 10.6) and a 0.001 M aqueous solution of TEA (pH 10.2).

The test samples were glucose, maltose, maltotetraose and an acid-hydrolysed potato starch syrup (5%, w/v).

Separations have been carried out at different temperatures between 30 and 90°C. However, for mobile phases containing TEA, a maximum column temperature of 70°C was applied because of the low boiling point of TEA.

RESULTS AND DISCUSSION

Glucose

The effects of both the composition of the mobile phase and the column temperature on the glucose chromatograms were studied.
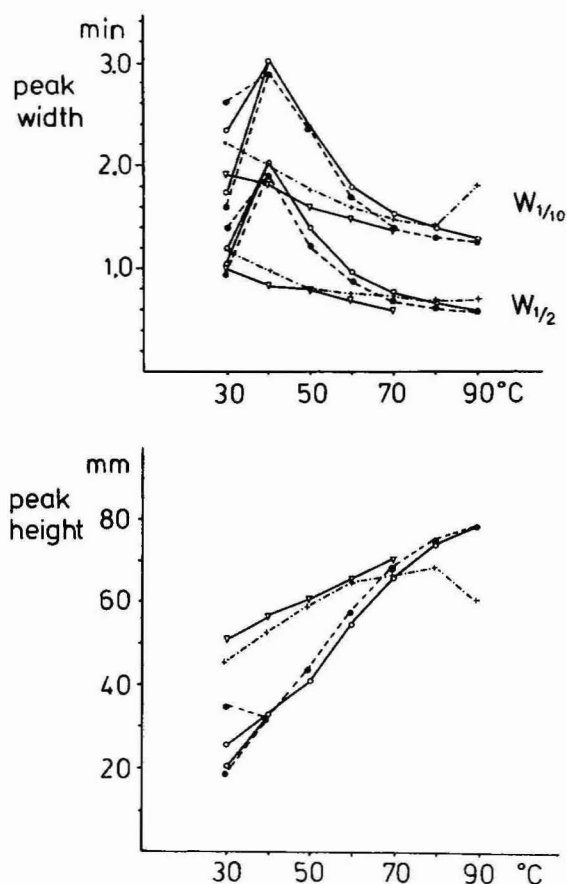
Fig. 1. Effect of the column temperature and the composition of the mobile phase on the peak height and the peak width at 50 % and 10 % of the peak height ($W_{1/2}$, $W_{1/10}$) in the glucose chromatograms. Composition of mobile phase: ●----●, water; O——O, 50 ppm CaEDTA; ▽——▽, 0.001 M TEA (pH 10.2); + .-. +, 50 ppm CaEDTA in Ca(OH)$_2$ (pH 10.6).

Fig. 1 shows the influence of column temperature on peak height and peak width for the different mobile phases and Fig. 2 shows the effect of the composition of the mobile phase on the chromatograms using a column temperature of 30°C. In Fig. 3 the effect of column temperature on the shape of the glucose peak, using a neutral or alkaline mobile phase, is demonstrated.

The catalytic effect of alkali on the mutarotation at room temperature is evident. However, at elevated temperatures the glucose peak in the chromatograms obtained with the alkaline mobile phases becomes distorted (Figs. 1 and 3). It is known that in aqueous alkaline solution transformation and degradation reactions with reducing sugars occur. The Lobry de Bruin-Alberda van Ekenstein transformation of glucose results in numerous products, including fructose, mannose and psicose[8,12–15]. As shown by Kainuma and Suzuki[16], higher temperatures accelerate the alkaline isomerization considerably. It is possible to isomerize about 24 % of the glucose originally present in 20 min at 71°C. Taking into account that it takes18–19
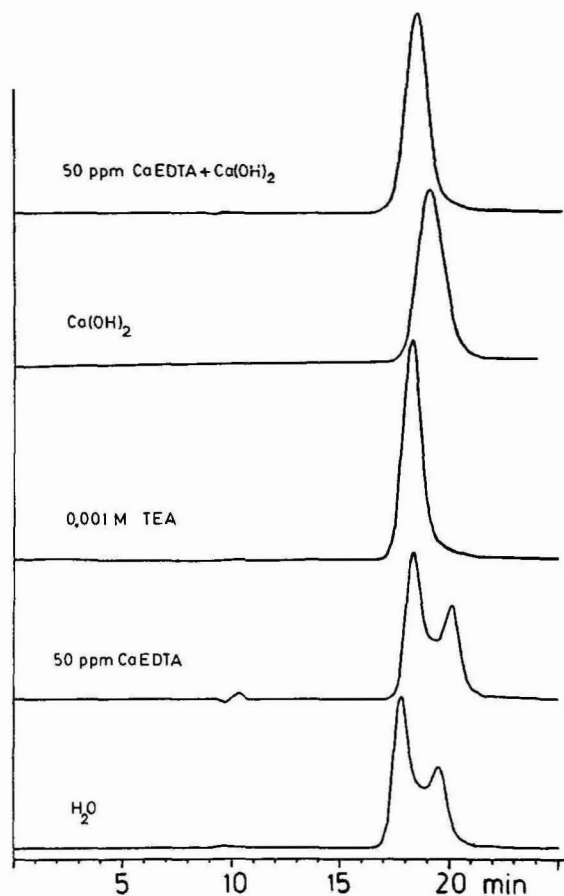
Fig. 2. Effect of the composition of the mobile phase on the glucose chromatogram at 30°C. Samples: 50 μl of 1 % glucose solution.

min to elute glucose from the chromatographic column (Fig. 3), it is obvious that the above-mentioned isomerization reactions in the alkaline mobile phase at elevated temperatures cannot be neglected.

Isomerization of glucose during the separation caused peak tailing, probably because of fructose formation (increase in $W_{1/10}$) and subsequently a decrease in the glucose peak height (Fig. 1). Fig. 3 shows the distorted glucose peaks that were obtained by using alkaline CaEDTA solution as the mobile phase. On the other hand, high temperatures increase the mutarotation considerably and, at the same time, decreases the viscosity of the aqueous mobile phase significantly. According to the Wilke–Chang equation[17], both a temperature rise and a decrease in viscosity result in higher diffusion coefficients of the compounds to be separated. Hence both the increased mutarotation and the decreased viscosity give rise to narrower and thus higher peaks. Therefore, applying a neutral mobile phase in combination with a column temperature of 80–90°C results in narrower and higher glucose peaks than applying a mobile phase with a mutarotation catalyst at room temperature (Fig. 1).
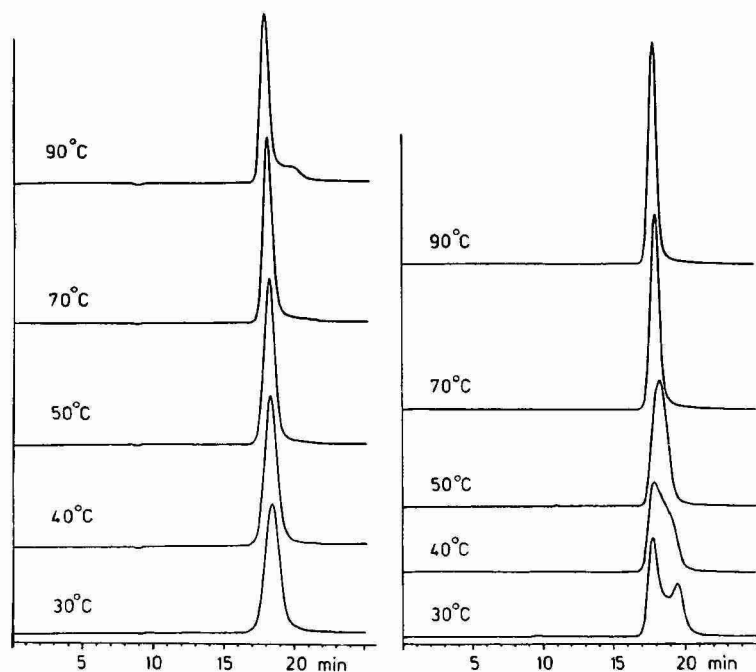
Fig. 3. Effect of the column temperature on the glucose chromatograms. Test sample: 50 μl of 1 % glucose solution. Right: pure water as mobile phase. Left: 50 ppm solution of CaEDTA in Ca(OH)$_2$ (pH 10.6).

A remarkable effect was that at neutral pH and temperatures lower than 40°C, addition of CaEDTA to the mobile phase improved the separation between the α- and β-glucose anomers (Fig. 2), especially at higher CaEDTA concentrations (e.g., 250 ppm).

Maltose

The effects of the composition of the mobile phase and the column temperature on the maltose chromatograms were similar to the effects found with glucose, although less pronounced. This is not unexpected, because the maltose anomers differ only in the geometric configuration of one hydroxyl group in one of the two glucose units. For a similar reason there is also less chromatographic difference between maltose (4-O-α-D-glucopyranosyl-D-glucose) and its isomerization product maltulose (4-O-α-D-glucopyranosyl-D-fructose) than between glucose and fructose. Under the chromatographic conditions usually applied[7], glucose and fructose are separated completely, but maltose and maltulose are not. Therefore, the isomerized maltose peak in the chromatograms seems less distorted than the isomerized glucose peak using a warm alkaline mobile phase.

A neutral mobile phase and a column temperature of less than 40°C result in the elution of a double peak or a peak with a shoulder. The catalytic effect of alkaline mobile phases on the mutarotation was demonstrated by the elution of nearly Gauss-shaped chromatographic peaks of maltose at room temperature. The peak appeared to be narrower and thus higher than those obtained by using a neutral mobile phase.

Almost the same plot as shown for glucose in Fig. 1 could be obtained for maltose. As for glucose, the best chromatograms for maltose were obtained by applying a neutral mobile phase in combination with a column temperature of 90°C.

Maltotetraose

The anomer forms of maltotetraose resemble each other even more closely than the maltose anomers. Therefore, the results were essentially the same as for maltose, but again less pronounced.

Hardly any peak deformation due to isomerization reactions at elevated temperatures in the alkaline mobile phases could be observed. This does not necessarily indicate that no isomerization of the maltotetraose occurs. One of the main isomerization products in aqueous alkaline solution will be maltotetraulose, in which the end-stranding glucose unit is transformed into a fructose unit. This transformation does not change the molecular weight of the molecule and thus the effect on the size-exclusion separation mechanism on the chromatographic column is negligible. Although the complexability of a fructose unit with the Ca^{2+} ions of the resin is stronger than that of a glucose unit, it is believed that for maltotetraose and maltotetraulose this is of minor importance. Hence it can be concluded that a transformation reaction of maltotetraose into maltotetraulose on the chromatographic column will not distort the peak shape.

The chromatograms of maltotetraose recorded at 30°C with a neutral mobile phase show a peak with a shoulder. This shoulder was again enhanced by the addition of CaEDTA to the mobile phase. The catalytic effect of the alkaline mobile phases at relative low column temperatures is obvious: the peak width is reduced and the peak height is increased compared with the use of a neutral mobile phase at the same column temperature. However, the highest and narrowest maltotetraose peak is obtained again by simply applying a column temperature of 90°C and a neutral (CaEDTA-containing) mobile phase.

Acid-hydrolysed potato starch

As shown above, addition of an alkaline mutarotation catalyst as TEA to the mobile phase results in the elution of a single Gaussian-shaped peak at room temperature for each malto-oligosaccharide tested. However, the eluted peaks were relatively broad compared with those obtained by applying a neutral mobile phase at 80 or 90°C. Therefore, it was not possible to obtain at room temperature a well developed chromatogram of an acid-hydrolysed potato starch solution containing a homologous series of malto-oligosaccharides (Fig. 4). If, however, a comparison is made between the use of TEA-containing and a neutral mobile phase at the same low temperature then, owing to the catalytic effect of TEA on the mutarotation, the chromatograms of a TEA-containing mobile phase were significantly better than those obtained with a neutral mobile phase. In the latter instance the α- and β-glucose anomers partly coincide with the maltose peak. At a column temperature of 50°C a TEA-containing mobile phase still results in a superior separation compared with a neutral mobile phase at 50°C. However, the best chromatograms of mixtures of malto-oligosaccharides were recorded by using a neutral (50 ppm CaEDTA-containing) mobile phase at 90°C, as described before[7]. Almost the same chromatographic resolution was achieved by applying the TEA mobile phase at 70°C, but owing to the
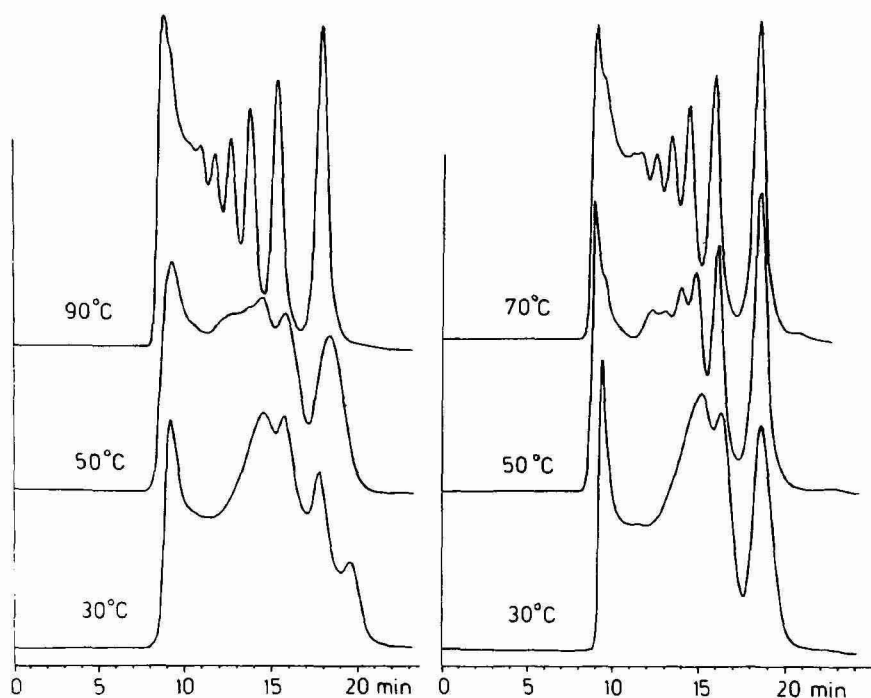
Fig. 4. Chromatograms of acid-hydrolysed potato starch solutions (5%, w/v) at different temperatures using pure water (left) or 0.001 M TEA solution (pH 10.2) (right) as the mobile phase.

formation of isomerization products (*e.g.*, at the foot of the glucose peak, Fig. 4) the chromatograms no longer represent the original composition of the samples being analysed.

CONCLUSIONS

Contrary to the experiences of Verhaar and Kuster, dealing with the separation of different monosaccharides[14], addition of an alkaline mutarotation catalyst to the mobile phase does not improve the chromatography of malto-oligosaccharides in such a way that separation can be performed at room temperature instead of 90°C.

Moreover, at elevated temperatures the formation of isomerization products occurs when an alkaline mobile phase is used and the TEA-containing mobile phase will gradually displace Ca^{2+} from the cation-exchange resin, which results in a continuous decrease in separation efficiency[14]. Therefore, a regular column regeneration procedure is required.

The best chromatograms of mixtures of malto-oligosaccharides were obtained by applying a neutral 50 ppm CaEDTA solution as the mobile phase and a column temperature of about 90°C, as described before[7]. Apparently, at neutral pH heat is as good a mutarotation catalyst without causing isomerization and/or degradation reactions. Moreover, owing to the elevated temperatures the viscosity of the mobile phase decreases significantly and thus the diffusion coefficients of the compounds

separated increase (Wilke–Chang equation), resulting in narrower and therefore higher peaks.

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ANALYTICAL MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY OF CELLULOLYTIC ENZYMES ON SPHERON ION EXCHANGERS[*]

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SUMMARY

Cellulolytic enzymes from the cultivation liquid of *Trichoderma viride-reesei* were submitted to a rapid medium-pressure liquid chromatography, analytical and semipreparative, on weakly, medium and strongly acidic cation exchangers and on medium and strongly basic anion exchangers Spheron 1000. The course of gradient elution was monitored by measurement of effluent absorbance at 285 nm and 254 nm, conductivity, pH and enzymatic activity assays (cleavage of crystalline cellulose, filter-paper, carboxymethylcellulose and *p*-nitrophenyl-β-D-glucopyranoside). On all ion exchangers tested, optimum conditions for separation were found. The chromatographic profiles and separated enzymatic activities of our preparation were compared with those of commercial technical cellulolytic preparations from the cultivation liquids of *Trichoderma viride* and *Aspergillus niger*, using Spheron DEAE-1000. The results are discussed from the point of view of the possible use of rapid column chromatography in the diagnosis of enzymatic preparations and as a modern analytical method for biotechnology.

INTRODUCTION

Cellulolytic enzymes belong to the group of enzymes which take part in the gradual degradation of cellulose to glucose[1]. They are produced by various types of organisms, *e.g.*, bacteria, fungi or actinomycetes. In the majority of cellulolytic organisms the cellulase complex is composed of several enzymes, which hydrolyse cellulose substrates synergically. The cellulase system contains *exo*-β-1,4-β-glucanases which hydrolyse the cellulose chain from the non-reducing end and split off either glucose or cellobiose, *endo*-β-1,4-glucanases which randomly cleave internal bonds of the cellulose fibres and β-glucosidase which hydrolyses cellobiose and lower cellodextrins to glucose[2]. The accessibility of highly active cellulases is a prerequisite for the successful application of enzymatic conversion of cellulose in industry.

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* Part of the thesis of Zuzana Hostomská, *Methods of Rapid Separation and Characterization of Cellulolytic Enzymes*, carried out in fulfilment of the biochemical sciences degree at the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences, Prague, 1982.

A number of screening methods has been developed for the selection of highly productive mutant strains[3]. The evaluation of the samples is usually carried out from the point of view of cellulase activity, the total activity of the non-fractionated systems being determined by the method of cleavage of filter-paper[4], known as filter-paper activity (FPA). Different preparations of cellulases differ in the proportions of individual components, depending on the source, cultivation and treatment of the sample. The FPA method provides no information on the levels of individual enzymes of the cellulolytic system or on their rôle in the hydrolysis of cellulose substrates. Such information is best obtained by fractionation of the cellulase complex and by purification of the sample. For the preparation of technical enzymes, modern biotechnology requires rapid, accurate and reproducible analytical methods.

The recent development of the modern high-performance liquid chromatography (HPLC) of biopolymers[5-8] has permitted the application of this method to the separation of cellulolytic enzymes of various origins[9-11]. In earlier papers we demonstrated the possibility of using the hydrophilic polymer Spheron for the preparation of macroporous ion exchangers[12,13]. These ion exchangers proved suitable for rapid chromatography of technical enzymes[14-16]. The aim of this work was to extend these studies to rapid separations of the cellulolytic enzyme complex on an analytical and semipreparative scale.

EXPERIMENTAL

Materials

Enzymes. The desalted lyophilized cellulase preparation from the *Trichoderma viride-reesei* mutant was prepared from a cultivation liquid obtained from the Research Institute of the Food Industry in Prague. The cultivation took place at 30°C for 6 days in a 5-l tank at 300 rpm and 1:1 aeration, on a medium composed of 1.5% of cellulose and 1% of wheat bran, at pH 6.2. The cellulase preparations Onozuka R-10 and *Aspergillus niger* were commercial samples supplied by Serva (Heidelberg, F.R.G.).

Chromatographic materials. For the separation of cellulases, commercially accessible (Lachema, Brno, Czechoslovakia) ion-exchange derivatives of Spheron 1000 were used, with a particle size 20–40 μm: weakly acidic cation exchanger Spheron C-1000, nominal capacity 1.85 mequiv./g; medium acidic cation exchanger Spheron Phosphate-1000, 3.1 mequiv./g; strongly acidic cation exchanger Spheron S-1000, 1.72 mequiv./g; medium basic anion exchanger Spheron DEAE-1000, 1.5 mequiv./g; strongly basic Spheron TEAE-1000, 1.4 mequiv./g.

Substrates. For the determination of cellulolytic activity, carboxymethyl (CM)-cellulose from Serva was used, having a substitution degree of 0.7%, a polymerization degree of 500–520 and molecular weight about 100,000. Avicel pH 105, a microcrystalline cellulose, was supplied by FMC Corporation (Philadelphia, PA, U.S.A.), Whatman No. 1 paper from Whatman (Springfield Mill, Maidstone, U.K.) and *p*-nitrophenyl-*β*-D-glucopyranoside from Lachema.

Methods

Chromatography. The preparation of ion-exchange derivatives of Spheron and the chromatographic apparatus were described earlier[12,13]. For the separation of

cellulases a combination of isocratic elution and elution with a linear gradient of the ionic strength of the buffer were used. The concentration of the buffers is always given in relation to the counter-ion. The chromatographic columns were packed by the slurry method, using the stepwise pulse-packing method[17], at 2.0–2.5 MPa. The effluent from the column was monitored by a tandem system of two flow-through photocells, recording absorbance at 254 and 285 nm. Conductivity, pH and enzymatic activity were measured in each fraction.

Enzymatic activity assays. After treatment with enzymes, the newly formed reducing ends were determined with the arsenomolybdate reagent of Somogyi[18] and Nelson[19]. The reaction mixture contained 2 ml of a 1 % solution of CM-cellulose in 0.05 M sodium citrate of pH 4.9 and 100 μl of the enzyme solution. The incubation lasted for 30 min at 40°C. The unit of enzymatic activity was defined as the amount of enzyme which sets free within 1 min an amount of reducing sugars corresponding to 1 μmol of glucose.

Filter-paper (1 × 1 cm) was incubated in 1 ml of 0.05 M citrate buffer pH 4.7 with 100 μl of sample for 3–14 h at 40°C. After the end of the incubation 200 μl of supernatant were withdrawn and the content of liberated sugars was determined using the method of Somogyi[18] and Nelson[19].

β-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucopyranoside as substrate. The reaction mixture contained 1 ml of a 1 mM solution of the substrate in 0.05 M acetate buffer pH 5 and 100 μl of the enzyme solution. Since the β-glucosidase activity was relatively low the incubation was carried out for 3–14 h at 40°C. The reaction was stopped by addition of 2 ml of 1 M Na_2CO_3. The unit of enzyme activity was defined as the amount of enzyme which sets free 1 nmol of *p*-nitrophenol within 1 min.

Avicelase activity was determined by suspending 16 mg of Avicel pH 105 in 1 ml of 0.05 M citrate buffer of pH 4.7 and incubating with 100 μl of an enzyme sample for 3 h under slow constant rotation of the test-tube at 37°C. [The equipment described earlier (ref. 20, Fig. 1) was used for this purpose.] The reducing sugars were determined by the Somogyi[18] and Nelson[19] method using 200 μl of the supernatant.

RESULTS

We carried out a systematic investigation of the possibilities of rapid medium-pressure ion-exchange chromatography of the cellulolytic complex of the imperfect fungus *Trichoderma viride-reesei*, known to be one of the most suitable microbial producers of technical cellulolytic enzymes. The experiments were carried out on all commercially available Spheron ion exchangers. Experiments with isoelectric focusing of the cellulolytic complex, which will be described in a subsequent paper[21], served as a partial guide in the selection of chromatographic conditions. Optimum conditions found for chromatography on Spheron DEAE-1000 were then used for the comparison of our cellulolytic preparation with commercially available cellulolytic preparations Onozuka R-10 (*T. viride*) and preparations from *Aspergillus niger*.

Chromatography on cation exchangers
The cellulases from *T. viride* were separated on an analytical scale on Spheron carboxyl-1000 at pH 3, 4 and 5, and with the same ionic strength gradient. The best
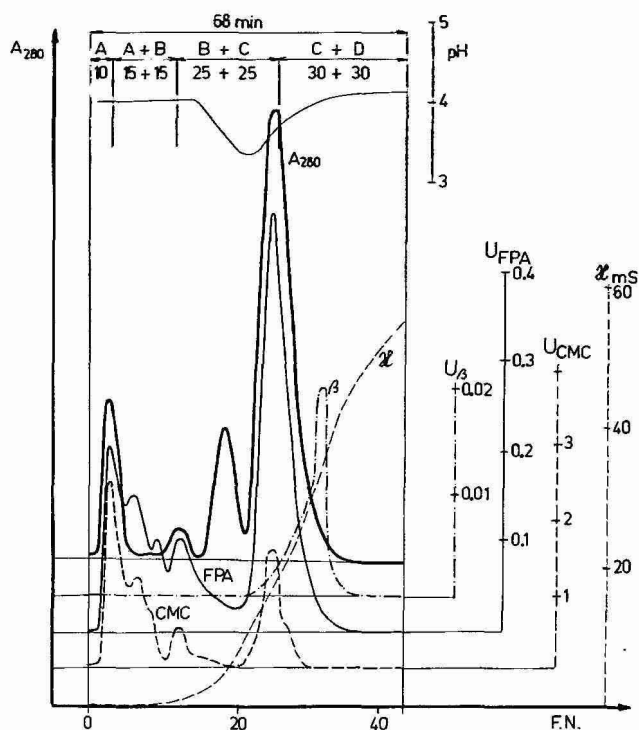
Fig. 1. Chromatography of 20 mg of a desalted lyophilized preparation of the cellulolytic system from *T. viride* on Spheron C-1000. Conditions: column, 20 × 0.8 cm; fractions, 3.3 ml at 90-sec intervals; pressure 0.3 MPa; flow-rate 2.2 ml/min; room temperature. Buffers (all of pH 4) for linear gradients: A, 0.025 M NaOH + citric acid; B, 0.05 M + citric acid; C, 0.25 M NaOH + citric acid + 1 M NaCl; D, 0.25 M NaOH + citric acid + 2 M NaCl. A_{280} = Detector response; κ = conductivity in mS; U_{CMC} = units of carboxymethylcellulase activity; U_{FPA} = units of filter-paper activity; U_β = units of β-glucosidase activity. The activities were determined in 0.8-ml aliquots of the effluent (see Experimental); F.N. = fraction number.

separation was achieved —contrary to the original assumption— in the proximity of the isoelectric point of the main fraction (pI = 4.2) at pH 4. The concentration of 0.025 M of the starting buffer was optimal. The chromatographic course under these conditions is illustrated in Fig. 1. The main fraction of the carboxymethylcellulase (CM-cellulase) activity and a part of the filter-paper cleaving activity (FPA) or the Avicel cleaving activity (not shown) were eluted isocratically. The gradient of increasing citrate molarity indicates at least three minor components, characterized both by CM-cellulase activity and by FPA. Using a linear NaCl gradient an apparently non-protein peak was obtained, which displayed a higher absorbance at 254 nm than at 280 nm but none of the investigated activities. Toward the end of this gradient, separate maxima of FPA and β-glucosidase activity were detected. In all the chromatograms (at various pH values and ionic strengths), a part of the protein material was eluted at approximately the hold-up volume of the column. It is possible that this is due to the presence of non-ionogenic admixtures which interact with the enzymes and transport them.

On medium acidic Spheron Phosphate-1000, chromatograms were obtained at
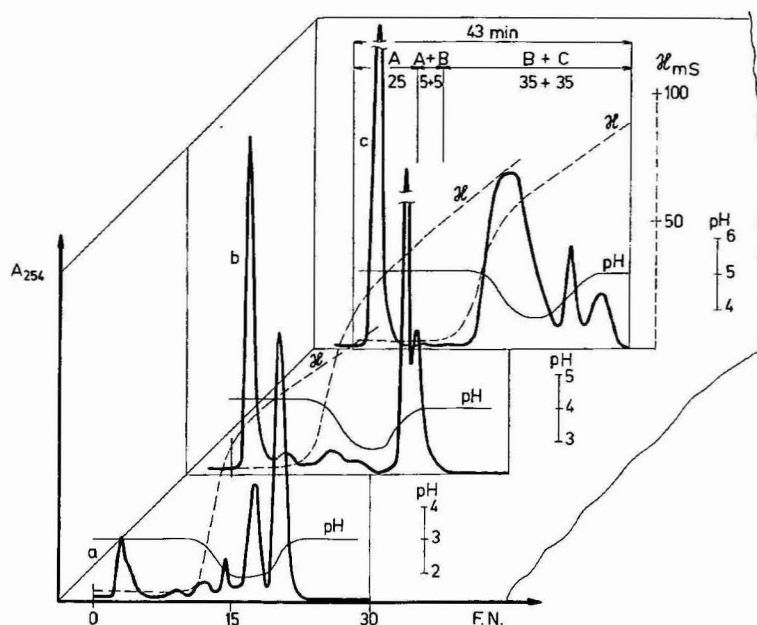
Fig. 2. Determination of the optimum pH for the separation of the cellulolytic system of *T. viride* on Spheron phosphate-1000. Conditions: 7.5 mg of lyophilized preparation; column, 20 × 0.8 cm; fractions, 3.5 ml at 85-sec intervals; pressure 0.2 MPa; flow-rate 2.4 ml/min; room temperature. Buffers: A, 0.05 M NaOH + citric acid; B, 0.05 M NaOH + citric acid + 1 M NaCl; C, 0.05 M NaOH + citric acid + 3 M NaCl. Citric acid was always added until the required pH value was achieved, *i.e.*, 3, 4 and 5 in a, b and c. A_{254} = Detector response.

pH 3–5, using similar combinations of the gradients (see Fig. 2). At a pH value one unit lower than the isoelectric point of the main fraction of cellulases, the mixture was strongly retained on the column (pH 3, see curve a). Experiments at still lower pH values were not carried out owing to the instability of the enzymes. As is evident from Fig. 2, the quality of separation decreased with increasing pH, and an increasing proportion of the mixture was not retained on the column. It is interesting that the absorbance of individual components increased with pH. Chromatography of the cellulolytic complex on Spheron Phosphate-1000 at pH 3 proved most suitable as the first step of fractionation of the complex on a preparative scale and therefore it was subsequently investigated in greater detail, including the evaluation of enzymatic activities[21].

Fig. 3 shows a chromatogram on the cation-exchange derivative Spheron sulfate-1000 at pH 4, with the mentioned gradient systems. Three types of activity were determined in the effluent, in accord with the detector record at 280 nm. The increased acidity of the sulphonic groups, in comparison with the carboxyl and phospho derivative, was most striking in the complete separation of the β-glucosidase activity, which was eluted towards the end of the chromatogram in the form of a single peak. At least four forms of the CM-cellulase activity were detected, of which the main fraction was eluted with the hold-up volume. The FPA and Avicelase activities (not shown) were determined in two forms; that in smaller amount was practically not bound to the column, and the main fraction was obtained on elution with buffers B + C.
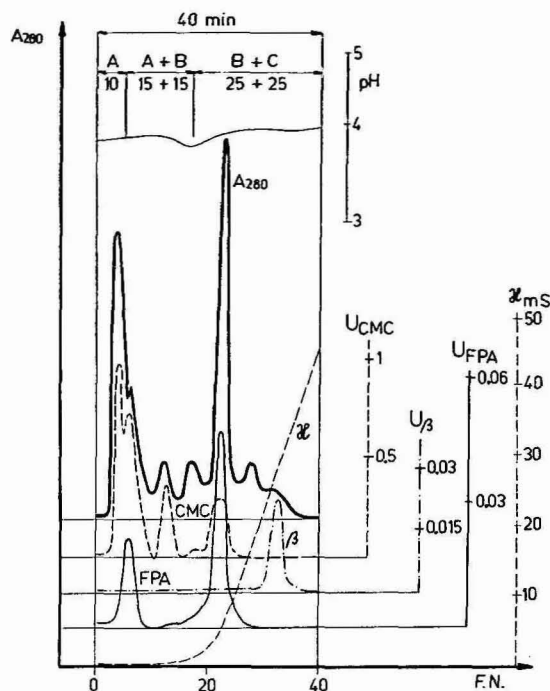
Fig. 3. Chromatography of 8 mg of the cellulolytic system from *T. viride* on Spheron S-1000. Conditions: column, 20 × 0.8 cm; fractions, 2.2 ml taken at 60-sec intervals; pressure 0.5 MPa; flow-rate 2.2 ml/min; room temperature. All buffers were of pH 4: A, 0.01 *M* NaOH + citric acid; B, 0.05 *M* NaOH + citric acid; C, 0.25 *M* NaOH + citric acid + 1 *M* NaCl. For enzymatic activities see Fig. 1.

Chromatography on anion exchangers

Cellulolytic enzymes are acidic proteins, and as such they are usually chromatographed on anion-exchange resins. We investigated the separation of the cellulolytic system of *T. viride* on a medium basic exchanger, Spheron DEAE-1000, at pH 4-7. The best separation was achieved at pH 5 and with the gradient systems given in Fig. 4. Similarly to cation exchangers, this ion-exchange derivative also did not afford a complete separation of any of the investigated activities for high-molecular-weight substrate; the protein peaks were characterized by the presence of several enzymatic activities. However, it is possible that this imperfect separation of the CM-cellulase activity and FPA is not a result of an incomplete chromatographic separation, but that it is generally caused by insufficient specification of individual activities, following from the choice of the substrate, and by the fact that in both cases the activity is determined via the reducing ends formed (for details see Discussion). The β-glucosidase activity, which was obtained in the form of a single peak on cation-exchange derivatives, was separated into four forms on the DEAE derivatives. CM-cellulase was obtained in four forms and FPA in two forms. The last three peaks in the chromatographic profile had no cellulase activity.

The series of chromatographic separations was completed by experiments on a strongly basic Spheron TEAE-1000 packing. Fig. 5 illustrates the chromatographic profile at two wavelengths; the proportions of the absorbances indicate that
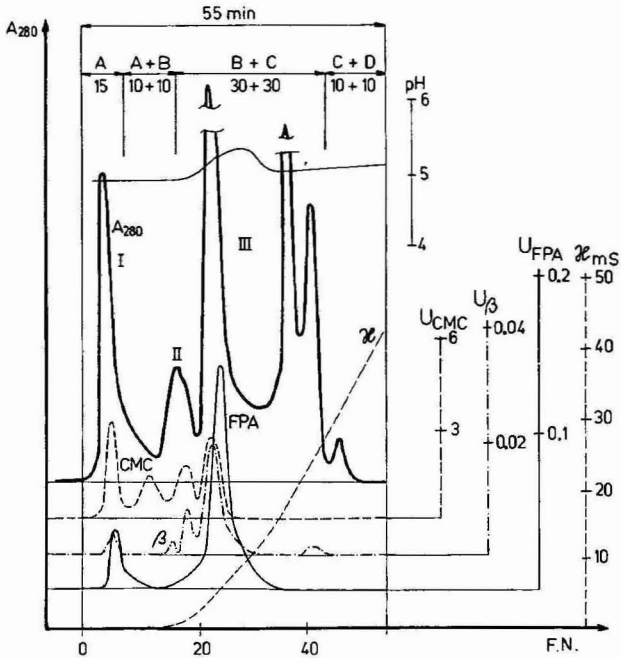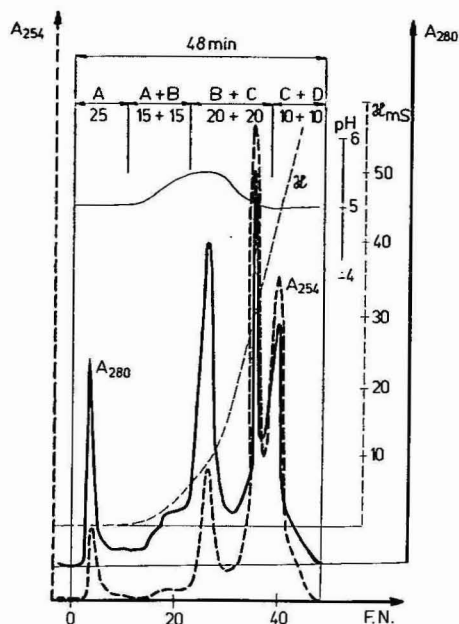
Fig. 4. Chromatography of the cellulolytic system of *T. viride* on Spheron DEAE-1000. Conditions: 20 mg of the preparation; column, 20 × 0.8 cm; fractions, 2.3 ml in 66-sec intervals; flow-rate 2 ml/min; pressure 0.5 MPa; room temperature. Buffers of pH 5: A, 0.005 *M* citric acid + NaOH; B, 0.1 *M* citric acid + NaOH; C, 0.25 *M* citric acid + NaOH + 1 *M* NaCl; D, buffer C + 3 *M* NaCl. For the enzymatic activities see Fig. 1.

the last two peaks are probably not proteins. Contrary to expectation, we could not obtain a better chromatographic profile than in the case of the DEAE derivative, despite the fact that the elution sequence of the peaks was similar. Therefore we did not evaluate the obtained fractions by activity assays.

Comparison of the chromatographic profiles of cellulases from T. viride-reesei and from two commercial preparations of technical cellulase

A comparison of our cellulolytic preparation from the mutant of *T. viride* with some commercial preparations was of great interest. Such a comparison is not only useful from the point of view of the evaluation of the enzymatic composition of the preparations themselves, but also as an evaluation of the efficiency of the conditions found for the distinction of the cellulolytic preparations according to their origins. All the chromatograms of the three different preparations were obtained on the medium basic anion exchanger Spheron DEAE-1000 in citrate buffers of pH 5 and with similar gradients of increasing molarity of the buffers.

Chromatography of the technical preparation Onozuka R-10 is shown in Fig. 6. Isocratic elution with the starting buffer gave the main protein fraction I which was little bound to the DEAE packing under these conditions. It contained a double peak of high β-glucosidase activity, another of CM-cellulase activity and the main fraction of the FPA. Using a linear gradient of citrate ions the minor peak of the CM-cellulase activity, Ia, was eluted. A further peak, II, was again characterized by the presence of

Fig. 5. Chromatography of the cellulolytic system of *T. viride* on Spheron TEAE-1000. Conditions: column, 20 × 0.8 cm; 7 mg of the preparation; flow-rate 2.4 ml/min; fractions collected in 60-sec intervals; pressure 0.4 MPa; room temperature. Buffers of pH 5: A, 0.005 *M* citric acid + NaOH; B, 0.1 *M* citric acid + NaOH; C, 0.25 *M* citric acid + NaOH + 1 *M* NaCl; D, 0.25 *M* citric acid + NaOH + 3 *M* NaCl. A_{254}, A_{280} = Detector responses.

two β-glucosides with one third of the activity of the first peak, CM-cellulase activity and FPA. Elution with an NaCl gradient gave peak III in which only FPA and a minor fraction of β-glucosidase activity could be detected. The last two peaks of the chromatographic profile did not display any cellulolytic activity.

Fig. 7 illustrates the chromatography of the commercial preparation from *Aspergillus niger*. The protein profile recorded as absorbance at 280 nm was different from that in Figs. 4 and 6. The determination of the enzymatic activities confirmed that this preparation was different from those originating from *T. viride*. The starting buffer eluted the main fraction of the CM-cellulase activity and FPA. No β-glucosidase activity could be found. Gradients of increasing ionic strength gave the asymmetric peak II in which a high β-glucosidase activity was found in the form of a simple peak as well as a double peak of low CM-cellulase activity and a simple peak of FPA.

In Figs. 4, 6 and 7 records of the absorbance at 280 nm are shown, from which basic information on chromatographed samples follows. In the case of the preparations from *T. viride* (Figs. 4 and 6) the mutual relationship is evident from the profiles of measured absorbance at 280 nm alone but different levels of individual enzymes are also evident without activity assays. In Figs. 4 and 6 the opposite ratio of the heights of the two main protein peaks I and III is recorded. When these records were completed by the determination of three different activities, a more detailed knowledge of these materials was obtained. The preparation Onozuka R-10 displays a 28 times higher total β-glucosidase activity (compared to our enzyme),which is practi-
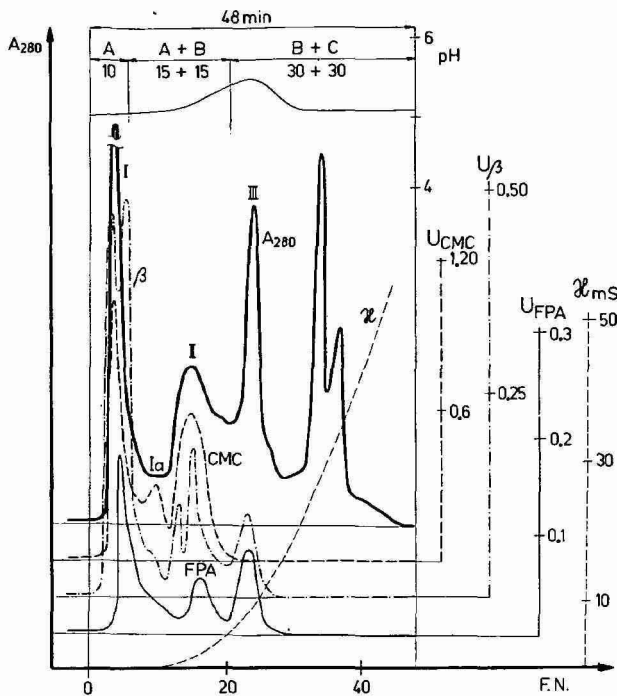
Fig. 6. Chromatography of 20 mg of the preparation Onozuka R-10 on Spheron DEAE-1000. Conditions: column, 20 × 0.8 cm; fractions at 60-sec intervals; flow-rate 2.1 ml/min; pressure 0.5 MPa; room temperature. Buffers of pH 5: A, 0.005 M citric acid + NaOH; B, 0.1 M citric acid + NaOH; C, 0.25 M citric acid + NaOH + 1 M NaCl. 0.5 ml of the effluent were withdrawn for the determination of enzymatic activities. For the enzymatic activities see Fig. 1.

cally all eluted with the hold-up volume. This form of the enzyme is almost absent in our preparation from *T. viride*. Further peaks of the β-glucosidase activity were eluted at the same values of buffer molarity, but they were present in different relative proportions. In the case of Onozuka R-10 three chromatographically different forms of filter-paper cleaving enzymes were found, while in the case of our preparation only two were found in opposite quantitative ratio in comparison with Onozuka R-10. The component which cleaved filter-paper and Avicel, which was eluted in both cases at the beginning of the gradient B + C, had the ability to liberate individual fibres from the filter-paper.

The determination of the activities in the preparation *A. niger* (Fig. 7) provided further information on this material. The preparation had an 83 times higher β-glucosidase activity than our material. This activity was eluted with the gradient B + C (see peak II in Fig. 7), in a position where, in the case of the preparation from *T. viride* (Fig. 4), an enriched FPA with the ability to liberate filter-paper fibres was found. The latter enzyme property was not found in the preparation from *A. niger*, where FPA indicates only the ability to liberate reducing sugars.

DISCUSSION

We examined the chromatography of cellulolytic enzymes on cation-exchange
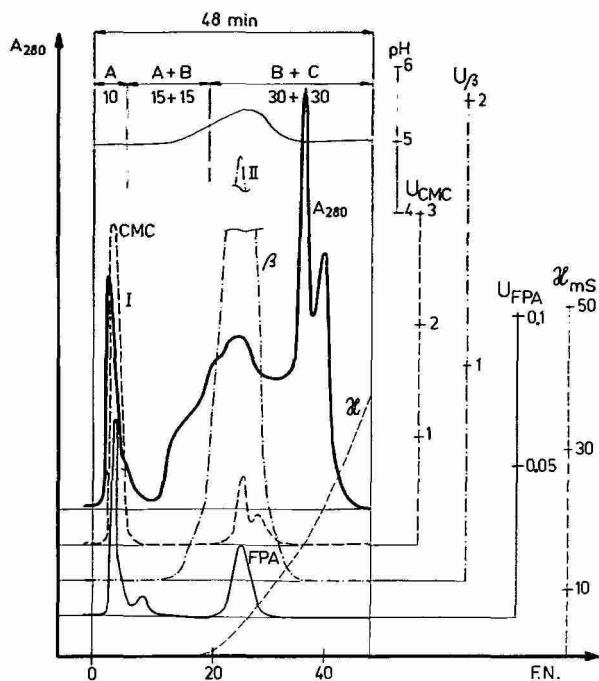
Fig. 7. Chromatography of 20 mg of the cellulase preparation from *Aspergillus niger* on Spheron DEAE-1000. Conditions as in Fig. 6.

and anion-exchange derivatives of Spheron at various pH values, using gradients of sodium or citrate ions and sodium chloride ionic strength gradients or pH gradients. The results of the pH-gradient separations are not shown because they did not contribute in any experiment to a better separation than that illustrated in preceding sections. Chromatography took 40–70 min. A good separation depended on the complexity of the gradient systems and the choice of the flow-rate. From the point of view of flow-rate the possibilities are not yet exhausted and the separation could be carried out at even higher rates, *i.e.*, in a shorter time.

The separations carried out with the preparation of the cellulolytic system from *T. viride* demonstrated the presence of five forms of CM-cellulase and at least two forms of the enzymes which cleaved filter-paper. It remains to be determined whether genetically different isoenzymes are involved, or whether these differences are cases of polymorphism of the same types of enzymes, due, for example, to subsequent changes in the polysaccharide composition, or to changes caused by a limited proteolysis.

In this study we could not completely separate the CM-cellulase activity from FPA. Both activities were always found in the fractions, but at different ratios. This fact could be ascribed to an imperfection of the separation process, probably due, for example, to the close proximity of the isoelectric points of individual components of the mixture, although this is improbable considering the choice of such a broad range of conditions. The quality of the chromatography is demonstrated by the complete separation of the β-glucosidase activity on the cation-exchange derivatives of Spheron (see Fig. 3).

The explanation for the observed multiple specificity of the separated fractions may lie in an imperfection in the determination of enzymatic activities, *i.e.*, in the fact that individual enzymes cannot be distinguished in principle when CM-cellulase and filter-paper are used as substrates. These macromolecular substrates cannot be defined as unambiguously as the relatively simple low-molecular-weight substrate for β-glucosidase activity —p-nitrophenyl-β-D-glucopyranoside. In addition, the activity was estimated in both cases by the determination of the reducing ends formed.

The problem may be considered from another point of view. The essence of the catalytic effect of all three types of the cellulolytic enzymes is the cleavage of the β-glucosidic bond in cellulose. The amounts of the individual detectable activities may reflect the polymorphism of the substrate, an expression of the adaptability of the microorganisms to the degradation of most types of arrangement of cellulose fibres in natural materials. From this it would follow that the tests used differentiate only quantitative differences in these activities, and that the whole spectrum of the cellulolytic enzymes does not display strictly qualitative differences in their specificities. If this hypothesis is correct, than any one enzyme of the cellulolytic system would be able, in principle, to cleave any cellulose, although with different efficiencies. This problem requires further study and HPLC separations represent a good approach.

Medium-pressure liquid chromatography of cellulolytic enzymes on ion-exchange derivatives of Spheron generally permits a comparison of preparations from various sources and from various mutant strains. The method elaborated consists of the initial comparison of elution profiles, which may be completed by the determination of enzymatic activities in the effluent, in order to increase our knowledge of the enzymatic properties of the preparations under investigation. This procedure may also be used to study the effect of various parameters during fermentation (temperature, pH, aeration, medium composition) on the individual activities, by the withdrawal of samples during fermentation and their rapid analysis, as well as the effect of storage.

ACKNOWLEDGEMENTS

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RELATIVE ABUNDANCE OF DIMETHYLNAPHTHALENE ISOMERS IN CRUDE OILS

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SUMMARY

A procedure has been developed for routine relative quantitative analysis of each of the ten isomeric dimethylnaphthalenes in crude oils. The method requires isolation of an alkylnaphthalene fraction by preparative thin-layer chromatography on activated alumina plates, followed by fused-silica capillary gas chromatography analysis on OV-1 and OV-1701 phases. Because the concentration of 1,8-dimethyl-naphthalene in crude oils is so low, it is necessary to carry out a preparative capillary gas chromatography step for analysis of this isomer. In some crude oils, the presence of dimethylbenzothiophenes interferes with the analysis: these components can be removed by treatment of the alkylnaphthalene fraction with *m*-chloroperbenzoic acid prior to analysis.

INTRODUCTION

As part of a study into the geochemical significances of changes in the distribution of alkylnaphthalenes, we required to be able to carry out rapid and accurate routine analyses of each of the ten isomeric dimethylnaphthalenes in crude oils. Tesařík *et al.*[1] have summarised attempts to separate dimethylnaphthalenes by both packed column and capillary gas chromatography (GC), and were in fact successful in separating all of the isomers, excepting the 1,6- and 1,7-pair, on a composite column comprised of one column coated with Bentone 34 and dodecyl phthalate (50:50) connected to a column coated with Ucon LB 550 X and tris(cyanoethoxy)-propane (TCEP) (85:15). There have also been a number of subsequent reports listing additional retention data on dimethylnaphthalenes and related aromatic compounds which are characteristically found in crude oils[2–4]. In this paper we report a procedure for quantitative analysis of all ten isomeric dimethylnaphthalenes in crude oils. This procedure involved a preliminary isolation of an alkylnaphthalene fraction by thin-layer chromatography (TLC), followed by capillary GC analysis using two columns with different stationary phases.

EXPERIMENTAL

Crude oil samples

The sample described as a Wyoming Pennsylvanian oil was recovered from the Pennsylvanian formation in the Nieber Dome Field in Wyoming, U.S.A. That described as Barrow Upper Jurassic was recovered from the 2040-m level in an Upper Jurassic formation in the Barrow Island oil field, Western Australia.

Dimethylnaphthalene standards

The 1,2-, 1,3-, 1,4-, 1,5-, 1,6-, 2,3- and 2,6-dimethylnaphthalenes were purchased commercially (Aldrich, Fluka). 1,8-Dimethylnaphthalene was prepared from acenaphthylene according to literature procedures[5]. 1,7-Dimethylnaphthalene was obtained as a mixture by isomerising the 1,8-isomer[6]. The 2,7-isomer was contained in an all-isomer mixture (Aldrich).

Isolation of alkylnaphthalenes

Crude oil (80 mg), diluted with dichloromethane if necessary, was applied as a thin band to freshly activated alumina TLC plates (0.75 mm, Merck Alumina G) using a Camag chromatocharger. A reference mixture of dimethylnaphthalenes was spotted at each end of the plate, the plates were developed with pentane and the required band ($R_F \approx 0.4$–0.5) was located using short wavelength UV light. The alkylnaphthalene fraction was extracted from the recovered band of alumina by extraction with dichloromethane (20 ml), and this solution was concentrated to 1–2 ml in a Kuderna–Danish apparatus. In one experiment carried out to assess the extent of fractionation in the procedure, the solution containing the alkylnaphthalene fraction was concentrated to a volume of 250 μl. A 50-μl sample was set aside for GC analysis and the remaining 200 μl were divided into two equal portions. One portion was subjected to TLC under the conditions outlined above, and the second portion was treated similarly, except that the TLC plate was left on the bench for 30 min prior to extraction of the alkylnaphthalene fraction. The two alkylnaphthalene fractions resulting from this procedure were then analysed by GC under standard conditions. Gas chromatograms were recorded using a Hewlett-Packard (HP) 5880 gas chromatograph fitted with a 50-m wall-coated open-tubular (WCOT) column coated with either OV-1 or OV-1701. The oven was held at 30°C for 5 min then temperature programmed at 1° min^{-1}, to 270°C. Hydrogen was used as a carrier gas at a flow-rate of 30 cm sec^{-1}, and peaks were detected using a flame ionization detector. Mass spectra were recorded using an HP 5985B GC–mass spectrometry (MS) system fitted with a 50-m OV-101 WCOT column operating under the same conditions as the HP 5880. The ion source temperature was 250°C and the electron ionizing voltage was 70 eV.

Isolation of 1,8-dimethylnaphthalene by preparative capillary GC

An alkylnaphthalene fraction was chromatographed on the HP 5880 system fitted with a 50-m OV-101 capillary column. When the 1,2-dimethylnaphthalene peak had just eluted, the detector end of the column was quickly removed from the FID, withdrawn through the oven door and the oven door was closed immediately to allow the temperature program to continue. The effluent from the column was bubbled

through dichloromethane (10 ml) for 3 min, then the section of the column protruding from the oven was broken off and any material condensed on its surface was collected by forcing dichloromethane (200 μl) through the tubing with a syringe. The combined dichloromethane solutions were concentrated to 10 μl using a Kuderna–Danish apparatus prior to analysis by GC–MS.

Oxidation of sulphides to sulphones

m-Chloroperbenzoic acid (2 mg) was added to the alkylnaphthalene fraction extracted from the alumina of the TLC plate with dichloromethane (20 ml) and the mixture was allowed to stand at ambient temperature for 10 min. The solution was then passed through a column of basic alumina (5 g, Woelm W200 Basic) to remove sulphones and acidic material.

RESULTS AND DISCUSSION

The complex composition of crude oils is evident from the partial GC trace of the Barrow Upper Jurassic oil which is shown in Fig. 1. Preparative TLC of this oil on a freshly activated alumina plate under carefully controlled and standardised conditions provided an alkylnaphthalene fraction. The capillary GC traces of this alkylnaphthalene fraction on a OV-1 column is shown in Fig. 1b. That carried out using an OV-1701 column is shown in Fig. 1c. Except for 2,7-dimethylnaphthalene, where published retention index data[2,4] were used, assignment of peaks was made by coinjection of authentic standards. The possibility of coelution of other compounds with the dimethylnaphthalenes was eliminated by GC–MS analysis. Examination by GC–MS of extracts of the regions of the plate immediately above and below the region isolated confirmed that this fraction contains all of the naphthalenes, methylnaphthalenes and dimethylnaphthalenes in the oil sample.

Excepting for 1,5- and 2,3-isomers, which coeluted, all of the dimethylnaphthalenes were resolved on the OV-1 column under the conditions employed. On the OV-1701 column, these isomers were resolved, but in this case, the 2,6- and 2,7-isomers coeluted, and the 1,3- and 1,7-isomers were poorly resolved. The distribution of dimethylnaphthalenes in the Barrow Upper Jurassic oil can therefore be obtained from these two chromatograms. The mean values obtained from five replicate analyses are shown in Table I, column 1. The reproducibility of the analysis is $\pm 3\%$.

As might have been expected on the basis of thermodynamic data[7], 1,8-dimethylnaphthalene is usually not detected in crude oils[8] or coal tars[1]. A tiny peak at a retention time very similar to that of 1,8-dimethylnaphthalene is indicated in Fig. 1b. Coinjection with authentic material showed that it was not the 1,8-isomer, and GC–MS analysis confirmed that this isomer was not present in sufficient quantity for direct analysis to be possible. A fraction containing 1,8-dimethylnaphthalene and acenaphthylene was therefore obtained by preparative capillary gas chromatography. This was analysed by GC–MS: the areas of the peaks due to 1,8-dimethylnaphthalene in the m/z 141 and 156 mass fragmentograms were compared with the areas of the peaks due to acenaphthylene in the m/z 153 and 154 mass fragmentograms; finally, after correction had been made for detector response, the level of 1,8-dimethylnaphthalene could be related to those of the other isomers via their ratios to the level
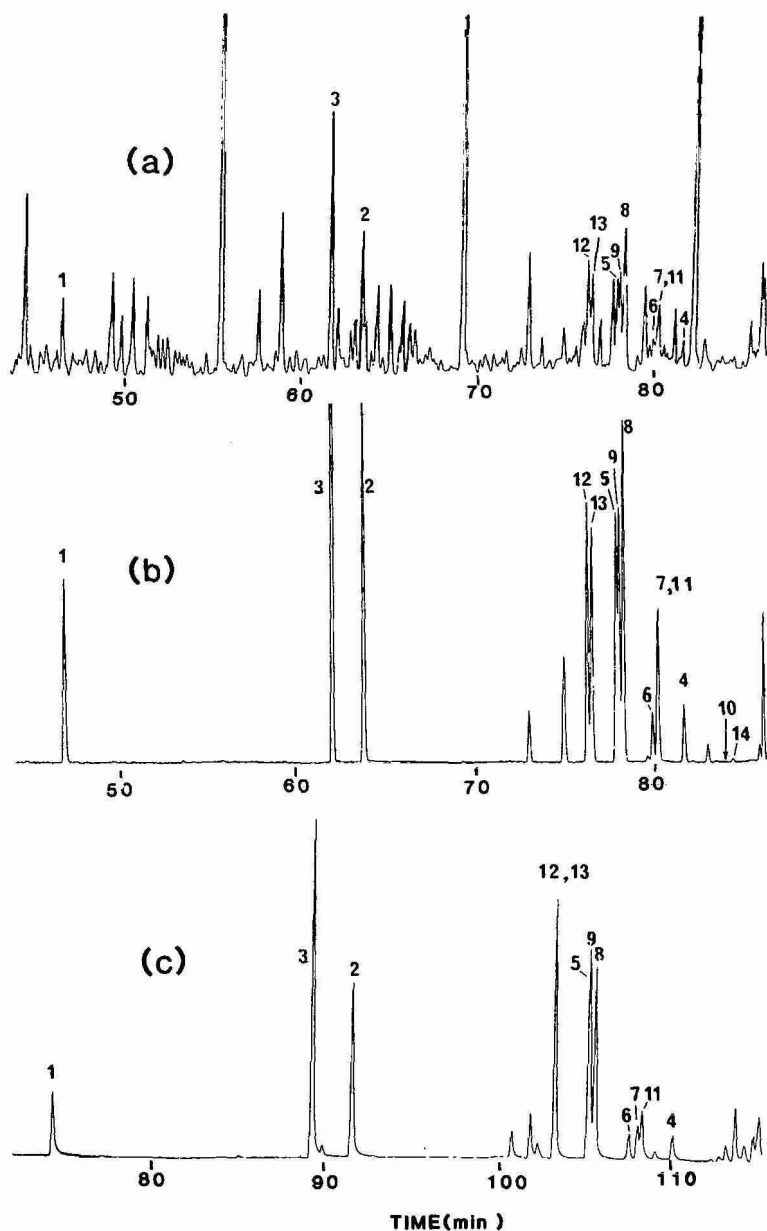
Fig. 1. Capillary GC traces of the Barrow Upper Jurassic crude oil (a) and its alkylnaphthalene fraction using OV-1 (b) and OV-1701 (c) columns. Peaks were assigned as: 1 = naphthalene; 2 = 1-methylnaphthalene; 3 = 2-methylnaphthalene; 14 = acenaphthylene; dimethylnaphthalenes (see Table I).

of acenaphthylene. In the case of the Barrow Upper Jurassic oil, the 1,8-isomer was found to account for approximately 0.003 % of the dimethylnaphthalenes.

The boiling points of dimethylnaphthalenes range from 262°C for the 2,6- and 2,7-isomers to 277°C for the 1,8-isomer. We were therefore concerned that fraction-

TABLE I

RESULTS FROM ANALYSES OF ALKYLNAPHTHALENES IN BARROW UPPER JURASSIC CRUDE OIL

Values are percentages of the sum of the areas of the peaks due to the dimethylnaphthalene isomers. Values for sample 1 are the means of five replicate analyses. Values for sample 2 were obtained after reduction of sample solution volume to 250 μl. Values for sample 3 were obtained by repeating the TLC procedure on sample 2. Sample 4 was obtained from sample 2 by repeating the TLC procedure and allowing the plate to stand at ambient temperature for 30 min before isolation of the alkylnaphthalene fraction and GC. n.d. = Not determined.

Compound	Peak No.	Alkylnaphthalene fractions			
		1	2	3	4
1,2-Dimethylnaphthalene	4	3.7	3.7	3.8	3.7
1,3-Dimethylnaphthalene	5	15.4	15.6	15.5	16.0
1,4-Dimethylnaphthalene	6	3.0	2.9	2.9	3.0
1,5-Dimethylnaphthalene	7	4.0	4.0	4.0	4.0
1,6-Dimethylnaphthalene	8	21.2	21.2	21.1	21.1
1,7-Dimethylnaphthalene	9	15.6	15.3	15.4	15.0
1,8-Dimethylnaphthalene	10	0.003	n.d.	n.d.	n.d.
2,3-Dimethylnaphthalene	11	6.6	6.7	6.8	6.7
2,6-Dimethylnaphthalene	12	16.0	15.9	15.9	15.9
2,7-Dimethylnaphthalene	13	14.5	14.6	14.5	14.5

ation might occur during our analytical procedure, particularly in the TLC step. In order to eliminate the possibility that losses occur during evaporation of the dichloromethane solvent an alkylnaphthalene fraction was evaporated to 250 μl, and the residue was analysed by GC; Table I, sample 2. The TLC procedure was repeated on two 100-μl portions of this residue: in one case, however, the TLC plate was allowed to stand on the bench for 30 min before the alkylnaphthalene band was removed. Analysis of these two fractions was carried out and the results are presented in Table I: samples 4 and 3 respectively. The values in this table show that there is no relative enrichment or depletion of any of the dimethylnaphthalene isomers.

Attempted quantitative analysis of the dimethylnaphthalene isomers in the alkylnaphthalene fraction of some oils is frustrated by the co-elution of dimethylbenzothiophenes with the dimethylnaphthalenes. This problem can be overcome by oxidising the interfering benzothiophenes to the corresponding sulphones using the method of Hunt and Shabanowitz[9]. The dichloromethane solution of the alkylnaphthalene fractions was simply treated with excess of m-chloroperbenzoic acid at room temperature for 10 min, then the reaction products and excess reagent were removed by passing the solution through a short column of basic alumina. The eluate was analysed by GC without further purification. Fig. 2a shows the GC profile of the alkylnaphthalene fraction of the Wyoming, Pennsylvanian oil; Fig. 2b shows the same region of the GC trace of the same fraction after treatment to remove the dimethylbenzothiophenes.

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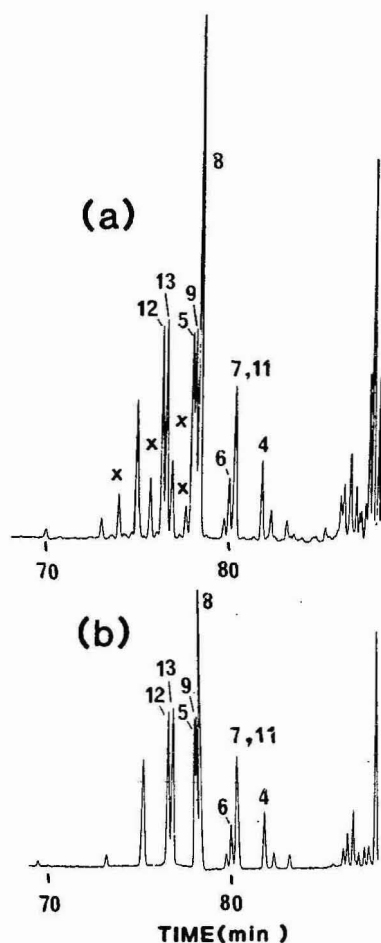
Fig. 2. Capillary GC traces of the alkylnaphthalene fraction of Wyoming Pennsylvania crude oil before (a) and after (b) peracid treatment. Peaks labelled X represent isomeric dimethylbenzothiophenes; for other peak assignments refer to Table I and Fig. 1.

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GAS CHROMATOGRAPHIC TECHNIQUE FOR ANALYSING PARTIALLY DEGRADED DIETHANOLAMINE SOLUTIONS

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SUMMARY

A simple and reliable gas chromatographic technique is presented for the quantitative analysis of partially degraded aqueous diethanolamine solutions. The samples are injected, without preparation, into a chromatograph equipped with a 6 ft. × 1/8 in. O.D. stainless-steel column (packed with 60–80-mesh Tenax GC) and a flame-ionization detector. Nitrogen is used as the carrier gas and temperature programming is necessary. Apart from mono-, di- and triethanolamine, eleven degradation compounds could be detected and measured at concentrations as low as 0.5 wt.-% with accuracies of typically ±5%. The degradation compounds are identified and retention times are given.

INTRODUCTION

Aqueous diethanolamine (DEA) solutions are widely used in the gas processing industry for removing carbon dioxide and hydrogen sulphide from light hydrocarbons[1-3]. The process is based on the reaction of a weak base (DEA) with acid gases (hydrogen sulphide or carbon dioxide) to give water-soluble salts. The overall reactions may be summarized as follows:

$$(HOC_2H_4)_2NH + H_2S \rightleftharpoons (HOC_2H_4)_2NH_2{}^+ \; HS^- \qquad (1)$$

$$(HOC_2H_4)_2NH + CO_2 + H_2O \rightleftharpoons (HOC_2H_4)NH_2{}^+ \; HCO_3{}^- \qquad (2)$$
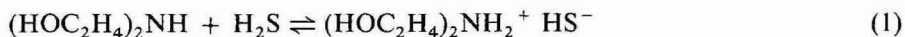
These reactions are reversible and the DEA is regenerated for repeated use. Unfortunately, certain side reactions may also occur which result in irreversible degradation of DEA into undesirable compounds. Degradation is undesirable because it leads to a loss of valuable DEA, fouling of the equipment with degradation products, reduction in the process efficiency and throughput and, possibly, corrosion. The direct and indirect costs resulting from degradation of DEA are, therefore, considerable to the oil and gas industry.

Owing to the industrial significance of DEA, a quantitative study of the degradation of DEA was initiated. Such a study was dependent on the availability of an

efficient analytical procedure for detecting and measuring the concentrations of degradation compounds. Unfortunately, reliable and simple techniques had not been reported for the detection of DEA and its degradation products in aqueous solutions. The analysis is difficult to perform because DEA and its degradation products have fairly low vapour pressures, decompose at elevated temperatures, are highly polar and usually occur in dilute aqueous solutions.

Many analytical methods have been suggested[4] for DEA and its degradation compounds, e.g., wet chemical techniques, infrared and ultraviolet spectroscopy and paper and thin-layer chromatography. However, they all suffer from various disadvantages such as lack of accuracy, specificity, reliability and simplicity. Therefore, in this study we concentrated on the development of gas chromatographic methods.

Brydia and Persinger[5] described a chromatographic technique for the analysis of ethanolamines. As direct gas chromatographic methods led to excessive peak tailing, they investigated derivatization with trifluoroacetic anhydride (TFA) prior to chromatographic separation. However, TFA also reacts with water and the resulting trifluoroacetic acid causes severe tailing in the chromatograms. Piekos et al.[6] eliminated these problems by converting the alkanolamines into trimethylsilyl derivatives by using N,O-bis(trimethylsilyl)acetamide, which reacts with both the amino and hydroxyl groups of the alkanolamines. This silylation process yielded fairly stable compounds which are more easily separated and identified by gas chromatography. Water concentrations of up to 5 % could be tolerated provided that the silylating agent was used in excess.

As the water content of industrial amine solutions typically range from 65 to 90 %, the method of Piekos et al.[6] is not directly applicable. Choy and Meisen[7] therefore modified the technique by stripping water from the degraded DEA solutions with air. The removal of water usually resulted in the precipitation of some of the degradation products. The dried sample was therefore dissolved in dimethylformamide and the resulting mixture silylated with N,O-bis(trimethylsilyl)acetamide. The silylated compounds were then separated using a 6 ft. × 1/8 in. O.D. stainless-steel column packed with 8 % OV-17 on 80–100-mesh Chromosorb. A flame-ionization detector was used.

The method developed by Choy and Meisen[7], although reliable, was felt to be too time consuming for wide industrial application because of the extensive sample preparation. Further, there is the problem of incomplete silylation of some compounds; silylation of hydrogen bound to nitrogen atoms in alkanolamines is known to be difficult[8].

An attempt was therefore made to find a simple and more direct technique for analysing DEA and its degradation products. A thorough review of the literature yielded a paper by Saha et al.[9], who used Tenax GC to separate alkanolamines. Tenax GC is a porous polymer based on 2,6-diphenyl-p-phenylene oxide[10], which has a weakly interacting surface and can be used at temperatures up to 450°C. No sample preparation is required as the column is unaffected by the presence of water.

EXPERIMENTAL

A temperature-programmable gas chromatograph (Hewlett-Packard Model 5830A) equipped with a 6 ft. × 1/8 in. O.D. stainless-steel column packed with Tenax

TABLE I

ANALYTICAL EQUIPMENT AND OPERATING CONDITIONS FOR GAS CHROMATO-
GRAPHIC ANALYSIS

Gas chromatograph:	
Manufacturer	Hewlett-Packard
Model	5830A
Detector	H_2 flame-ionization
Chromatographic column:	
Material	Stainless steel
Dimensions	6 ft. × $\frac{1}{8}$ in. O.D.
Packing	Tenax GC, 60–80 mesh
Operating conditions:	
Carrier gas	N_2 at 25 ml/min
Injection port temperature	300°C
Detector port temperature	300°C
Column temperature	Isothermal at 150°C for 0.5 min, then increased at 8°C/min to 300°C
Syringe:	
Manufacturer	Hamilton
Model	701 (10 μl) with fixed needle and Chaney adapter
Injected sample size	1 μl

GC (purchased from Alltech, Arlington Heights, IL, U.S.A.) was used. Initial trials
were performed with aqueous solutions of monoethanolamine (MEA), diethanol-
amine (DEA) and triethanolamine (TEA). Using a hydrogen flame-ionization detec-
tor, nitrogen as the carrier gas and temperature programming, excellent separation
was obtained. One reference[11] indicated that stainless steel causes ethanolamines to
undergo catalytic degradation. No evidence of degradation within the column how-
ever, was, observed by us for any of the compounds tested.

After several initial trials, optimum conditions were found for the separation of
DEA and its degradation compounds; the conditions are summarized in Table I.
Temperature programming was used in order to achieve good separation of all deg-
radation products, as these compounds varied considerably in molecular weight and
polarity. A maximum temperature of 300°C was adopted to ensure that all com-
pounds were volatilized.

Typically, 1.0-μl samples were injected directly into the column with a precision
syringe fitted with a Chaney adaptor. The adaptor was used to ensure that a constant
volume of sample was introduced into the column. To improve the accuracy, a needle
guide was used at the injection port. This guide not only protected the fragile syringe
needle, but also served as a spacer for needle penetration and lengthened the septum
life.

The analysis was usually performed for 30 min in order to ensure the elution of
heavy degradation compounds. After each run, the column had to be cooled, from
300 to 150°C, which took about 5 min. A complete run therefore required about 35
min.
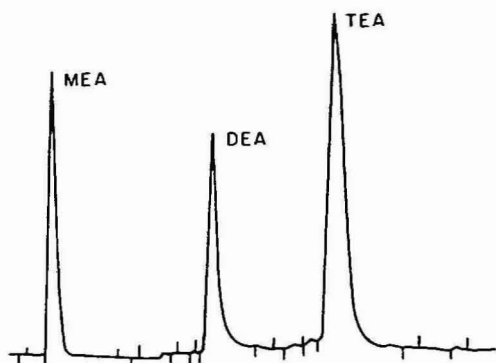
Fig. 1. Typical chromatogram for an aqueous solution of MEA, DEA and TEA.

Column performance

The column itself required no special care and was conditioned simply by passing nitrogen through it at the maximum operating temperature (300°C) for 8–10 h. The columns have a fairly long life; for example, one column was in continual use for nearly a year. However, when a column fails, it fails rapidly and becomes incapable of separating the heavy compounds.

Maintenance of the chromatographic equipment

Generally very little maintenance is required. In some instances deposits tend to build up in the injection port and must be removed. Further, deposits may accumulate on the detector jets and result in excessive noise on the chromatograms. Mechanical cleaning of the flame-ionization detector is difficult and the removal of the probes is not recommended unless absolutely necessary. A simpler method is to inject 10–30 μl of Freon 113 into the chromatograph with the equipment operating under normal conditions. Freon elutes from the column and produces hydrogen fluoride as the cleaning agent when burnt in the hydrogen flame.

As the column performance may vary somewhat, it is good practice to check the calibration after every 100 injections with standard samples. Septa should also be replaced at least every 30 injections as they tend to accumulate deposits and eventually begin to leak.
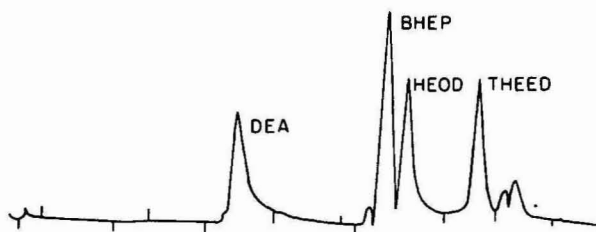
Fig. 2. Chromatogram of an aqueous DEA solution partially degraded with carbon dioxide under laboratory conditions.
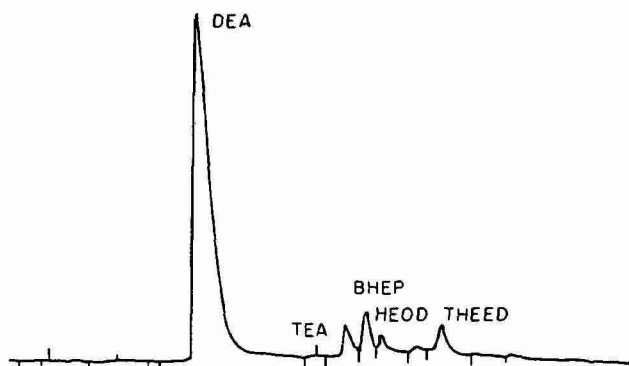
Fig. 3. Chromatogram of a partially degraded DEA solution from a gas plant in Western Canada.

RESULTS

Fig. 1 shows a typical chromatogram of a sample produced by mixing distilled water, MEA, DEA and TEA. The peaks are sharp and distinct.

From the literature it was apparent that the main degradation compounds of DEA are 3-(hydroxyethyl)-2-oxazolidone (HEOD), N,N,N-tris(hydroxyethyl)ethylenediamine (THEED) and N,N-bis(hydroxyethyl)piperazine (BHEP). Fig. 2 shows a typical chromatogram of a DEA solution degraded in the presence of carbon dioxide. As can be seen, there is good separation between DEA, HEOD, BHEP and THEED. Each compound gives a fairly sharp peak with little tailing. The smaller peaks are due to minor degradation products. Better separation especially between HEOD and BHEP, may be achieved by using a 9-ft. column. However, the longer column inevitably increases the elution times.
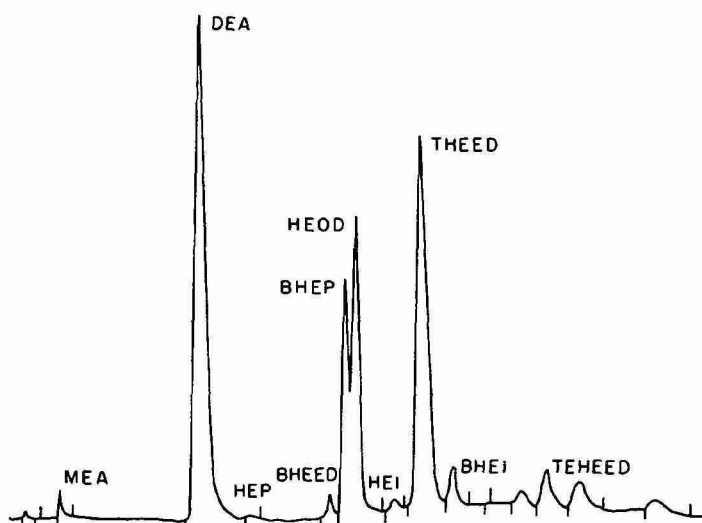
Fig. 4. Chromatogram of an aqueous DEA solution severely degraded with carbon dioxide under laboratory conditions.

TABLE II

COMPOUNDS FOUND IN DEGRADED DEA SOLUTIONS
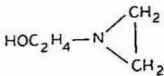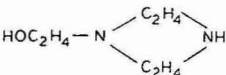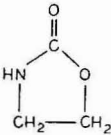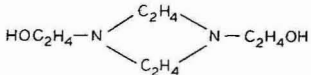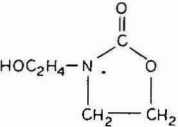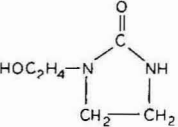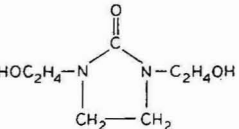
Compound	Structural formula	Typical retention time (min)
Monoethanolamine (MEA)	$HOC_2H_4NH_2$	1.4–1.5
N-(hydroxyethyl)-ethylenimine (HEM)	$HOC_2H_4-N\begin{smallmatrix}CH_2\\ \| \\ CH_2\end{smallmatrix}$	3.1–3.5
N-(hydroxyethyl)-ethylenediamine (HEED)	$HOC_2H_4NHC_2H_4NH_2$	6.8–7.2
Diethanolamine (DEA)	$(HOC_2H_4)_2NH$	7.4–8.0
N-(Hydroxyethyl)-piperazine (HEP)	$HOC_2H_4-N\overset{C_2H_4}{\underset{C_2H_4}{\diagup\diagdown}}NH$	9.8–10.2
Oxazalidone (OZD)	(ring: $HN-C(=O)-O-CH_2-CH_2$)	10.4–10.6
Triethanolamine	$(HOC_2H_4)_3N$	12.0–12.5
N,N-Bis(hydroxyethyl)-ethylenediamine (BHEEP)	$(HOC_2H_4)_2NC_2H_4NH_2$	12.8–13.2
N,N-Bis(hydroxyethyl)-piperazine (BHEP)	$HOC_2H_4-N\overset{C_2H_4}{\underset{C_2H_4}{\diagup\diagdown}}N-C_2H_4OH$	13.0–13.6
3-(Hydroxyethyl)-2-oxazalidone (HEOD)	(ring: $HOC_2H_4-N-C(=O)-O-CH_2-CH_2$)	13.4–14.0
N-(Hydroxyethyl)-imidazalidone (HEI)	(ring: $HOC_2H_4-N-C(=O)-NH-CH_2-CH_2$)	15.5–16.0
N,N,N-Tris(hydroxyethyl)-ethylenediamine (THEED)	$(HOC_2H_4)_2NC_2H_4NHC_2H_4OH$	17.2–17.4
N,N-Bis(hydroxyethyl)-imidazalidone (BHEI)	(ring: $HOC_2H_4-N-C(=O)-N-C_2H_4OH$, CH_2-CH_2)	18.2–18.5
N,N,N,N-Tetra(hydroxyethyl)-ethylenediamine	$(HOC_2H_4)_2NC_2H_4N(C_2H_4OH)_2$	20.4–20.6

Fig. 3 shows a typical chromatogram of a partially degraded DEA sample from an industrial source. Peaks of HEOD, BHEP and THEED are clearly evident. A peak caused by TEA is also noted. TEA is frequently present as an impurity in industrial DEA solutions. These result show that industrial samples can easily be analysed by this method.

Calibration

The peak areas were found to be linear functions of the concentration of each component, which greatly simplified the calibration of the chromatograph. "Direct calibration" rather than an internal standard was used. Calibration plots of concentration *versus* peak area were produced by simply injecting known concentrations of the various amines or degradation products and noting the peak area, which was automatically calculated by the chromatograph's computer. At least five samples were injected for each concentration and the peak area averaged.

Using this method, DEA and its known degradation products could be detected accurately at concentrations as low as 0.5 wt.-%. The reproducibility was good (typically within ± 5.0% with a new column). An internal standard could be used to reduce errors arising from changes in sample injection, carrier gas flow-rate and detector sensitivity.

Identification of degradation products

As this study was undertaken to examine the degradation of DEA, it was necessary to identify the compounds causing the various peaks on the chromatogram. Some degraded DEA solutions contained up to eighteen compounds in significant concentrations (> 1 wt.-%). Using a gas chromatograph coupled to a mass spectrometer (Hewlett-Packard Model 5985B) it was possible to identify up to fourteen peaks[12] (a typical example is shown in Fig. 4). Table II lists the compounds and their retention times (based on the conditions given in Table I). Some other compounds were also detected but their concentration was very low (< 1 w.t.-%) so that their identification was neither warranted nor easily accomplished. It may be noted that this is the first time that many of these compounds have been unequivocally identified in degraded DEA solutions.

CONCLUSIONS

A gas chromatographic technique has been developed that is capable of detecting DEA and its degradation products quantitatively. It can therefore be used to monitor the quality of DEA solutions under industrial conditions. Eleven major degradation compounds have been indentified.

The advantages of the present technique are no sample preparation, the column is unaffected by water, simplicity, speed and reliability.

ACKNOWLEDGEMENT

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RAPID SCREENING METHOD FOR METHAMPHETAMINE IN URINE BY COLOUR REACTION IN A SEP-PAK C_{18} CARTRIDGE

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SUMMARY

A simple screening method for methamphetamine in urine by colour reaction was developed. Methamphetamine, which is quantitatively retained in a Sep-Pak C_{18} cartridge, is (after a clean-up procedure) coloured by Simon's reagent (consisting of sodium nitroprusside solution, sodium carbonate solution and acetaldehyde gas). The detection limit was 0.5 μg/ml using 5 ml of urine sample. The results of the screening method agreed with those of thin-layer chromatography and gas chromatography–mass spectrometry.

INTRODUCTION

Abuse of methamphetamine is spreading throughout the world and is a serious social problem, especially in Japan. Therefore, a rapid and specific screening procedure for methamphetamine in urine is required.

Until recently, the most common methods for the detection of methamphetamine, which is the major compound excreted in human urine after administration of methamphetamine[1,2], have been based on thin-layer (TLC) and gas chromatography (GC). These methods require a large volume of organic solvent for the extraction of methamphetamine and metabolites, and the procedures take many hours. With the development of immunoassay screening methods for the drugs of abuse, radioimmunoassay[3–6], enzyme multiple immunoassay[7–10] and the haemagglutination inhibition test[11] for methamphetamine have been investigated. Although these procedures have high sensitivity and specificity, they require special instruments, antisera and sensitized blood cells.

We have attempted to develop a more simple screening method for methamphetamine in urine by a colour reaction using a Sep-Pak C_{18} cartridge. In this procedure methamphetamine, retained selectively on the resin, is coloured with Simon's reagent[12], consisting of sodium carbonate, sodium nitroprusside and acetaldehyde.

EXPERIMENTAL

Materials

Methamphetamine hydrochloride and amphetamine sulphate were purchased from Dainippon Pharmaceutical (Osaka, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. *p*-Hydroxymethamphetamine hydrochloride was synthesized essentially by the method of Buzas and Dufour[13], and the purity of the product (m.p. 142–143°C) was checked by TLC, GC, mass spectrometry (MS) and IR spectrometry. Trifluoroacetic anhydride was purchased from Wako (Osaka, Japan) and the other chemicals used were of analytical-reagent grade. The Sep-Pak C_{18} cartidge was obtained from Waters Assoc. (Milford, MA, U.S.A.).

Screening test for methamphetamine in urine

After connecting a 10-ml glass syringe to the Sep-Pak C_{18} cartridge, the resin in the cartridge was activated by passing 5 ml of methanol and 10 ml of water through the glass syringe. Unless indicated otherwise, urine samples, aqueous solutions, water and methanol were passed through the cartridge at a flow-rate of 5 ml/min.

A urine sample (5 ml) was adjusted to pH 9.0 with sodium carbonate–sodium hydrogen carbonate buffer and poured into an activated Sep-Pak C_{18} cartridge; subsequently 2 ml of 20% (w/v) sodium carbonate solution and 5 ml of water–methanol (1:1, v/v) were poured into the cartridge to eliminate selectively coloured components of urine. After an additional 1 ml of water, 0.2 ml of the colour-producing reagent solution, consisting of 1.2% (w/v) sodium nitroprusside solution–20% (w/v) sodium carbonate solution (5:1, v/v) was poured in, then 20 ml of acetaldehyde gas, which was obtained with a syringe from the headspace of a 5-ml vial containing 1 ml of acetaldehyde, was introduced into the cartridge. Finally, 1 ml of water was poured into the cartridge at a flow-rate 0.5 ml/min and the colour developed was observed.

Extraction of methamphetamine and metabolites in urine

A 5-ml urine sample was adjusted to pH 9.0 with sodium carbonate and extracted four times with 10 ml of chloroform–isopropanol (3:1, v/v). The combined organic extracts were dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo* after adding a drop of acetic acid to prevent evaporation of amines. The residue obtained was subjected to TLC, GC and GC–MS.

Analytical methods

TLC was carried out on 0.25 mm thick silica gel GF_{254} plates (E. Merck, Darmstadt, G.F.R.); the solvent systems used for development were (I) isopropanol–28% aqueous ammonia (95:5, v/v) and (II) methyl ethyl ketone–dimethylformamide–28% aqueous ammonia (13:1.9:0.1, v/v/v). After development, the plates were examined under UV light (254 nm) and sprayed with either (A) 1% (w/v) iodine–methanol solution or (B) 20% (w/v) sodium carbonate solution and 1% (w/v) sodium nitroprusside solution as detection reagent, and the plate was exposed to acetaldehyde gas for 1 min.

For GC, a Shimadzu GC-4CM gas chromatograph equipped with a flame-ionization detector was used. The glass column (1 m × 3 mm I.D.) was packed with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh). The carrier gas was

nitrogen (50 ml/min). The column, injection and detector temperatures were 125, 160 and 160°C, respectively. The extract obtained from urine was dissolved in 200 μl of ethyl acetate and 200 μl of trifluoroacetic anhydride were added. The vessel containing the mixture was sealed tightly and heated at 55°C for 20 min. The solvent was evaporated *in vacuo,* the residue obtained was dissolved in 100 μl of ethyl acetate and 1 μl was injected into the gas chromatograph. For the determination of methamphetamine, amphetamine and *p*-hydroxymethamphetamine, 0.25 μg/μl of fluorene in ethyl acetate was used as an internal standard.

GC–MS was carried out with a Hitachi Model M-80 double focusing mass spectrometer by the chemical ionization method using isobutane as the reactant gas. A 1 m × 3 mm I.D. glass column packed with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh) was used. The column, injector and separator temperatures were 125, 165 and 180°C, respectively. The flow-rate of the helium carrier gas was 50 ml/min. The ionization voltage was 100 eV and the ionization current was 110 μA.

RESULTS AND DISCUSSION

Effect of pH of urine on retention of methamphetamine and metabolites in a Sep-Pak C_{18} cartridge

To 5 ml of control urine obtained from a healthy man, 50 μg of methamphetamine hydrochloride, amphetamine sulphate or *p*-hydroxymethamphetamine hydrochloride were added and the pH was adjusted to 3, 5, 7, 9 or 11 by adding 4.5 ml of hydrochloric acid–sodium acetate buffer (pH 3.0), acetic acid–sodium acetate buffer (pH 5.0), disodium hydrogen phosphate–potassium dihydrogen phosphate buffer (pH 7.0) or sodium carbonate–sodium hydrogen carbonate buffer (pH 9.0 and 11.0), respectively. The treated urine sample was poured into the activated Sep-Pak C_{18} cartridge. The effluent solution from the cartridge was adjusted to pH 9 and extracted four times with chloroform–isopropanol (3:1, v/v). The combined organic extracts were treated to determine methamphetamine, amphetamine and *p*-hydroxymethamphetamine. Table I shows that methamphetamine and amphetamine are completely retained in the cartridge in the pH range 5–11. On the basis of the results urine samples were adjusted to pH 9 in subsequent experiments.

TABLE I

EFFECT OF pH OF URINE ON RETENTION OF METHAMPHETAMINE AND METABOLITES IN A SEP-PAK C_{18} CARTRIDGE

50 μg of methamphetamine hydrochloride, amphetamine sulphate or *p*-hydroxymethamphetamine hydrochloride were added to 5 ml of control urine. Results are averages ± standard deviation ($n = 5$).

Compound	Compound not retained (%)				
	pH 3	*pH 5*	*pH 7*	*pH 9*	*pH 11*
Methamphetamine	0.5 ± 0.6	—*	—	—	—
Amphetamine	8.0 ± 1.0	—	—	—	—
p-Hydroxymethamphetamine	33.4 ± 3.6	10.3 ± 1.0	3.2 ± 1.0	—	—

  * —, Not detected.

*Effect of clean-up procedure on retention of methamphetamine and metabolites in a
Sep-Pak C$_{18}$ cartridge*

To 5 ml of control urine, 0.5–500 μg of methamphetamine hydrochloride, am-
phetamine sulphate and *p*-hydroxymethamphetamine hydrochloride were added. The
treated urine sample was adjusted to pH 9, poured into the activated cartridge and
the effluent solution from the cartridge was collected (effluent 1). Coloured compo-
nents, which were contained in urine and retained in the cartridge, were eliminated
by pouring in 2 ml of 20% (w/v) sodium carbonate solution, 5 ml of water–methanol
(1:1, v/v) and 1 ml of water. Each effluent solution (effluents 2, 3 and 4) was com-
bined together with effluent 1. A few drops of acetic acid were added, methanol was
evaporated and the aqueous solution remaining was adjusted to pH 9 and treated to
determine methamphetamine, amphetamine and *p*-hydroxymethamphetamine by
GC. The results showed that methamphetamine was sufficiently retained in the car-
tridge after the clean-up procedure but amphetamine and *p*-hydroxymethamphetam-
ine were eluted with increasing amounts of the compounds added to urine (Table II).

When 5 ml of urine containing 500 μg of methamphetamine hydrochloride, 50
μg of amphetamine sulphate and *p*-hydroxymethamphetamine hydrochloride were
applied to the cartridge and the clean-up procedure was carried out, the compounds
eluted in effluents 1, 2, 3 and 4 were determined. The results showed that 7% of the
methamphetamine added, 60% of the amphetamine added and 98% of the *p*-hy-
droxymethamphetamine added were eluted in effluent 3. It was concluded that this
clean-up procedure is suitable for the detection of methamphetamine by the colour
reaction.

Coloration in Sep-Pak C$_{18}$ cartridge

Urine samples were prepared by adding various amounts of methamphetamine
hydrochloride to control urine, and 5 ml of the treated urine sample were poured
into the Sep-Pak C$_{18}$ cartridge and treated as described above. For coloration, 0.2
ml of 1.2% (w/v) sodium nitroprusside solution–20% (w/v) sodium carbonate solu-
tion (5:1, v/v) was poured into the cartridge and acetaldehyde gas was introduced.
When 5 μg/ml of methamphetamine was present in urine sample, a blue colour was

TABLE II

EFFECT OF CLEAN-UP PROCEDURE ON RETENTION OF METHAMPHETAMINE AND METAB-
OLITES IN A SEP-PAK C$_{18}$ CARTRIDGE

Results are averages ± standard deviation (*n* = 5).

Compound added to 5 ml of control urine (μg)			Compound eluted in effluents 1+2+3+4 (%)		
Methamphetamine hydrochloride	*Amphetamine sulphate*	*p-Hydroxymeth-amphetamine hydrochloride*	*Methamphetamine*	*Amphetamine*	*p-Hydroxymeth-amphetamine*
5	0.5	0.5	—*	—	27.6 ± 3.1
50	5	5	2.6 ± 1.0	48.2 ± 9.2	90.2 ± 6.1
500	50	50	12.6 ± 1.0	56.9 ± 0.9	99.6 ± 0.7

* — = Not detected.

Sep-Pak C$_{18}$ cartridge

Activation ——⌈ 1 | methanol
            ⌊ 2 | water

Sampling ——— 3 | urine

Clean-up ———⌈ 4 | 20% Na$_2$CO$_3$ solution
            | 5 | methanol—water (1:1, v/v)
            ⌊ 6 | water

Colouration ——⌈ 7 | 1.2% sodium nitroprusside solution—
             | | 20% Na$_2$CO$_3$ solution (5:1, v/v)
             | 8 | acetaldehyde gas
             ⌊ 9 | water
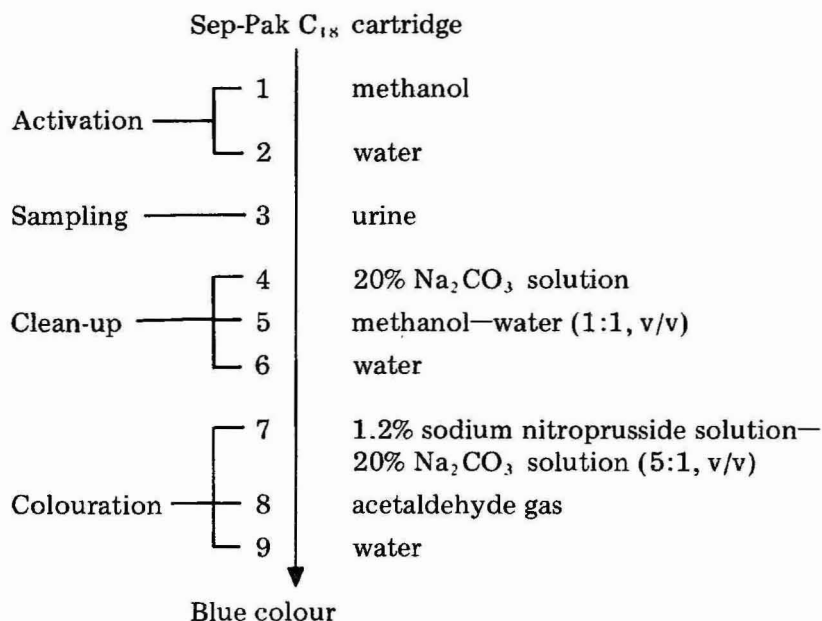
Blue colour

Fig. 1. Screening test for methamphetamine in urine.

clearly observed in the upper part of the resin in the cartridge, and with a control urine a reddish colour was observed in the upper part of the resin. The blue colour developed by less than 2 μg/ml of methamphetamine in urine sample was rendered indistinct by the underlying reddish colour. However, the reddish colour could be selectively eluted by pouring 1 ml of water into the cartridge at a flow-rate of 0.5 ml/min after introducing acetaldehyde gas, and the blue colour developed by methamphetamine could then be clearly observed. The screening test is summarized in Fig. 1. The detection limit of this method is 0.5 μg/ml of methamphetamine in urine when 5 ml of urine is used as the sample.

Screening test for suspect urine

Fifteen urine samples obtained from methamphetamine abuse suspects were examined by the screening method and subjected to TLC, GC and GC–MS, and the amounts of methamphetamine in the same samples were determined by GC. The results of the screening test agreed with those of TLC, GC and GC–MS, as shown in Table III.

Although urine sample 4 in Table III gave a positive coloration with this screening test, methamphetamine was not detected by TLC and GC–MS. It has been found that Simon's reaction is applicable to the detection of secondary aliphatic amines[12] and that methoxyphenamine in drugs possessing a secondary aliphatic amino group gives a positive coloration with similar sensitivity to that of methamphetamine[14,15]. Further, it should be noted that some metabolites, which are produced *in vivo* by N-dealkylation of drugs having a tertiary amino group and excreted in urine, give a positive coloration with Simon's reagent, for example, 1-

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TABLE III

COMPARISON OF RESULTS OBTAINED BY THE SCREENING TEST WITH THOSE OB-
TAINED BY TLC, GC AND GC-MS

− = Negative; + = positive.

No.	Screening test	TLC	GC-MS	GC: methamphetamine in urine ($\mu g/ml$)
1	−	−	−	0
2	−	−	−	0
3	−	−	−	0
4	+	−	−	0
5	+	+	+	0.1
6	+	+	+	0.2
7	+	+	+	2.5
8	+	+	+	3.3
9	+	+	+	25.0
10	+	+	+	29.5
11	+	+	+	31.7
12	+	+	+	32.6
13	+	+	+	72.8
14	+	+	+	115.4
15	+	+	+	130.2

phenyl-2-(N-benzylamino)propane (benzylamphetamine), an N-demethylated
metabolite of 1-phenyl-2-(N-methyl-N-benzylamino)propane (benzphetamine),
showed positive coloration[16,17]. Positive coloration of sample 4 is considered to be
derived from such compounds.

In order to identify methamphetamine in urine, therefore, urine samples that
give a positive coloration by this screening method must be confirmed by GC-MS.

ACKNOWLEDGEMENT

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Note

Preparation of efficient packed columns with a polyperfluoroalkyl ether stationary phase

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Fluorocarbon stationary phases (*e.g.*, perfluoroalkanes, Kel-F oils, fluoroalkyl esters) have been used for the gas chromatographic (GC) separation of compounds with high reactivity (*e.g.*, UF_6, ClO_2F, SF_5Cl, etc.)[1–6]. For the above applications the fluorocarbon stationary phases were selected because of their chemical inertness. Columns prepared with these fluorocarbon phases on PTFE or diatomaceous supports invariably exhibited poor column efficiencies and generally restricted temperature operating ranges compared to silicone and polyester phases introduced at about the same time. Typical column efficiencies were stated to be in the range of 30–225 theoretical plates per meter[1–6], a value which would be considered too low for general use. More recently, Chromatographic Specialties Ltd. have introduced column packings coated with Fluorad FC-431 which provides moderately efficient columns suitable for separating alcohols, phenols and amines at temperatures up to 200°C[7]. Fluorad FC-431 is a fluoroalkyl ester produced by the 3M Company (St. Paul, MN, U.S.A.). Its exact composition is proprietory information. The perceived and real difficulties anticipated in the preparation of efficient and thermally stable perfluorocarbon stationary phase packings have diverted attention away from their potential usefulness as general phases in gas chromatography.

Chemical inertness is only one useful property of fluorocarbon compounds. Intermolecular forces in fluorocarbon compounds are very weak resulting in lower-than-expected boiling points[8] and low retention on non-polar and polar organic stationary phases. For many years chromatographers have taken advantage of this property by preparing perfluorinated derivatives for the analysis of polar compounds of low volatility[9,10]. The perfluorinated derivatives invariably being more volatile than the parent compounds of similar hydrocarbon-containing derivatives. It should, therefore, be possible to chromatograph organic compounds on perfluorocarbon stationary phases at lower temperatures than is currently possible with conventional stationary phases. In such circumstances, it might be feasible to chromatograph higher-molecular-weight organic compounds than is presently possible or to chromatograph thermally labile compounds at a sufficiently low temperature that they are stable. To test the above hypothesis a thermally stable fluorocarbon stationary phase having reasonable selectivity for organic compounds and acceptable coating efficiency is required.

As a candidate stationary phase the polyperfluoroalkyl ether, Fomblin YR, the structure of which is

$$
\begin{array}{c}
CF_3 \\
| \\
(O\text{-}CF\text{-}CF_2)_n\text{-}(O\text{-}CF_2)_m
\end{array}
$$
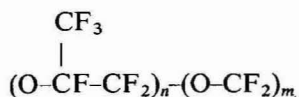
was selected. It has an average molecular weight of 6000–7000, is a liquid with a pour point of $\approx -20°C$ and is thermally stable to temperatures in excess of 300°C. The polyperfluoroalkyl ethers are commercially available, synthetic, inert, functional fluids used as engine lubricants and vacuum pump oils[11-13]. Fomblin-L, a lower-molecular-weight homologue of Fomblin YR, has been used as a reference standard for calibrating mass spectrometers in the high mass range[14].

EXPERIMENTAL

Test compounds were available in kit form (Thetakits, Anspec, Ann Arbor, MI, U.S.A.). Perfluorokerosene-L, b.p. $\approx 90°C$ was obtained from PCR Research Chemicals (Gainesville, FL, U.S.A.), and WD-40 oil from WD-40 Company (San Diego, CA, U.S.A.). Trichlorofluoromethane (Freon 11) was obtained from Matheson Gas Products (East Rutherford, NJ, U.S.A.) and 1,1,2-trichlorofluoroethane (Freon 113) from Aldrich (Milwaukee, WI, U.S.A.). Fomblin YR was obtained from Montedison (New York, NY, U.S.A.).

Column packings were prepared by the rotary evaporator coating technique using Freon 113 as solvent. The air-dried packings were sieved before use. Glass columns of various lengths were packed by vacuum suction and gentle vibration. On-column silylation was performed before use by injecting slowly 100 μl of silyl-8 or bis(trimethylsilyl)trifluoroacetamide (BSTFA) into the column at 150°C with a nitrogen carrier gas flow-rate of 30 ml min^{-1}.

For GC a Varian 3700 gas chromatograph with an on-column heated injector and flame ionization detector was used. Separation conditions are given in the figure legends.

RESULTS AND DISCUSSION

Fluorocarbon stationary phases have not been widely used in GC due to their poor coating characteristics and low maximum operating temperatures. Fomblin YR is a chemically and thermally stable functional fluid with a wide liquid range; properties desired in a stationary phase for GC. The presence of ether oxygens in Fomblin YR should also enhance partitioning and/or adsorption of organic compounds as well as providing an anchor site to enable efficient columns to be prepared on diatomaceous supports.

In contrast to the difficulties reported by other workers for the preparation of efficient packings with perfluorocarbon stationary phases[1-6], efficient columns are relatively simple to prepare with Fomblin YR. With a phase loading of 3–15% (w/w) of Fomblin YR on Chromosorb P (100–120 mesh), columns yielding 2000–2500 theoretical plates per meter have been prepared routinely using the rotary evaporator
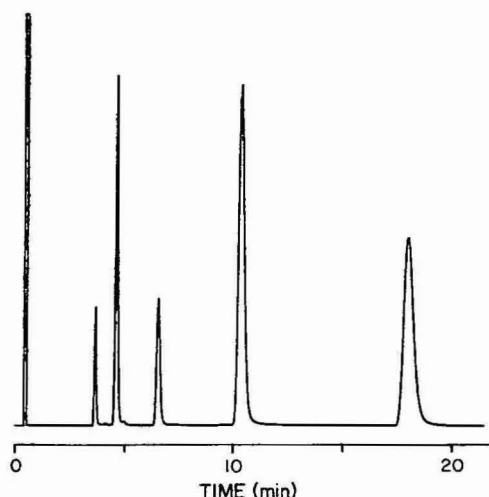
Fig. 1. Separation of C_5 through C_9 n-alkanes on a 3 m × 2 mm I.D. glass column packed with 10% (w/w) Fomblin YR on Chromosorb P (100–120 mesh), temperature 45°C, nitrogen carrier gas flow 20 ml min⁻¹.

coating technique. Fig. 1 shows a test chromatogram of one such column. Fomblin YR is almost insoluble in most common organic solvents excepts for Freon 113, which is the solvent recommended for column preparation. Packings prepared by stirring in an open dish using an infrared lamp for solvent evaporation invariably produced columns of lower efficiency. Typical values being 800–1500 theoretical plates per meter. Columns which were not silylated after preparation always showed lower efficiency, peak asymmetry and poor column stability at high temperatures. Silylation seems to be necessary to remove active sites and also to provide a stable liquid film at elevated temperatures. However, persilylation prior to coating may be an undesirable feature. All attempts to prepare packings with Gas-Chrom Q resulted in columns of low efficiency having 200–300 theoretical plates per meter. Columns tested at 45°C gave symmetrical peaks for the hydrocarbon test mixture (Fig. 1), but

TABLE I

COMPARISON OF THE ADJUSTED RETENTION TIME OF n-HYDROCARBONS ON FOMBLIN YR AND SQUALANE

All measurements were made with a 3 m × 2 mm I.D. column packed with 10% (w/w) stationary phase on Chromosorb P (100–120 mesh), temperature 45°C and carrier gas nitrogen flow of 30 ml min⁻¹.

Hydrocarbon	Adjusted retention time (min)		Ratio squalane to Fomblin YR
	Fomblin YR	Squalane	
Pentane	0.3	3.9	13
Hexane	0.7	10.9	16
Heptane	1.4	29.8	21
Octane	3.3	85.1	26
Nonane	6.2	223.0	36
Decane	11.2		
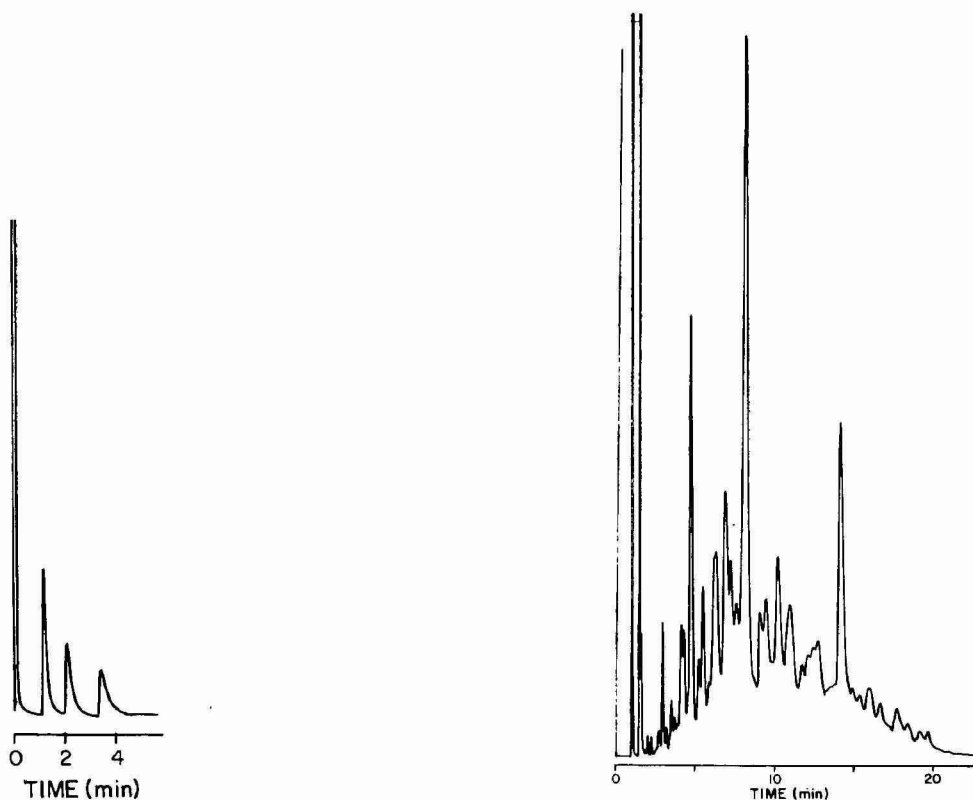
Fig. 2. Separation of C_{36}, C_{38} and C_{40} *n*-alkanes on a 1 m × 2 mm I.D. glass column packed with 10% (w/w) Fomblin YR on Chromosorb P (100–120 mesh), temperature 255°C, nitrogen carrier gas flow 20 ml min^{-1}.

Fig. 3. Separation of WD-40 oil (a petroleum product used for lubrication, rust proofing and to displace moisture from electrical systems). The column is the same as in Fig. 1. Temperature programmed from 35°C to 100°C at 5°C min^{-1}, and nitrogen carrier gas flow 30 mol min^{-1}.

after heating briefly to above 100°C, and returning to 45°C for re-evaluation, asymmetric peaks with diminished retention were obtained. This suggests that the stationary phase film is not stable on Gas-Chrom Q and redistributes itself on the packing surface unevenly at higher temperatures. A similar phenomenon occurred with the packings prepared with Chromosorb P and on-column silylated prior to use, but only at much higher temperatures. Packings containing 3% (w/w) Fomblin YR can safely be used up to 200°C and columns with a 10% phase loading up to 255°C. Both temperatures are below the maximum allowable operating temperature of the phase which was established as 275°C. Thus, for Fomblin YR the upper operating temperature for the column is set by the stability of the liquid phase film on the support and not by column bleed.

    The upper operating temperature of the Fomblin YR columns prepared with Chromosorb P is sufficient to allow a wide range of organic compounds to be separated, the more so, as the absolute retention of organic compounds on Fomblin YR

is a great deal less than is found for conventional liquid phases. For example, the adjusted retention time of a series of *n*-alkanes on Fomblin YR is more than an order of magnitude lower than observed on an identical squalane column, Table I. This facilitates the elution of high-molecular-weight hydrocarbons at low column operating temperatures. Fig. 2 illustrates the rapid separation of C_{36}, C_{38} and C_{40} hydrocarbons at a column temperature of 255°C. Some tailing can be observed in this chromatogram, perhaps because the upper temperature limit of the liquid phase film stability had been reached, or because of low solubility in the stationary phase (assuming a partition mechanism) of the hydrocarbons, or because the column temperature was too low to adequately vaporize the sample. A more representative application of the stationary phase is shown in Fig. 3 for the separation of a sample of petroleum oil distillate. Fig. 4 is a temperature programmed separation of a mixture of C_6–C_{15} *n*-alkanes and 1-alkenes. Not all members of the series are separated and the separation obtained cannot simply be accounted for by boiling point difference.
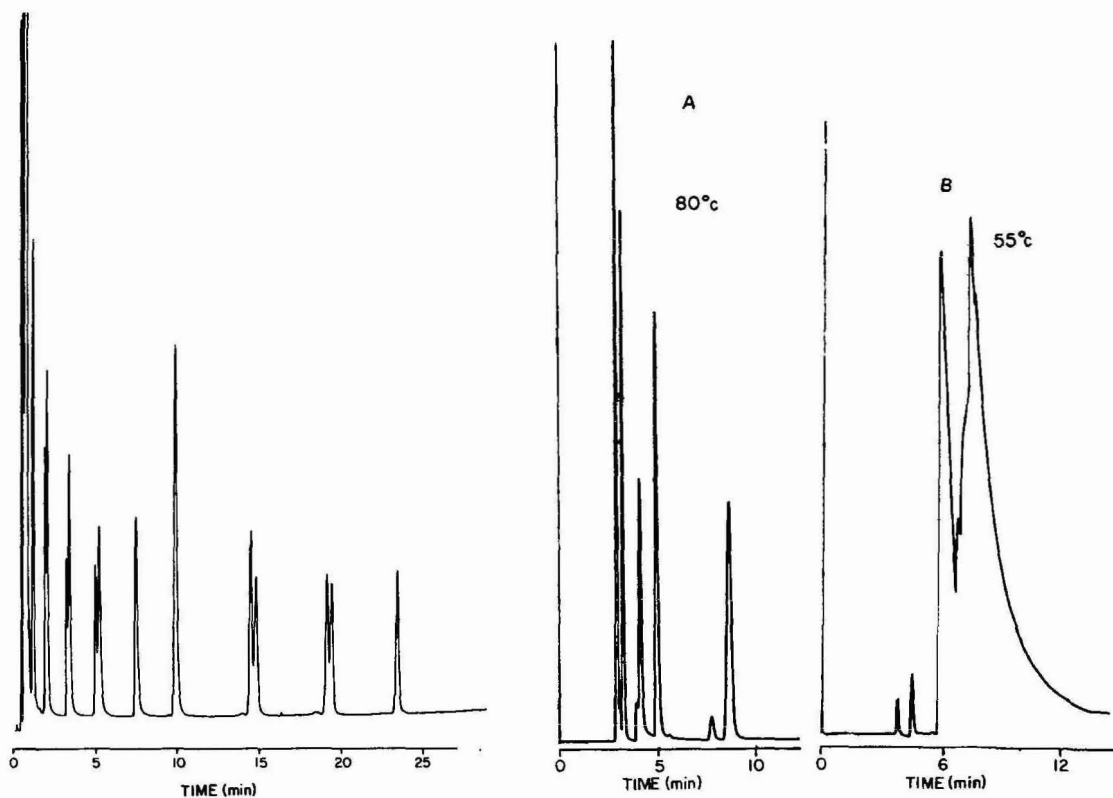
Fig. 4. Separation of a mixture of C_6 through C_{15} alkanes and 1-alkenes on the same column as used in Fig. 1. Temperature programmed from 45°C to 175°C at 5°C min⁻¹, and nitrogen carrier gas flow 30 ml min⁻¹.

Fig. 5. Separation of McReynolds test probes on the same column as used in Fig. 1, carrier gas flow 30 ml min⁻¹. A, Symmetrical test probes in order of elution: benzene, 1,4-dioxane, 1-iodobutane, 2-octyne and *cis*-hydrindane at 80°C. B, Asymmetric polar test probes at 55°C.
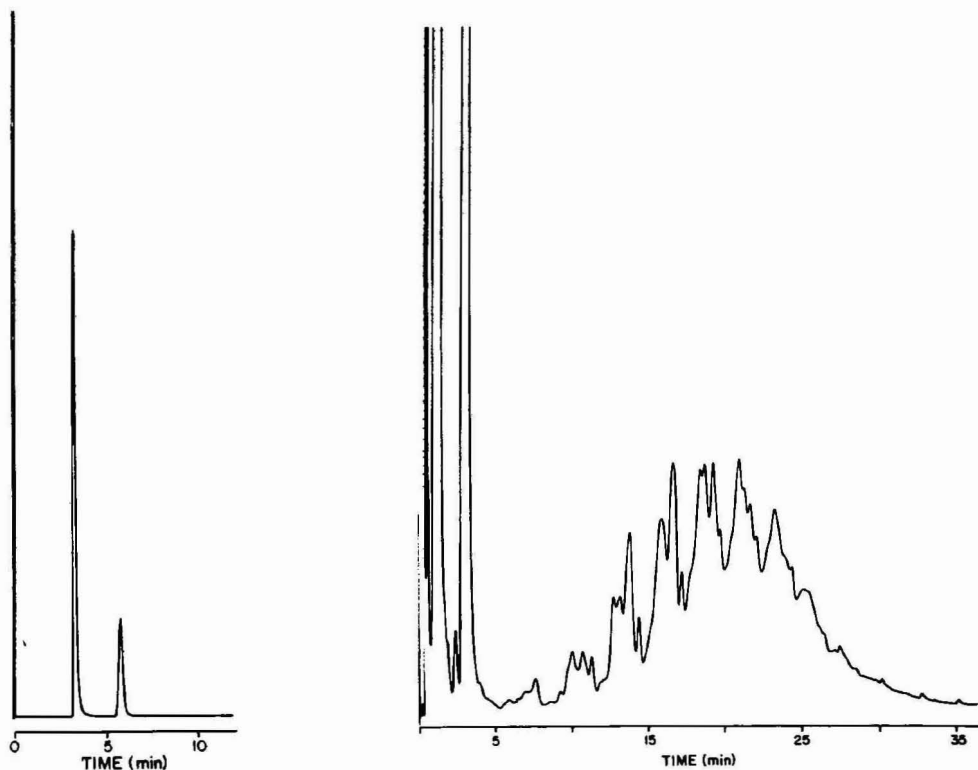
Fig. 6. Separation of Freon 11 and Freon 113 on the same column as used in Fig. 1, nitrogen carrier gas flow 30 ml min⁻¹ and column temperature 35°C.

Fig. 7. Separation of impurities in a sample of perfluorokerosene-L. Same column as used in Fig. 1, nitrogen carrier gas flow 30 ml min⁻¹ and column temperature isothermal (22°C) for 4 min, and then programmed at 5°C min⁻¹ to 180°C.

This would indicate that Fomblin YR shows some selectivity for the separation of saturated and unsaturated hydrocarbons.

The McReynolds test probes can be divided into two groups based on their chromatographic properties on Fomblin YR. Benzene, 1,4-dioxane, 1-iodobutane, 2-octyne and *cis*-hydrindane can be chromatographed with good peak shape (Fig. 5A). The more polar test probes butanol, nitropropane, pyridine, 2-methyl-2-pentanol and 2-pentanone produce asymmetric peaks with very low retention (Fig. 5B). Poor solubility of the polar probes in the stationary phase and/or the possibility that Fomblin YR provides poor shielding of active sites on the support is probably the reason for the results obtained. Fomblin YR is not a suitable stationary phase for the separation of polar organic compounds.

Perfluorocarbon compounds are often difficult to separate on conventional liquid phases due to a lack of selectivity. Perfluoroalkyl ether phases might prove useful for separating these compounds. Freons can be chromatographed with good peak shape on Fomblin YR, Fig. 6. The minor impurities in perfluorokerosene-L are also reasonably well separated on Fomblin YR, Fig. 7.

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Note

Immobilization of silicone stationary phases for capillary chromatography through the action of azoisobutyronitrile

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There are a variety of reasons for the continuing development of immobilized stationary phases for capillary gas chromatography (GC). The thermal stability of immobilized layers is often enhanced as compared to mechanically deposited films. Phase displacement at the column inlet due to large solvent injections is eliminated because of the low solubility of the stationary phase. Proper synthesis of the polymer can impart mechanical stability to the coating allowing either thick films or highly polar phases to be evenly coated without droplet formation. An additional impetus for such developments in this laboratory is the use of capillary columns in super-critical fluid chromatography (SFC) as described in previous communications[1,2].

The phase immobilization methods reported in the literature for use in capillary GC involve either prepolymerized fluids attached to the glass surface via the reactive surface silanol groups[3,4] or *in situ* cross-linking of conventional, mechanically coated stationary phases, as reported by Grob *et al.*[5] and others[6–9]. The latter approach, using techniques widely employed in rubber vulcanization processes, appears to have the distinct advantage of simplicity. Because cross-linking occurs in the polymer rather than to the substrate, this immobilization technique is applicable to both glass and fused-silica column technology.

Several cross-linking agents have been described for phase immobilization efforts. *In situ* generation of free radicals from various organic peroxides, such as benzoyl peroxide[5–9], dicumyl peroxide[8,9], *tert.*-butyl peroxide[9] has been investigated. Lee *et al.*[7] also recommended the use of azo-compounds to meet similar goals. In addition, gamma rays were found effective[10] in inducing free-radical polymerization of coated polysiloxanes.

The above procedures for polymer immobilization vary in degree of difficulty. The generation of free radicals during the phase treatment inside the column and along the entire column length should be both homogeneous and controllable. Introduction of some volatile peroxides in the vapor phase during the column treatment is inconvenient due to the hazard[11] as well as possible non-uniform decomposition along the column length. Solid initiators are considerably more convenient to use as additives to the stationary phase solution so long as they do not influence the wettability of the column wall or deposit undesirable reaction products upon decomposition. While the former problem was observed by Lipsky and McMurray[9] with benzoyl peroxide, the same agent is blamed for the generation of products[12] harmful

to the column. Lee *et al.*[7] have chosen azo-compounds as cross-linking agents to avoid similar difficulties as only volatile decomposition products were formed.

This communication reports the successful use of azoisobutyronitrile (AIBN) as a convenient initiator of cross-polymerization of conventional silicone stationary phases. While this solid substance has been known[13] for its easy generation of free radicals, its major decomposition products do not adversely affect column performance and can be easily removed at elevated temperatures. Simultaneously, its use as a phase additive during the coating process does not seem to alter wettability of the surfaces of glass or fused-silica capillaries. Its low decomposition temperature ($t_{1/2} =$ 1.3 h at 80°C) makes AIBN superior to other commonly used free-radical initiators.

In our numerous experiments, AIBN was employed to effectively immobilize commercially available silicone stationary phases (SE-30, SE-52 and SE-54) to the inner wall of either glass or fused-silica capillary columns. The glass capillaries (15–20 m × 0.25 mm I.D.) were first dynamically leached[14] and silylated at 400°C[15], while the fused-silica columns were used as received. Coating solutions for the static coating procedure contained 0.5% (w/v) of the silicone gums in *n*-pentane and 0.5
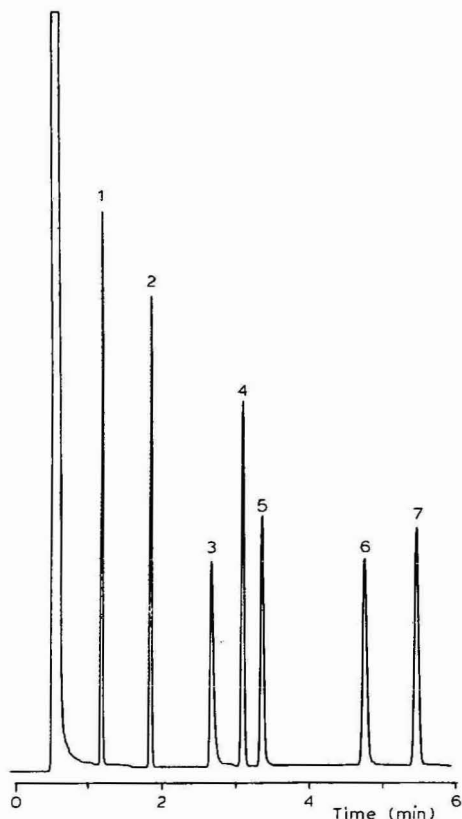
Fig. 1. Capillary gas chromatogram of a polarity mixture after extensive methylene chloride washing. Sample: 0.4 ng per component on column after split of 1 to 150. Column: 14 m × 0.25 mm, *i.e.*, 0.3-μm film thickness of SE-54, cross-linked with 10% (w/w) AIBN. Column temperature: 100°C. For peak labeling see Table I.

TABLE I

COMPARISON OF RETENTION INDICES BEFORE AND AFTER A METHYLENE CHLORIDE
WASH OF AN SE-54 COLUMN CROSS-LINKED WITH AIBN

Compound	I (before wash)	I (after wash)
1 Nonane	900.0	900.0
2 Decane	1000.0	1000.0
3 1-Octanol	1073.9	1073.9
4 Undecane	1100.0	1100.0
5 2,6-Dimethylphenol	1118.8	1119.2
6 2,6-Dimethylaniline	1178.3	1178.9
7 Dodecane	1200.0	1200.0

mg/cm^3 of AIBN. Following the coating step, the columns were sealed under vacuum
and cured at 80°C for 3 h. The capillary columns were then conditioned in a con-
ventional manner. Their performance and capacity ratios were measured before and
after a wash with 25 ml methylene chloride. The usual "polarity mixture" was used
to compare characteristics of the immobilized phases with those conventionally pre-
pared.

A typical loss of retention after the extensive wash with methylene chloride
was found to be only 4% (an average of five columns). A relatively high percentage
of AIBN was found to be essential; lesser amounts produced inferior results. This
fact might explain the difference between our results and those of Lee and co-work-
ers[16] who recently reported less positive results with the same agent.

Column efficiencies were typically between 2500 and 3000 effective plates per
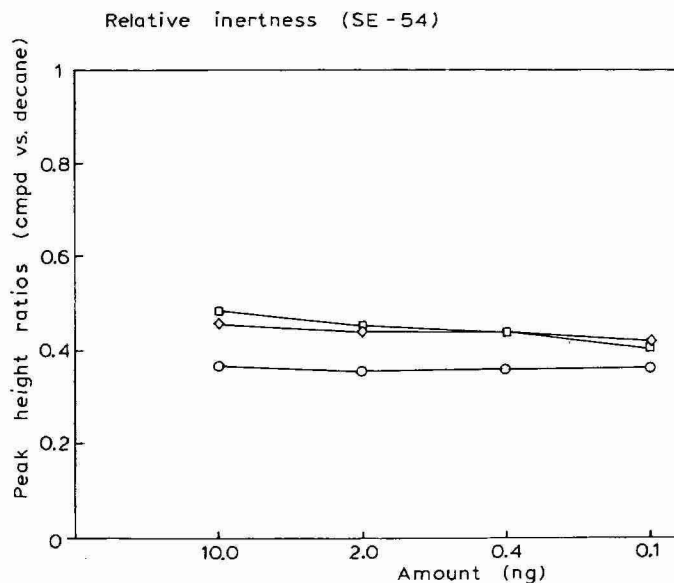
Fig. 2. Plots showing the relationship between peak height ratio of an active compound to decane *vs.* the
amount injected. Active compound: octanol (□); 2,6-dimethylphenol (◇) and 2,6-dimethylaniline (○).

meter (which corresponds to efficiencies of 75–80%). A typical chromatogram of the polarity mixture at a subnanogram level, obtained with an immobilized and excessively extracted film of SE-54 elastomer, is shown in Fig. 1, while Table I lists the average values of retention indices for the solutes chromatographed prior to and after methylene chloride extraction. Comparisons with retention values obtained with conventionally coated columns (without AIBN addition) indicated that neither the substance nor any of its degradation products measurably affect chromatographic retention. In addition, AIBN does not appear to adversely affect the column wettability with the stationary phases under study.

To determine adequately the degree of irreversible column adsorption, a polarity test mixture should be applied, starting with concentrations close to the minimum detectable amount and, eventually, spanning the concentration range of analytical interest. This procedure, described by Purcell[17], is not always sufficiently appreciated in the literature. When the peak heights of more polar (and, relatively more sensitive) mixture components are ratioed to a near alkane peak, for different amounts, the slopes of corresponding curves should provide an adequate description of column inertness. This method assumes that adsorption of alkanes is negligible. As shown in Fig. 2, adsorptive properties of the glass columns prepared through the action of AIBN are minimal. Very similar behavior was exhibited by fused-silica columns.

ACKNOWLEDGEMENTS

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Note

Correlation of pK_{BH^+} of acetanilides with liquid chromatographic behaviour[*]

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The effect of molecular structure on chromatographic behaviour has been extensively investigated[1]. However, with the exception of thin-layer chromatographic (TLC)[2] and high-performance liquid chromatographic (HPLC)[3] studies, the correlation of basicity with retention time has received limited attention. The liquid–liquid partition chromatography of methyl derivatives of aniline in acidic systems indicated that their chromatographic behaviour is determined by the basicity of the amino group in addition to steric effects and their molecular volumes[2]. HPLC investigations[3] of weakly basic aromatic amines using slightly acidic silica gel adsorbent columns revealed an empirical and linear correlation amongst the *o-*, *m-* and *p-*isomers of the amines, but such a correlation between amines containing different substituents was not clearly established.

The direct correlation of basicity constants (pK_{BH^+}) of acetanilides with HPLC retention times has now been established and is discussed in this paper. Substituted acetanilides are very weak bases, much weaker than the corresponding anilines. Spectroscopic or potentiometric determinations of their pK_{BH^+} values are difficult, as they lack adequate spectral differences in their acid and base forms and have insufficient solubility. Therefore, we have attempted to calculate the pK_{BH^+} values of acetanilides by methods based on a linear free energy relationship and by extrapolation of the available data.

EXPERIMENTAL

Preparation of monoacetyl derivatives

A mixture of the aniline (1 mole), pyridine (1 mole) and acetic anhydride (1.1 mole) was stirred and heated in a boiling water-bath for 2 h. The monoacetyl derivatives were isolated by precipitation on pouring in water, filtered, washed well with water and purified by recrystallization.

HPLC procedure

A Waters Model ALC/GPC-202R401 liquid chromatograph with a UV detector (280 nm) and a dual pen recorder was used. A Corasil-II silica gel column (61 cm

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[*] NCL Communication No. 3139.

TABLE I

RELATIVE BASICITIES OF SUBSTITUTED ACETANILIDES AND HAMMETT σ-VALUES OF SUBSTITUENTS

Substituent	$-pK_{BH}+$	$\sigma^\star$	Ref.
4-OH	1.02	−0.38	4
4-OCH$_3$	1.21	−0.28	4
3-OCH$_3$	1.66	0.11	5
2-OCH$_3$	1.45	0.00	5
4-OC$_2$H$_5$	1.18	−0.24	4
4-CH$_3$	1.27	−0.14	4
3-CH$_3$	1.67	−0.06	5
2-CH$_3$	1.63	0.10	5
4-H	1.59	0.00	4
4-F	1.70	0.06	4
4-Cl	1.88	0.24	4
3-Cl	2.05	0.37	4
4-Br	1.91	0.22	4
4-I	2.07	0.21	4
3-NH$_2$	1.68	0.00	5
4-COOH	2.12	0.44	4
4-NO$_2$	2.59	0.78	4

$\star$ Ref. 6.

$\times$ 2 mm I.D.) was used with chloroform as the mobile phase at a flow-rate of 2.0 ml/min at ambient temperature and a column pressure of 650 p.s.i. Spectral-grade chloroform was filtered prior to use as the mobile phase. The sample concentrations were adjusted to 1.0 mg/ml in the mobile phase (chloroform) and injections of 25 μl were made. The capacity factor (k') for each compound was calculated from the equation $k' = t_R - t_o/t_o$, where t_R is the sample retention time and t_o is the hold-up time, which was recorded by injecting pure carbon tetrachloride in the mobile phase. The retention time and hold-up time were means of at least ten consecutive injections.

RESULTS AND DISCUSSION

Correlation with Hammett σ-values of substituents

The p$K_{BH}+$ values of acetanilides and the σ-values of their substituents are summarized in Table I. A linear correlation was found (Fig. 1), and by regression analysis the equation

$$pK_{BH}+ = -1.549 - 1.394\ \Sigma\sigma$$

was derived. The p$K_{BH}+$ values of disubstituted acetanilides utilized in the study of HPLC behaviour were obtained using this regression equation.

Correlation with HPLC retention time

The p$K_{BH}+$ values of substituted acetanilides with their HPLC retention data are summarized in Table II. As a measure of the reproducibility, the relative standard deviations of the mean k' values (means of ten determinations) from the actual values
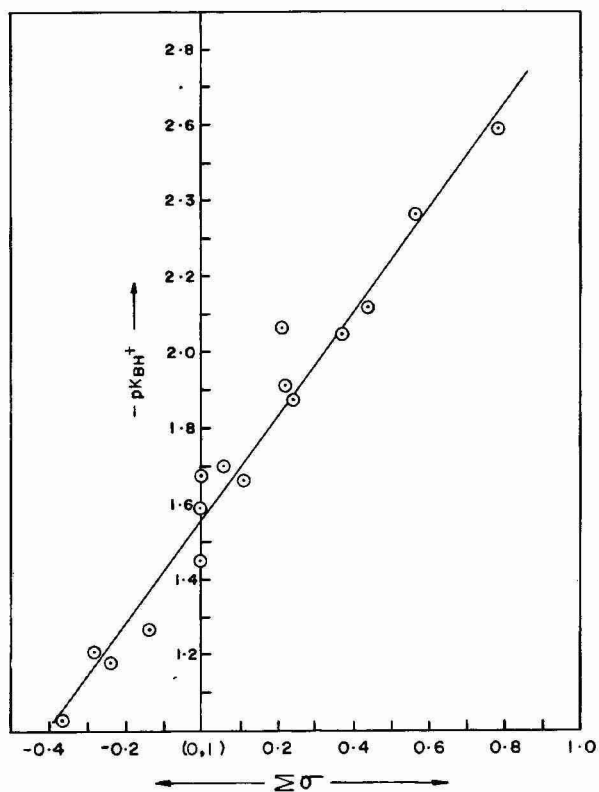
Fig. 1. Variation of pK_{BH^+} of acetanilides with Hammett σ-values of substituents.
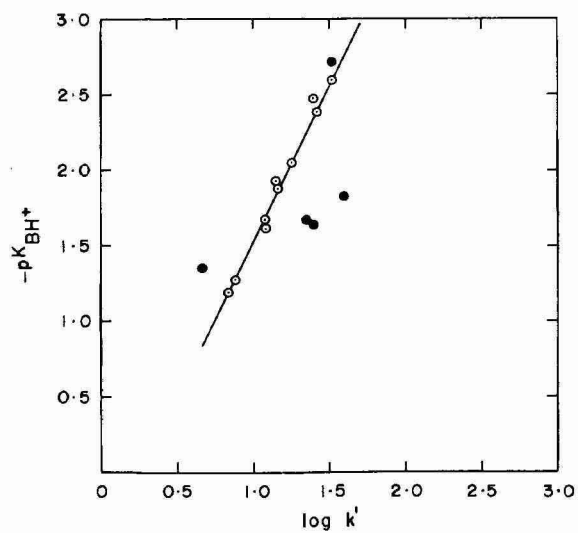
Fig. 2. Variation of basicity constants of acetanilides with capacity factor. O, *meta*- and *para*-substituted acetanilides; ●, *ortho*-substituted acetanilides.

TABLE II

COMPARISON OF BASICITY CONSTANTS (pK_{BH}^+) OF SUBSTITUTED ACETANILIDES AND CAPACITY FACTORS (k')

Substituent	$-pK_{BH}^+$	k'	Log k'*	Relative standard deviation of k' (%)
—	1.59	11.9	1.0755	0.6
4-OCH$_3$	1.21	6.7	0.8261	.0.3
4-CH$_3$	1.27	7.5	0.8751	0.4
3-CH$_3$	1.67	11.8	1.0750	0.4
2-CH$_3$	1.63	24.7	1.3927	1.2
4-Cl	1.88	14.1	1.1500	0.2
3-Cl	2.05	17.7	1.2500	1.0
2-Cl	1.35	4.6	0.6628	0.2
4-Br	1.91	14.1	1.1500	0.5
4-NO$_2$	2.59	33.1	1.5185	1.5
3-NO$_2$	2.46	25.1	1.4000	2.0
4-CH$_3$, 3-NO$_2$	2.38	26.0	1.4150	1.9
2-CH$_3$, 5-NO$_2$	2.72	32.5	1.5118	2.0
2,6-Di-CH$_3$	1.83	36.2	1.5587	1.8
2,6-Di-C$_2$H$_5$	1.69	22.5	1.3521	1.2

* In Fig. 2, log k' values rounded up to the second decimal place were utilized for plotting.

for each of the fifteen monoacetyl derivatives were estimated and were found to be in the range 0.2–2.0%. The graph of pK_{BH}^+ against logarithm of the capacity factor (Fig. 2) is linear for the *meta-* and *para*-substituted acetanilides, but the *ortho*-substituted acetanilides show some deviations. This behaviour suggests that basicity is a predominant factor in the adsorption process. The deviations shown by *ortho*-substituted acetanilides may be attributed to the additional field and steric effects exhibited by *ortho*-substituents in addition to the inductive and resonance contributions[7–10].

Apart from their contribution towards a better understanding of the liquid chromatographic behaviour of very weak bases, such as acetanilides, the significance of such linear correlations lies in the fact that they can be used to predict the values of basicity constants of compounds whose experimental determination is difficult for the reasons mentioned above.

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Note

Adsorption chromatography on Bio-Gel P-2*

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In order to resolve a complex mixture of biological compounds present in the ultrafiltrate of patients with chronic uraemia, a gel chromatographic method on polyacrylamide gels (Biogel P-2; Bio-Rad Labs.) has been developed in our laboratories[1]. This method offers better results and reproducibility than those reported by others[2–4].

These results have been explained in terms of an optimization of the experimental conditions: a linear flow-rate lower and a higher ionic strength of the mobile phase than those use by others are believed to be the two factors favourably influencing the separation.

As the substances involved in the separation possess a low molecular weight, other possibilities associated with the partition and adsorption properties of the Bio-Gel P-2 could be considered. Actually, in our experience and in that of other authors[4–7], it was impossible to obtain a calibration graph with Bio-Gel P-2 at any linear flow-rate for molecular weights ranging from 1800 and 100 daltons (the value given by the manufacturer for this gel). It therefore appeared reasonable to assume that, with low-molecular-weight compounds, the partition and adsorption properties of this stationary phase were also important in determining the separation of the mixture.

The adsorption properties of Bio-Gel P-2 towards sulphanilamide, phenol and butanol have been studied[8,9]. Scattered examples of separations of tetracyclines[10], monomeric oxo anions of phosphorus[11] and aromatic molecules[12], presumably based on the affinity properties of this gel phase, have also been described. A systematic study of the limits and possibilities of this stationary phase in the separation of small molecules has not been carried out, however.

In this investigation, a wide variety of small organic molecules have been separated, and it is shown that the separations achieved were not based on molecular weights; a study was made of this unconventional use of a chromatographic column filled with Bio-Gel P-2.

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* Dedicated to the memory of Eraldo Antonini prematurely deceased on March 19th, 1983.

This chromatography, as previously observed for Sephadex LH-20 and G-10[13,14], does not follow the rules of gel chromatography, but appears to be very useful in practical laboratory work. Good reproducibilities and high efficiencies in separating a large variety of compounds can be achieved using very simple apparatus.

EXPERIMENTAL

Bio-Gel P-2 (150–300, 80–150, 40–80 and < 40 μm) was purchased from Bio-Rad Labs., Richmond, CA, U.S.A.; the column was a K 16/100 with thermostatted jacket and two adaptors obtained from Pharmacia (Uppsala, Sweden); the bed height was 83 cm. A peristaltic pump was a· Varioperpex from LKB (Stockholm, Sweden).

Elution was performed at a flow-rate of 0.5 ml/min by monitoring at 254 nm with a Uvicord S (LKB) and collection in 1-ml fractions on an UltroRac II fraction collector (LKB).

Analytical-reagent grade products were used. The elution volumes reported are averages of at least three experiments, each differing from the other by about 1%. All the samples were introduced into the column in a 2-ml volume by suction through the peristaltic pump.

RESULTS AND DISCUSSION

Bio-Gel P is a chromatographic support made by cross-linking acrylamide with a bifunctional acrylamide such as N,N′-methylenebisacrylamide.

Bio-Gel P-2 has a declared resolving power as a molecular sieve between 1800 and 100 daltons at optimal linear flow-rates. However, a mixture of calcein, cyano-cobalamin, uric acid, phenylalanine and tyrosine (with molecular weights of 622, 1355, 168, 165 and 181 daltons, respectively) is well resolved in a Bio-Gel P-2 column at different linear flow-rates but not according to their molecular weights, reported in Fig. 1.

In Table I elution volumes (V_e) of many organic molecules are reported; it can be seen that the column is able to separate very similar compounds such as 4-nitro-benzoic acid (V_e = 67 ml) and 3-nitrobenzoic acid (V_e = 73 ml). Amino acids such as phenylalanine and tyrosine are also separated.
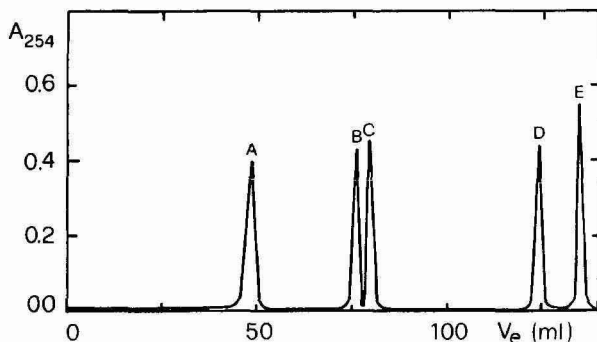
Fig. 1. Elution pattern of a mixture of calceine (A), cyanocobalamin (B), uric acid (C), phenylalanine (D) and tyrosine (E). The molecular weights are 622, 1350, 168, 165 and 181 daltons, respectively. Column, 83 × 1.6 cm I.D., Bio-Gel P-2, 80–150 μm; 50 mM NH₄HCO₃, pH 8; 20°C; flow-rate, 0.5 ml/min.

TABLE I

ELUTION VOLUMES ON A COLUMN (83 × 1.6 cm) OF BIO-GEL P-2

Conditions: particle size, <40 μm; 50 mM NH_4HCO_3; pH 8; 20°C.

Sample	Molecular weight	V_e (ml)	Sample	Molecular weight	V_e (ml)
Insulin	6000	47	Uric acid	168	79
Inulin	5000	47	Phenylalanine	165	124
Calcein	622	47	Tyrosine	181	135
2-Methoxybenzoic acid	152	62	Creatinine	113	159
4-Methoxybenzoic acid	152	64	Methyl benzoate	136	178
Benzoic acid	122	64	Methylguanidine	73	184
1-Chlorobenzoic acid	156	65	Benzaldehyde	106	188
4-Nitrobenzoic acid	167	67	Benzamide	121	188
2-Nitrobenzoic acid	167	68	Tryptophan	204	193
3,5-Diaminobenzoic acid	170	68	Resorcinol	110	210
3-Aminobenzoic acid	137	69	Phenol	94	215
4-Aminobenzoic acid	137	70	Benzene	78	445
2-Aminobenzoic acid	137	72			
3-Nitrobenzoic acid	167	73			
Cyanocobalamin	1355	76			

According to the structure of the stationary phase (*i.e.*, the gel is not penetrable by large molecules), high-molecular-weight compounds are eluted with the void volume of the column. In contrast, small molecules (those lying in the range 100–1800 daltons) can interact with the stationary phase, as they pass through the gel.

Actually, V_e is not strongly influenced by the size of the particles: in adsorption at the surface of the particles, an increase in the elution volumes would be expected with smaller particles. However, this does not appear to occur, as shown in Fig. 2, and this result must be interpreted in terms of interactions inside the gel network.
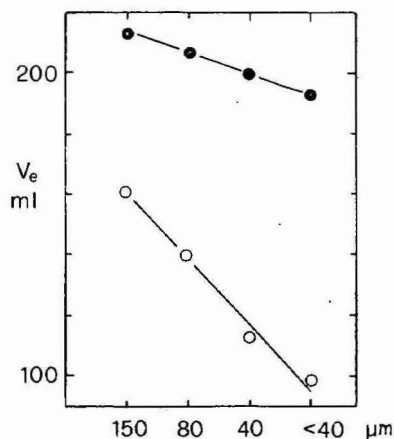
Fig. 2. Elution volumes (V_e) of benzamide (●) and benzoic acid (○) as a function of the diameter (μm) of the particles of the stationary phase (150 = 150–300 μm; 80 = 80-150 μm; 40 = 40–80 μm). Column, 83 × 1.6 cm I.D., Bio-Gel P-2; 50 mM NH_4HCO_3, pH 8; 10°C; flow-rate, 0.5 ml/min.
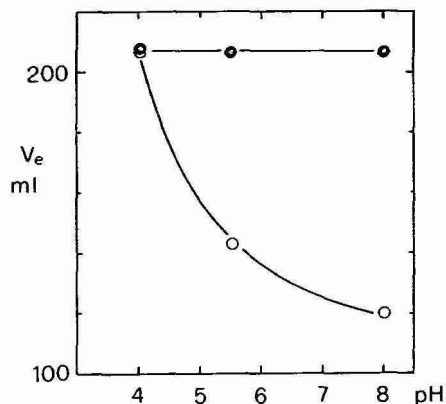
Fig. 3. Elution volumes (V_e) of benzamide (●) and benzoic (○) as a function of pH of the mobile phase. Column, 83 × 1.6 cm I.D., Bio-Gel P-2, 80–150 μm; 10°C; flow-rate, 0.5 ml/min.
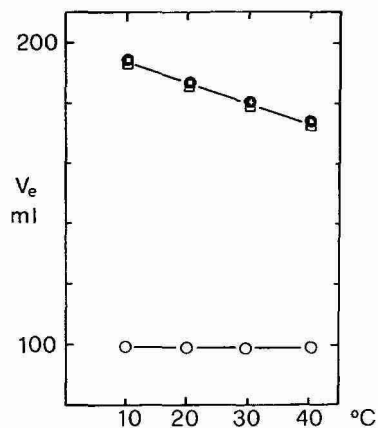
Fig. 4. Elution volumes (V_e) as a function of temperature of benzamide (●) and benzoic acid (○) at pH 8 (50 mM NH$_4$HCO$_3$) and benzoic acid (□) at pH 3.2 (50 mM acetic acid). Column, 83 × 1.6 cm I.D., Bio-Gel P-2, <40 μm; flow-rate, 0.5 ml/min.

Many workers have pointed out that these gels, such as the dextran gels, may form either hydrogen or hydrophobic bonds with adsorbates, thus modifying the elution volumes of the molecules passed through a gel chromatographic column[5-9].

Thermodynamic studies carried out on Bio-Gel P-2 have shown that the adsorbate–adsorbent linkages are first hydrogen bond and then hydrophobic in nature[8,9]. Then the mass of the adsorbent exerts an important role in the separation of two molecules having similar elution volumes. As an example, a column with a bed height of 83 cm and I.D. 1.6 cm (as reported in Table I) is able to take advantage of the differences in elution volumes due to the different partition and adsorbing properties of the gel, to obtain a satisfactory separation between two molecules even when they are very similar. A short column (12.5 cm) does not give an effective separation because the adsorbent mass is not sufficient.

In separations with Bio-Gel P-2, the most important role seems to be played by the hydrogen bonds formed between the adsorbate and the gel.

The elution volume of benzamide does not depend on the pH, as reported in Fig. 3, whereas V_e for benzoic acid decreases as the pH increases. The faster elution of ionizable compared with neutral molecules is presumably related in part to the presence in the polyacrylamide gels, as for dextran gels, of carboxylic groups[15].

Actually, the data in Fig. 4 suggest that the adsorption phenomena given by benzamide might be related to hydrogen bonds, as can be inferred from the strong dependence of V_e on temperature, whereas the interaction of ionized benzoic acid definitely has a different origin, not being dependent on temperature. On the other hand, the elution volume of benzoic acid is itself influenced by temperature when the chromatography is performed at pH 3.2, where the acid is largely undissociated. Under these conditions the behaviour of benzoic acid becames identical with that of benzamide, as shown in Fig. 4.

The large elution volume of benzene is not surprising. Benzene has a very poor
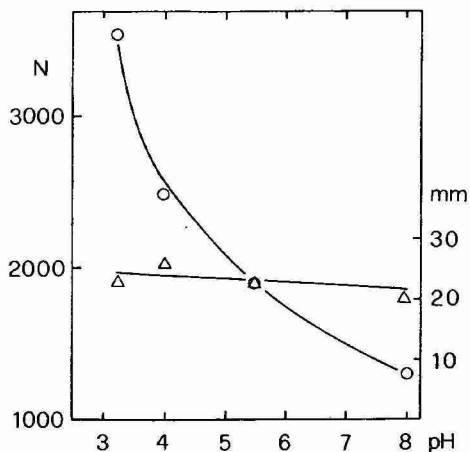
Fig. 5. Number of theoretical plates, N (left-hand ordinate, ○) and peak width (right-hand ordinate, △) in mm for benzoic acid of a column (83 × 1.6 cm I.D.) of Bio-Gel P-2, <40 μm, as a function of the mobile phase. Elution was performed at a flow-rate of 0.5 ml/min at 10°C.
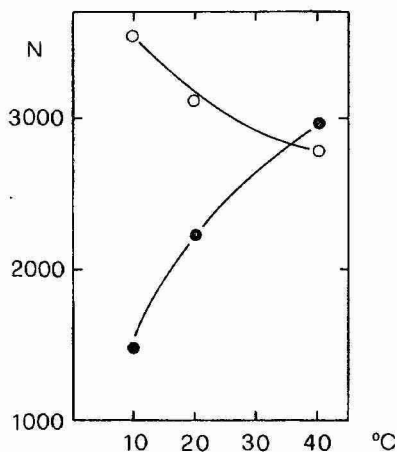
Fig. 6. Number of theoretical plates, N, for benzoic acid of a column (83 × 1.6 cm I.D.) of Bio-Gel P-2, <40 μm, as a function of temperature. Elution was performed at a flow-rate of 0.5 ml/min in 50 mM NH_4HCO_3 at pH 8 (●) and in 50 mM acetic acid at pH 3.2 (○).

affinity for water and can strongly interact with the stationary phase through hydrophobic bonds and/or hydrogen bonds. Accordingly, phenol and resorcinol, which are more soluble in water, have lower elution volumes than benzene although they have stronger absolute interactions than benzene with the stationary phase.

It is interesting that the number of theoretical plates, N, of the column used is rather high, ranging from 1000 to 3500 depending on the experimental conditions that were investigated for benzoic acid. As expected, N decreases with increasing pH of the eluent, as shown in Fig. 5: in fact, an increase in pH increases the percentage of the ionized form of benzoic acid, which further interacts with the gel matrix, giving a lower value of N for the system. It should be noted, however, that the peak width is almost invariant. The fact that the interactions between benzoic acid and the gel matrix are different in nature according to the pH of the mobile phase is apparent also from Fig. 6.

The number of theoretical plates increases with increasing temperature at pH 8 (hydrophobic interactions between ionized benzoic acid and the gel), but decreases with increase in temperature at pH 3.2 (hydrogen bonds).

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Note

Reversible immobilization of molybdenum cofactor on a gel matrix via sulphydryl groups

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The molybdenum cofactor (Mo-co) is a low-molecular-weight, oxygen-sensitive component common to numerous molybdoenzymes (*e.g.*, nitrate reductase, xanthine oxidase, sulphite oxidase, aldehyde oxidase) where it constitutes part of the catalytically active centre[1]. Mo-co bound to cofactor-containing proteins can be released in a functionally active form by heat denaturation of these proteins in the presence of Mo-co-protecting agents (ascorbic acid and/or thiol reagents)[2,3]. Recently, Mo-co from rat liver sulphite oxidase was suggested to contain sulphur[4].

Very recently, Mo-co released from milk xanthine oxidase was shown to contain free sulphydryl groups which are involved in the cofactor-mediated process of subunit dimerization of *Neurospora crassa* nitrate reductase as well as in the correct liganding of molybdenum[5]. The hitherto unknown presence of sulphydryl groups on Mo-co made it possible to investigate conditions for the covalent chromatography of Mo-co in order to immobilize the cofactor via sulphydryl groups on a gel matrix.

Reduced glutathione (GSH) was reported to be a highly efficient stabilizer of Mo-co activity, being superior to other commonly used thiol reagents[5]. Commercially available Thiol-Sepharose 4B (Sepharose–glutathione-2-pyridyldisulphide) contains GSH as an active ligand covalently coupled to the gel matrix. In this work we made use of the appearant high affinity of Mo-co's sulphydryl groups to GSH and a method is described for reversible covalent immobilization of Mo-co on Thiol-Sepharose 4B. Gel-bound Mo-co exhibited a very high stability and could be specifically eluted in a highly active form.

EXPERIMENTAL

Preparation of Mo-co

Mo-co was released from xanthine oxidase by heat treatment as described recently[3]. A 10-μl volume (= 0.1 mg of protein) of milk xanthine oxidase (Serva, Heidelberg, F.R.G.) was diluted in 1 ml of buffer A (50 mM potassium/sodium phosphate buffer, pH 7.5, 0.5 mM EDTA) containing 1 mM ascorbic acid and 25 mM sodium molybdate. The solution was thoroughly evacuated, flushed with nitrogen,

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stoppered, incubated for 90 sec at 80°C and chilled on ice. Precipitated protein was removed by centrifugation.

Assay of Mo-co

Mo-co has been assayed by restoration of NADPH-nitrate reductase (E.C. 1.6.6.2) activity in extracts of *Neurospora crassa* mutant *nit*-1. Strain *nit*-1 was grown on NH₄ (as a nitrogen source), induced by nitrate for 5 h and extracted as described earlier[3,6]. The assay of Mo-co was carried out exactly as described previously[3]. A 100-μl volume of *nit*-1 extract and 50 μl of Mo-co were used per assay. The times for complementation and for the nitrate reductase test were 40 and 15–30 min, respectively. One unit of Mo-co activity will reconstitute the *nit*-1 nitrate reductase to 1 nmol/min.

Coupling of Mo-co to the gel matrix

For analytical purposes, about 0.5 g of activated Thiol-Sepharose 4B (Pharmacia, Uppsala, Sweden) was mixed with 10–20 volumes (w/v) of deaerated buffer A containing 0.3 M sodium chloride, evacuated, flushed with nitrogen and swollen overnight at 2°C. After extensive washing with the same buffer, about 200 μl of wet settled gel were mixed with 1 ml of Mo-co solution (containing 0.3 M sodium chloride), evacuated, flushed with nitrogen and incubated for 20 min at 2°C with slight shaking. Subsequently, the gel particles were sedimented by centrifugation (for 1 min at 1000 g) and the supernatant was carefully sucked off. The gel was aerobically washed twice with buffer A (*i.e.*, resuspended in 10 ml of buffer A and sedimented by centrifugation, in each instance). Bound Mo-co could be eluted by resuspending the sedimented gel for 5 min in 1 ml of buffer A containing 5 mM GSH and 25 mM sodium molybdate. Used gel can be regenerated according to the manufacturer's procedure[7].

RESULTS AND DISCUSSION

The ratio of gel matrix to Mo-co was chosen so that more than 95% of the Mo-co applied was coupled to the gel, *i.e.*, up to 1000 units of Mo-co per millilitre of swollen gel (Table I). Neither a second and third wash of the loaded gel with buffer A nor washing with 1 M potassium chloride solution removed detectable amounts of Mo-co, thus demonstrating that non-specific binding did not occur. Addition of 1–5 mM GSH and 25 mM molybdate to the gel caused immediate and quantitative release of catalytically highly active Mo-co (Table I). Molybdate without GSH is not able to elute bound Mo-co from the gel, but the presence of molybdate is essential for obtaining maximal activity of Mo-co in the subsequent *nit*-1 complementation assay. Therefore, molybdate was added to the elution buffer.

The coupling rection between Mo-co and Thiol-Sepharose was complete within 20 min at 2°C and was not impaired by performing it at room temperature and/or under aerobic conditions (Table II). Coupling was routinely performed in the presence of 0.3 M sodium chloride (a range of 0.2–0.5 M is advisable), as it was observed that omission of at least 0.2 M sodium chloride resulted in an approximately 10% lower efficiency of coupling under standard conditions.

Preparation of Mo-co by heat treatment of xanthine oxidase as the Mo-co source requires the presence of reductants in order to preserve the sulphydryl groups

TABLE I

COVALENT CHROMATOGRAPHY OF Mo-co ON THIOL-SEPHAROSE 4B

Step	Mo-co activity (units/ml)
Mo-co before coupling	165
Supernatant after coupling	8
2nd wash with buffer A*	0
Elution with:	
25 mM Na$_2$MoO$_4$, 1 mM Asc**	0
25 mM Na$_2$MoO$_4$, 1 mM Asc, 1 M KCl	0
25 mM Na$_2$MoO$_4$, GSH: 0.5 mM	80
1 mM***	149
5 mM***	156
10 mM***	147

* 5 mM GSH and 25 mM Na$_2$MoO$_4$ added after elution.

** Asc = ascorbic acid.

*** Separate elutions with the same gel batch and Mo-co preparation.

of Mo-co[3]. The most powerful reductant for this purpose was found to be 5 mM GSH[5] which, however, owing to its interference with the covalent chromatography of Mo-co, had to be replaced with 1 mM ascorbic acid, which is also known as a potent Mo-co protector[2,3]. If xanthine oxidase was heat treated in the absence of any reductant, the Mo-co lost its catalytic activity irreversibly. However, if xanthine oxidase was heat treated in the presence of Thiol-Sepharose, i.e., the heat release of Mo-co and the coupling rection were combined into one step, Mo-co was fully stabilized and eluted highly active from the gel (Table III). This protective effect of Thiol-Sepharose is obviously due to the matrix-bound GSH which serves as an active coupling ligand and even in the bound state seems to be strong enough to protect Mo-co. Although attractive, the Mo-co preparation and the coupling reaction were not combined for routine experiments as the gel matrix Sepharose 4B underwent gradual degradation during repeated cycles of heating.

TABLE II

TIME COURSE AND CONDITIONS OF COUPLING OF Mo-co TO THIOL-SEPHAROSE 4B

Coupling conditions*	Mo-co activity** (units/ml)
5 min	120
10 min	144
15 min	152
20 min	160
30 min	160
30 min, aerobic	159
30 min at 20°C	160
30 min at 20°C, aerobic	161

* Separate couplings with 200 μl of swollen gel and 1 ml of Mo-co in each instance.

** Determined after elution with GSH and molybdate.

TABLE III

PROTECTING EFFECT OF REDUCTANTS ON Mo-co ACTIVITY DURING HEAT RELEASE

Additive to buffer A	Mo-co activity (units/ml)
25 mM Na$_2$MoO$_4$ + 1 mM ascorbic acid*	166
25 mM Na$_2$MoO$_4$*	0
25 mM Na$_2$MoO$_4$ + Thiol-Sepharose**	159

* Mo-co was heat-released in the presence of the additives indicated (conditions exactly as described under Experimental) and immediately tested for activity.

** 200 μl of swollen gel per 1 ml of heat-treatment mixture (NaCl omitted). After heat treatment, coupling was continued for 30 min as described under Experimental. Mo-co activity was determined after elution with GSH and molybdate.

The coupling of Mo-co to matrix-bound GSH increased the stability of the Mo-co several-fold. At 2°C, gel-bound Mo-co exhibited at least a ten-fold higher stability compared with that of the free form (half-life more than 5 days *versus* 12 h) (Table IV). At 20°C, a similar ratio between the stabilities of free and gel-gound Mo-co was observed, although the half-lives were generally lower. The data in Table 4 show that tempereature has a remarkable effect on the oxygen sensitivity of bound Mo-co. The same observation has been reported for free Mo-co in non-aqueous solvents[8]. Under aerobic and anaerobic conditions, molybdate exerted a stabilizing effect on Mo-co. At 20°C, anaerobic storage of gel-bound Mo-co in the presence of 25 mM molybdate turned out to be the most efficient variant for preserving the high activity of Mo-co.

TABLE IV

HALF-LIVES OF FREE AND GEL-BOUND Mo-co UNDER DIFFERENT CONDITIONS OF STORAGE

Storage conditions	Half-life (h)
2°C:	
Free*, −O$_2$, Asc + Mo**	12
GSH + Mo	26
Bound***, +O$_2$, + Mo	⩾120
20°C:	
Free*, −O$_2$, Asc + Mo	4
GSH + Mo	20
Bound***, −O$_2$	81
−O$_2$ + Mo	>120
+O$_2$	54
+O$_2$ + Mo	68

* Free = heat-released Mo-co not coupled to gel.

** Concentrations used throughout: Asc = 1 mM ascorbic acid; Mo = 25 mM Na$_2$MoO$_4$; GSH = 5 mM.

*** Bound = heat-released Mo-co coupled to Thiol-Sepharose 4B as described under Experimental and stored under the conditions indicated. Mo-co activity was determined after elution with GSH and molybdate.

In conclusion, the highly stable gel-bound Mo-co could serve as a means for studying the interactions between immobilized Mo-co and Mo-co-binding proteins or protein domains, as well as for elucidating the physical and chemical properties of immobilized Mo-co.

ACKNOWLEDGEMENTS

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Note

Post-column derivatization of vitamin B₆ using 2,6-dibromoquinone-4-chlorimide

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The analysis of vitamin B_6 is of considerable importance in various fields such as nutrition science, pharmaceutics and clinical chemistry. The introduction of high-performance liquid chromatography (HPLC) for the analysis of vitamin B_6 made its rapid separation and quantitation possible. Many authors have indicated the advantages of HPLC for vitamin B_6 analysis[1–7].

As conventional HPLC detection by ultraviolet absorbance is affected by impurities in the sample, it is necessary to separate vitamin B_6 completely from other impurities, which is time consuming, and/or remove them by extraction before injection.

Automatic derivatization of vitamin B_6 after elution from the column, which gives high selectivity together with high sensitivity, reduces the analysis time and makes sample preparation easier. 2,6-Dibromoquinone-4-chlorimide, a conventional colour reagent for use with pyridoxine hydrochloride in spectrophotometry, which gives an absorption maximum at 650 nm in the presence of ammonia solution, has been applied to the post-column derivatization of vitamin B_6. Hydrochloride forms of pyridoxine, pyridoxal, pyridoxamine and 4-pyridoxic acid were separated by the reversed-phase ion-pair chromatography, derivatized and selectively detected using by this system.

EXPERIMENTAL

An LC-3A liquid chromatograph equipped with a SPD-1A spectrophotometric detector, SIL-1A sample injector, CTO-2A column oven, two PRP-1 pumps as the reagent pump, a reaction coil made of stainless-steel tubing of various lengths and of 0.5 mm I.D. and a UV-240 spectrophotometer were used (all products from Shimadzu, Kyoto, Japan).

The column (25 cm × 4.6 mm I.D.) contained Zorbax C 8 of particle size 5 μm (DuPont Company, Wilmington, DE, U.S.A.). The mobile phase was distilled water containing 5 mM of sodium perchlorate and 5 or 10 mM of sodium 1-hexanesulphonate, which is an ion-pair reagent. The pH of the mobile phase was adjusted to 2.5 with perchloric acid, and 10–20% of methanol was added in order to adjust the retention time of vitamin B_6.

Standard samples were prepared by dissolving pyridoxine hydrochloride, pyr-

LC-3A
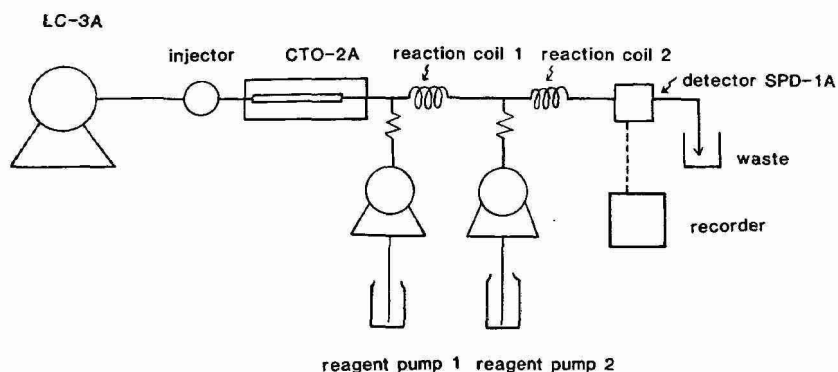
Fig. 1. Flow diagram of the system. Pump 1: 2,6-dibromoquinone-4-chlorimide (colour reagent). Pump 2: ammonia solution (2.5%).

idoxal hydrochloride (Tokyo Kasei, Tokyo, Japan), pyridoxamine dihydrochloride (P-L Biochemical, Milwaukee, WI, U.S.A.), deoxypyridoxine hydrochloride (Wako, Osaka, Japan) and 4-pyridoxic acid, pyridoxamine 5'-phosphate, pyridoxal 5'-phosphate (Sigma, St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the flow diagram of the system. Vitamin B_6 compounds are separated on the Zorbax C_8 column by reversed-phase ion-pair chromatography and
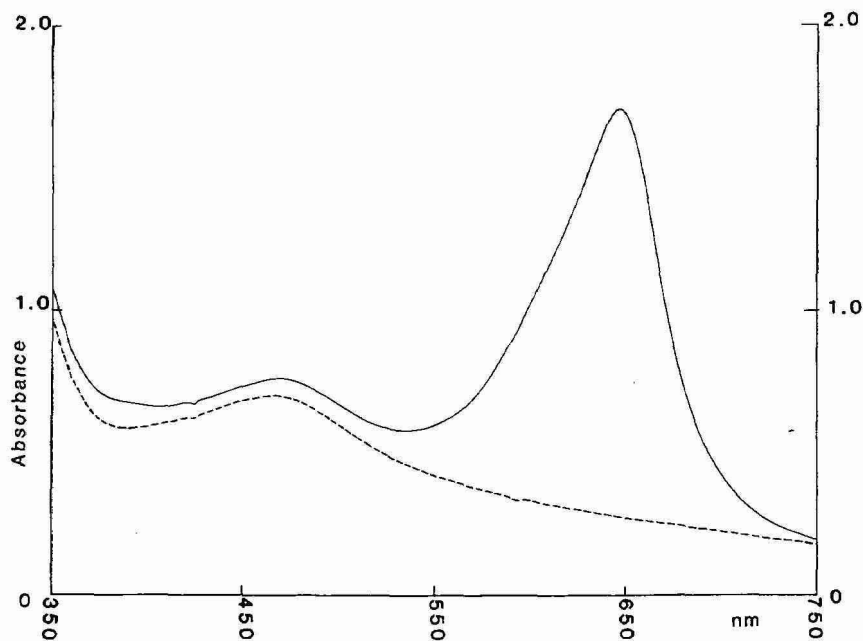
Fig. 2. Absorption spectrum of pyridoxine hydrochloride (solid line), and the reagent blank (broken line).

mixed with the colour reagent in reaction coil 1. The colour reagent is pumped to the reaction coil 1 by pump 1; 2.5% ammonia solution pumped by pump 2 is then added to the mixture and reacted in coil 2. The reaction product is detected by the SPD-1A detector at a wavelength of 650 nm.

Fig. 2 shows the absorption spectrum of pyridoxine hydrochloride (solid line) and the reagent blank (broken line). A 10-μl volume of a solution of pyridoxine hydrochloride at a concentration of 1.03 mg/ml (in a blank test, 10 μl of distilled water) and 100 μ of 25% ammonia solution were poured into 2 ml of reagent solution (50 mg dissolves in 200 ml of ethanol) in the cuvette, and the absorbance spectrum was obtained with the UV-240 spectrophotometer. As shown in Fig. 2, pyridoxine hydrochloride has an absorption maximum at 650 nm, but reagent blank solution also has almost one sixth of this absorbance at this wavelength, which causes a rise of the baseline.

In order to obtain the spectrum, 25% ammonia solution was poured into the cuvette, following the original method adopted in the Japanese Pharmacopoeia[8]. However, in this system, in order to avoid harmful ammonia vapour, a 2.5% concentration of ammonia solution was used as a regent, pumped by pump 2. The difference in the ammonia concentration can be ignored (measured with the UV-240; results not shown); we used 2.5% ammonia solution so as to make handling easier.

The effect of reagent concentration, length and temperature of reaction coil and flow-rate of the reagent on the peak height of pyridoxine hydrochloride was studied. The optimal conditions obtained from these experiments are presented in Table I.

The increase in peak height with increase in concentration or flow-rate attains a plateau at a concentration of 0.5 mg/ml or at a flow-rate of 1.4 ml/min. Concentrations higher than 0.5 mg/ml do not give any further increase in peak height and cause baseline fluctuations. A decrease in peak height, caused by dilution owing to the increase in flow-rate, did not occur in the range of flow-rates examined (0.2–1.8 ml/min). Temperatures of the reaction coil higher or lower than room temperature (25°C) resulted in smaller peak heights.

The calibration graph obtained under the optimal conditions is linear over the range of 50–1000 ng injected, and 10 ng of pyridoxine hydrochloride is the lower limit that can be identified as a peak. As the reagent blank shows absorption at the

TABLE I

OPTIMAL CONDITIONS

Mobile phase: 10 mM sodium 1-hexanesulphonate, 5 mM sodium perchlorate, 10% methanol (pH 2.5, adjusted with perchloric acid); flow-rate, 0.8 ml/min. Injection volume: 10 μl of an aqueous solution of pyridoxine hydrochloride (1.0 mg/ml). Pump 1, 0.8 ml/min and pump 2 1.2 ml/min for reagent concentration experiments; pump 1 1.4 ml/min and pump 2 1.0 ml/min for other experiments.

Parameter	Optimal value
Reagent concentration	0.5 mg/ml
Flow rate of pump 1	1.4 ml/min
Coil length (coil 1)	2 m (I.D. 0.5 mm; coil 2 is fixed to 2 m)
Reaction temperature	25°C

TABLE II

PEAK AREAS CORRESPONDING TO AN INJECTED AMOUNT OF 500 ng OF EACH COMPOUND

Compound	Peak area ($\mu V \cdot sec$)	Compound	Peak area ($\mu V \cdot sec$)
Pyridoxine hydrochloride	8201	Pyridoxamine 5'-phosphate	13917
Pyridoxal hydrochloride	11154	Pyridoxal 5'-phosphate	1616
Pyridoxamine dihydrochloride	9415	Catechol	1615
Deoxypyridoxine hydrochloride	10560	o-Xylenol	415
4-Pyridoxic acid	4036	p-Aminophenol	1016

wavelength used, pulsation of the reagent pump causes vibration of the baseline, so identification of peaks representing less than 10 ng is difficult.

Peak areas corresponding to injected amounts of 500 ng of five compounds of vitamin B_6, 4-pyrioxic acid (metabolite of vitamin B_6), deoxypyridoxine (used as an internal standard) and catechol, o-xylenol and p-aminophenol which may interfere with the detection are presented in Table II.

4-Pyridoxic acid, which is an excretion metabolite of vitamin B_6, has almost half the sensitivity of vitamin B_6 and pyridoxal 5'-phosphate is less sensitive than other vitamin B_6 compounds. Phenols have much lower sensitivity than vitamin B_6 and will not interfere with the determination of vitamin B_6 even if they are present in the sample.

Fig. 3 shows the chromatogram of three compounds of vitamin B_6 and 4-pyridoxic acid. Because of the separation selectivity of reversed-phase ion-pair chromatography[7], the four compounds are well separated and were eluted within 14 min.
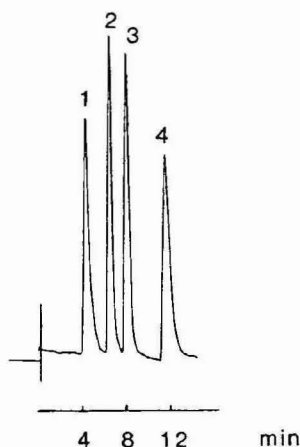
Fig. 3. Chromatogram of three compounds of vitamin B_6 and 4-pyridoxic acid. The mobile phase does not contain methanol; other conditions as in Table I. Amounts injected were 5.25 μg of pyridoxine hydrochloride, 5.2 μg of pyridoxal hydrochloride, 9.9 μg of 4-pyridoxic acid and 4.7 μg of pyridoxamine dihydrochloride; attenuation 0.64 a.u.f.s. Column temperature, 45°C. Peaks: 1 = 4-pyridoxic acid; 2 = pyridoxal hydrochloride; 3 = pyridoxine hydrochloride; 4 = pyridoxamine dihydrochloride.

UV 254nm   this system
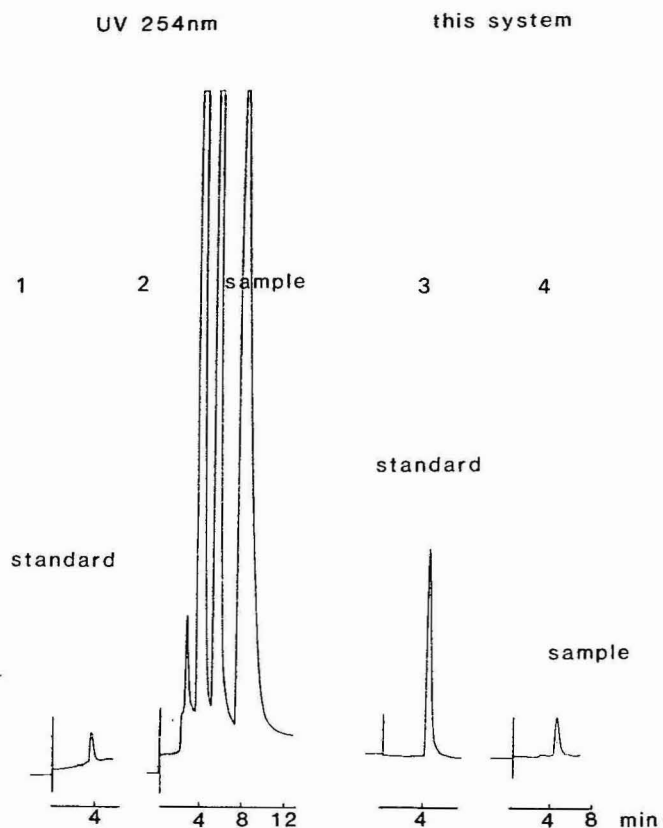
Fig. 4. Application of the system to the analysis a vitamin B tablet. The mobile phase contains 5 mM sodium 1-hexanesulphonate, 5 mM sodium perchlorate and 20% methanol (pH 2.5). Flow-rate, 0.8 ml/min; pumps 1 and 2, 1.0 and 1.2 ml/min, respectively. Pyridoxine hydrochloride: 0.95 mg/ml; 5.0 μl injected. Vitamin B tablet: 34.1 mg/ml, 100 μl injected. Attenuation 0.64 a.u.f.s.

Fig. 4 shows the application of this system to the analysis of a vitamin B tablet; the two chromatograms on the left are examples of detection by conventional UV (254 nm) absorption, and the two on the right are examples of the present system. The present system is almost seven times more sensitive than UV (254 nm) detection (see chromatograms 1 and 3). In the analysis of a vitamin B tablet, using UV (254 nm) detection (chromatogram 2) it is difficult to indentify pyridoxine hydrochloride because of interfering substances (other vitamin B compounds, caffeine or impurities sensitive at the wavelength used), but in the present system, no peak except pyridoxine hydrochloride is eluted, which indicates the high selectivity.

Hence when analysing one vitamin B_6 compound, it is not necessary to separate it from other interfering compounds because of the high selectivity; this system may therefore be applicable to flow injection method.

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CHROM. 15,951

Note

Adsorption chromatographic separation of ^{125}I-labelled estriol and estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester

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Steroid molecules labelled with ^{125}I are being increasingly used instead of tritium-labelled molecules as tracer in the radioimmunoassay. Although radioiodine can be incorporated via electrophilic substitution into steroids with an aromatic A-ring in the 2- and/or 4-positions[1,2], the iodination of the steroid itself in the A-ring is considered to result in the complete loss of tracer affinity to the antibody[3,4]. Therefore, radioiodine is usually incorporated via electrophilic substitution into a side-chain [tyrosine methyl ester (TME), histamine, etc.] coupled to the steroid skeleton. In steroids with an aromatic A-ring (*e.g.*, estriol and 17-β-estradiol), electrophilic substitution takes place simultaneously in the 2- and/or 4-position of the A-ring and in the 3- and/or 5-position of the TME side-chain.

In order to avoid the formation of the A-ring-labelled 17-β-estradiol-6-(O-carboxymethyl)oxime histamine. Nars and Hunter[3] conjugated the already iodinated [^{125}I]histamine to the 6-(O-carboxymethyl)oxime derivative of the 17-β-estradiol, extracted the tri-*n*-butylamine and isobutyl chloroformate activators with toluene and separated the radioiodinated 17-β-estradiol-6-(O-carboxymethyl)oxime by thin-layer chromatography (TLC). Finally, the ^{125}I-labelled 17-β-estradiol-6-(O-carboxymethyl)oxime histamine was eluted from the silica powder scraped from the plate[3].

The aim of this paper is to show that estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester (ETME) labelled with ^{125}I in the tyrosine methyl ester (TME) side-chain can be separated from the inactive starting material and from an unidentified labelled by-product using Sephadex LH-20 dextran gel as the adsorbent and ethanol–water as the eluent.

When ETME (see Fig. 1) is labelled with ^{125}I by the use of the chloramine-T method, the simultaneous formation of labelled 2-, 3'- and 4-iodo-ETME and 2,3'-,
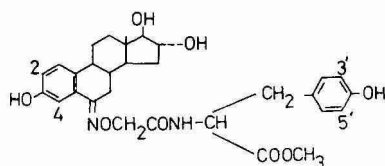
Fig. 1. Estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester. The figures indicate the positions into which radioiodine can be introduced via electrophilic substitution.

2,4- and 3',5'-diido-ETME has to be taken into account. Nevertheless when the amount of ^{125}I used in the chloramine-T labelling procedure is sub-stoichiometric compared with that for ETME, the formation of the diiodo derivatives can be precluded. Of the monoiodo-ETME derivatives, only 3'-iodo-ETME is expected to be bound to the antiserum raised against the same derivative, *i.e.*, estriol-6-(O-carboxymethyl)oxime bovine serum albumin (BSA).

EXPERIMENTAL

The apparatus and adsorbent used have been described previously[5-8]. ETME and estriol were labelled with ^{125}I by the use of a slightly modified version of the chloramine-T method which will be published later. To 10–20 μg (20–40 nmole) of ETME (mol.wt. = 552) or estriol dissolved in aqueous ethanol 100 μl of phosphate buffer (pH 7.6) were added, then 1–2 mCi (37–74 MBq) of carrier-free [125]I (0.5–1.0 nmole) followed by the addition of 20–50 μl of an aqueous solution containing 100–200 μg of chloramine-T. The labelling reaction was quenched after 30–60 sec with 50 μl of an aqueous solution containing 350 μg of sodium metabisulphite.

Sephadex LH-20 dextran gel was swollen in citrate buffer (pH 4) prior to being packed in the column (130 × 10 mm I.D.). The height of the packing was 100 mm. In order to check the separation of the ETME from the ^{125}I-labelled ETME, tritium-labelled ETME was also chromatographed separately from the chloramine-T labelling mixture.

In all instances the sample (0.1–0.2 ml) from the chloramine-T labelling procedure was placed on the top of the column and allowed to soak in it. After 10–20 min, *i.e.*, when adsorption equilibrium had been attained, the elution was performed with aqueous ethanol, the pH of which was adjusted to 4 so as to supress the dissociation of the phenolic hydroxyl groups. The effluent was passed over a NaI(Tl) scintillation crystal and the count rate monitored by a ratemeter and registered by an *x–y* plotter. A peristaltic pump, flow-rate 22–24 ml/h, delivered the eluent.

When tritium-labelled ETME was chromatographed the effluent was collected with a fraction collector and its radioactivity was determined by liquid scintillation counting.

The distribution coefficient was calculated according to

$$K = \frac{V_e - V_0}{W} \tag{1}$$

where V_e, V_0 and W are the elution volume, the dead volume and the weight of the adsorbent, respectively.

RESULTS AND DISCUSSION

The elution pattern obtained when tritium-labelled ETME was chromatographed is shown in Fig. 2. Omitting the elution peak of the free radioiodine, the elution pattern recorded when the chloramine-T labelling mixture of ETME was ·chromatographed is shown in Fig. 3. Of the two elution peaks the first was assigned to an unidentified labelled by-product. The second peak is attributed on the basis of the immunoreactivity (see later) to 3'-iodo-ETME.
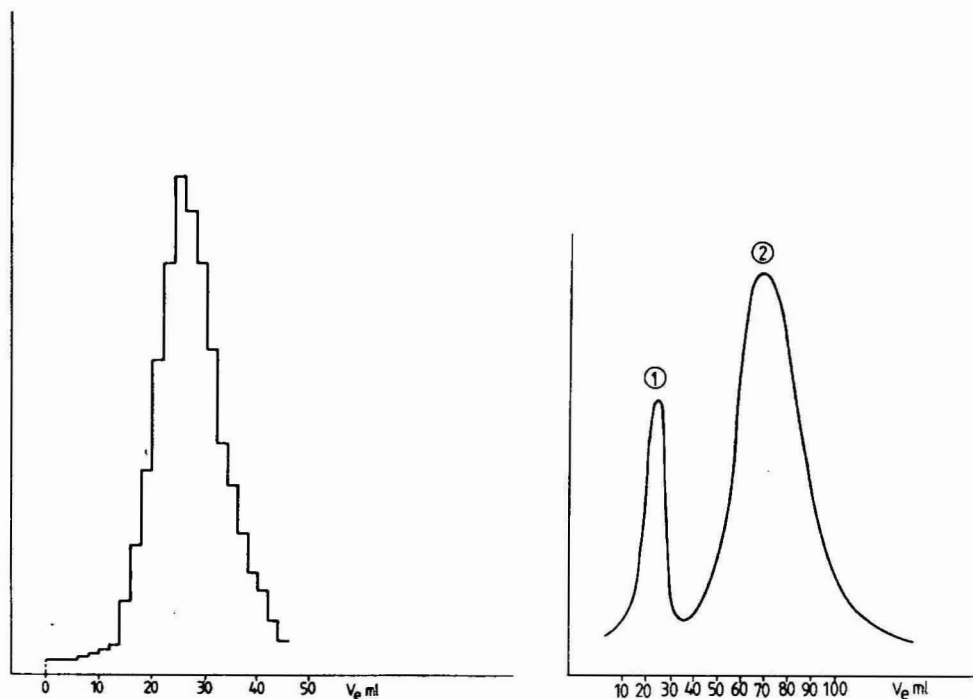
Fig. 2. Elution pattern of [³H]ETME. Eluent: 30% aqueous ethanol (pH 4).

Fig. 3. Elution pattern of the chloramine-T labelling mixture for ETME. Eluent: 30% aqueous ethanol (pH 4). Peak 1 = unidentified labelled compound; peak 2 = [3'-^{125}I]ETME.

The elution volume of the labelled by-product and that of the 3'-iodo-ETME decreases monotonously with increasing ethanol concentration of the eluent. This is illustrated in Fig. 4, which shows the elution patterns obtained at different ethanol concentrations. It can be seen from these elution curves that with a 40% ethanol concentration the separation is unsatisfactory whereas with 50% ethanol no separation of the two labelled products can be achieved at all. With 30% ethanol the difference in the elution volumes of ETME (26 ml) and 3'-iodo-ETME (78 ml) is large enough to achieve complete separation of the starting material and the 3'-iodo-ETME (see Figs. 2 and 3).

Estriol was also labelled with ^{125}I using the same procedure as in the case of ETME. The elution pattern obtained when the labelling mixture of estriol was chromatographed is shown in Fig. 5. As the formation of 2,4-diiodoestriol can be precluded owing to the large excess of the starting material compared with the amount of ^{125}I (*i.e.*, 20–40 nmole to 0.5–1.0 nmole) the elution peak seen in Fig. 5 can only be attributed to 2- or 4-iodoestriol.

Immunoreactivity of the ^{125}I-labelled estriol and ETME

In order to check the immunoreactivity of [^{125}I]estriol and [^{125}I]ETME, the labelled compounds corresponding to the elution peaks shown in Figs. 3 and 5 were incubated with antiserum raised against estriol-6-(O-carboxymethyl)oxime bo-
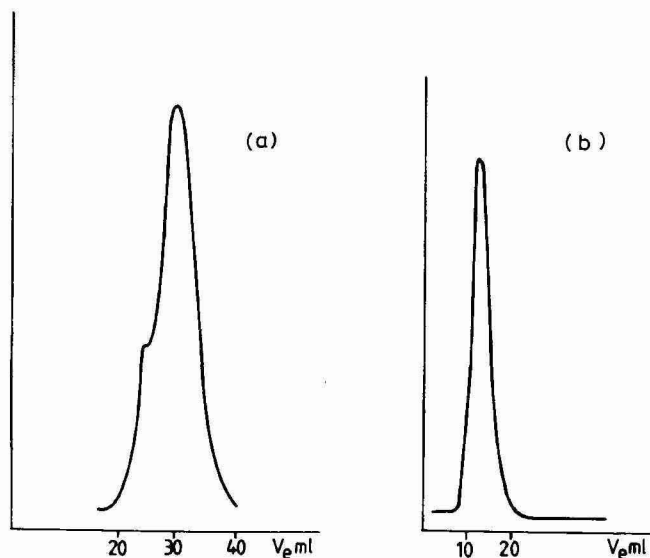
Fig. 4. Effect of ethanol concentration on the separation of the chloramine-T labelling mixture for ETME. Eluent: (a) 40% aqueous ethanol (pH 4); (b) 50% aqueous ethanol (pH 4).

vine serum albumin conjugate. As the antiserum does not distinguish between estriol and [^{125}I]ETME (labelled in the tyrosine residue) on the one hand, and does not bind A-ring-labelled estriol or ETME on the other, it was expected that only 3'-iodo-ETME (*i.e.*, the second elution peak in Fig. 3) would exhibit immunoreactivity. This is clearly supported by the data in Table I.

Effect of the organic solvent concentration on separation

The distribution coefficient (K) *versus* ethanol concentration in the eluent relationship is

$$\log K = \log K_0 - n \log x \tag{2}$$

where x is the ethanol concentration in the eluent expressed as a molar fraction, K_0 is the distribution coefficient extrapolated to $x = 1$ and n is a constant. Inserting actual K_0 and n values, K can be expressed as

$$\log K = -0.69 - 2.4 \log x \text{ for 2- or 4-iodoestriol} \tag{3}$$

$$\log K = -0.52 - 2.3 \log x \text{ for 3'-iodo-ETME} \tag{4}$$

TABLE I

BINDING OF ^{125}I-LABELLED ESTRIOL AND ETME DERIVATIVES

^{125}I-labelled derivative	Binding (%)
2- or 4-iodoestriol	< 1
3'-Iodo-ETME	60–70
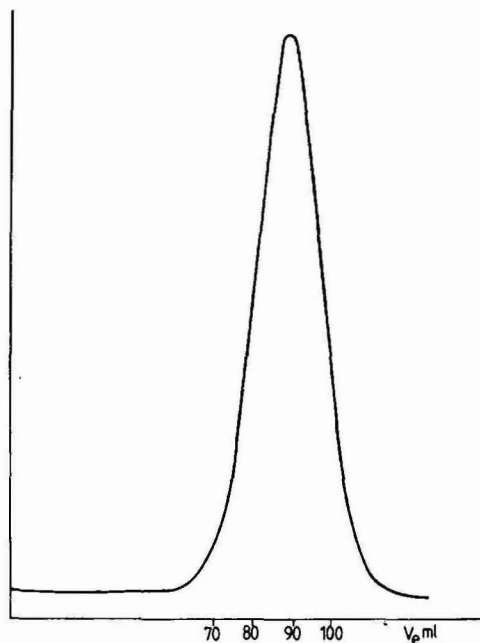
Fig. 5. Elution pattern of the chloramine-T labelling mixture for estriol. Eluent: 30% aqueous ethanol (pH 4).

Eqn. 2 also holds for ^{125}I-labelled iodothyronines[5], prostaglandins[6], testosterone[7] and progesterone[8].

The log K *versus* log x relationship for 2- and 4-iodoestriol and 3'-iodo-ETME is shown in Fig. 6.

CONCLUSIONS

2 and/or 4-iodoestriol and 3'-iodo-ETME are reversibly adsorbed on Sephadex LH-20 dextran gel and their selective elution can be performed by the use of water–ethanol as the eluent. The distribution coefficient defined by eqn. 1 varies with the organic solvent concentration of the solvent according to eqn. 2.

According to expectation, chloramine-T labelling of estriol results in the formation of 2- or 4-iodoestriol only if the amount of ^{125}I is sub-stoichiometric compared with that of the estriol. As the Sephadex LH-20 dextran gel does not distinguish between 2- and 4-iodoestriol, only a single elution peak appears when the labelled mixture of estriol is chromatographed. With ETME the electrophilic substitution of ^{125}I may take place either in the A-ring or in the tyrosine residue. Thus two labelled ETME derivatives can be expected to be formed in the chloramine-T labelling procedure.

Actually, apart from an unidentified labelled by-product there is only a single labelled product formed, which because of its immunoreactivity is attributed to 3'-iodo-ETME. The lack of formation of A-ring-labelled ETME might be due to the steric hindrance caused by the 6-(O-carboxymethyl)oxime TME side-chain or by the
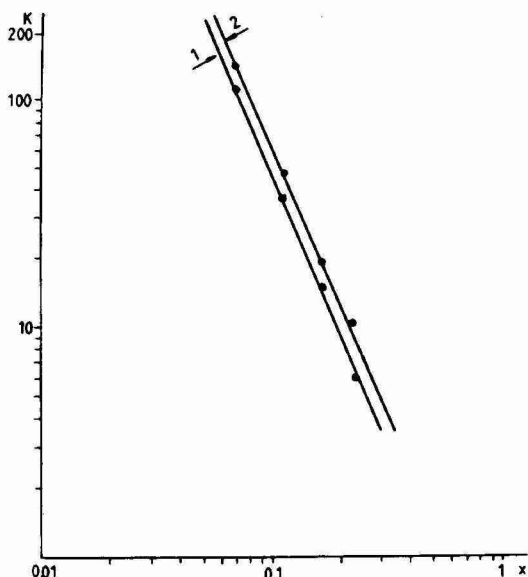
Fig. 6. Distribution coefficient *versus* ethanol concentration. 1 = 2- or 4-iodoestriol; 2 = 3'-iodo-ETME.

lower rate of substitution of iodine into the A-ring compared with that into the TME side-chain.

When the chromatography is aimed at the production of high specific activity and radiochemically stable ETME labelled in the tyrosine residue, it is recommended that free radioiodine and the ^{125}I-labelled by-product is eluted with 30% aqueous ethanol followed by elution of the immunoreactive [3'-^{125}I]ETME with 50% aqueous ethanol. The elution pattern thus obtained is shown in Fig. 7.

The [3'-^{125}I]ETME thus obtained can be used as a tracer in the radioimmunoassay of estriol at least for 3 month. The long shelf-life is attributed to the low
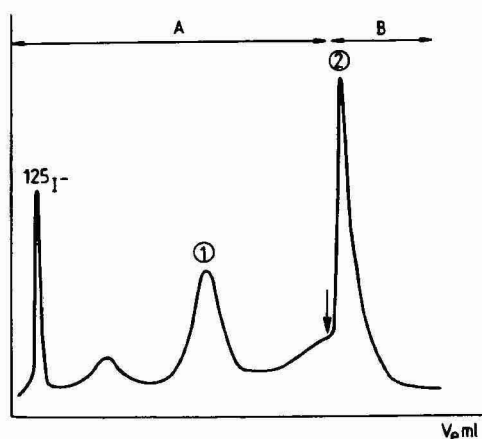
Fig. 7. Optimization of the separation of [3'-^{125}I]ETME. Eluent: (A) 30% aqueous ethanol; (B) 50% aqueous ethanol. Peak 1 = unidentified labelled compound; peak 2 = [3'-^{125}I]ETME.

dielectric constant (relative permittivity) of the solvent, which substantially increases the radiochemical stability[9].

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Note

Gas–liquid chromatographic method for determining propylenthiourea in rat tissues and fluids

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Alkylenebis(dithiocarbamates), widely used as fungicides in agriculture, are degraded to several compounds which play an important toxic role[1]. Ethylenebis(dithiocarbamates) (EBDC) have been investigated in depth, in particular their main degradation product, ethylenthiourea (ETU), which has been shown to be mutagenic and carcinogenic[2–4]. This led us to examine the toxic effects of propylenebis(dithiocarbamates), the degradation of which has not been widely investigated[5,6] although these fungicides are used for agricultural purposes.

A comprehensive study of the toxic effects of these compounds has to take into account the quantitative determination of the degradation products, which can be formed *in vivo*. For this purpose, a simple analytical method had to be developed to detect nanogram amounts of degradation products in tissues and biological fluids.

At present, we have limited our study to the analysis of the main metabolite of Propineb, propylenethiourea (PLTU), previously poorly investigated[7] with regard to its toxic effects *in vivo*. Several methods[8–13] have been described for determining the ETU concentration in vegetable samples; all are based on a derivatization procedure followed by gas chromatographic analysis. The chemical structures of PLTU and ETU are so similar that we thought that the ETU derivatization procedures would also be suitable for PLTU.

A detailed examination of the methods described led us to choose two of them: the method proposed by Nash[12], which is based on a double derivatization procedure using *o*-chlorobenzyl chloride and pentafluorobenzyl chloride or trifluoroacetic anhydride, and the method proposed by King[13], who used *m*-trifluoromethylbenzyl chloride. In a preliminary step, we tried to derivatize PLTU following the Nash procedure, which was claimed to be more sensitive than that of King, but the possibility of obtaining two isomers, owing to the presence of an acyl group alternatively in the 1- or 3-position on the imidazoline ring, led us to select the method proposed by King.

EXPERIMENTAL

Standard preparation techniques

PLTU was prepared according to the method described by McKay and Hat-

ton[14]. S-(m-Trifluoromethylbenzyl)propylenethiourea hydrochloride was prepared according to the method described by Boyd and Meadow[15]: m.p., 149–150°C; found, C 46.33, H 4.64, N 8.92%; calculated for $C_{12}H_{14}ClF_3N_2S$, C 46.38, H 4.54, N 9.02%; NMR (δ) (solvent $CDCl_3$), 1.2 (d, J = 3.0 Hz, CH_3), 3.0–4.4 (m, CH_2CH), 4.52 (s, CH_2), 7.0–7.63 (m, aromatic), offset (broad d, J = 8.0, NH · HCl; disappeared with D_2O).

Propineb was prepared according to reported procedure[16].

Reagent and apparatus

Trifluoromethylbenzyl chloride was supplied by EGA-Chemie. All solvents were of special grade for pesticide analysis from Merck.

A Varian 3700 gas chromatograph, equipped with a ^{63}Ni detector, a Varian CDS 111 integrator and a Varian A25 recorder was used.

Experimental procedures

The rat organs and fluids tested were fortified with a detectable amount of PLTU in order to examine the applicability of the derivatization procedures to real samples. All of the samples were derivatized following the procedure described below.

The sample was homogenized in 95% ethanol (1:5, w/v) and then centrifuged at 8000 g for 15 min at 5°C. Four drops of derivatization agent were added to 5 ml of the supernatant and the mixture was refluxed for 1 h in a 10-ml graduated tube. After cooling the mixture, the condenser was washed with 1–2 ml of 95% ethanol, then 1–2 drops of 6 N hydrochloric acid were added to the reaction tube; the ethanol was then removed by a Rotavapor apparatus (Büchi, Switzerland) at 35°C.

To the residue was added 2 ml of distilled water and the mixture washed twice with 1 ml of diethyl ether, which was subsequently removed with a Pasteur pipette; the remaining ether was removed in a water-bath at 50°C.

After cooling the mixture, 0.5–1.0 ml of benzene was added, followed by 0.3–0.5 ml of 10% sodium hydroxide solution. The mixture was immediately shaken and centrifuged and 1–5 μl of the organic layer were injected in the gas chromatograph using a 2 m × 2 mm I.D. glass column packed with 3% OV-275 on 80–100-mesh Chromosorb G. The operating conditions were as follows: column temperature, 195°C; detector temperature, 300°C; injector temperature, 200°C; nitrogen flow-rate, 30–35 ml/min.

RESULTS AND DISCUSSION

The above method was used to determine PLTU in liver, brain, kidney, heart, spleen, thyroid gland, muscle, adipose tissue, ovary, uterus, placenta, foetus, serum and urine. No interfering peaks were observed, as can be seen in Fig. 1.

A recovery test was performed on rat urine, adding PLTU to a test sample in order to obtain a final concentration of 5 ppm. This fortified sample was then derivatized. The recovery was 93.2 ± 8.7% (mean ± S.D., n = 5). The linearity range was tested from 2 to 20 ng injected.

The PLTU concentration in the urine of rats that had been given different amounts of Propineb was determined in order to obtain some preliminary information on Propineb metabolism. The method proposed by Cullen[17] was applied to the
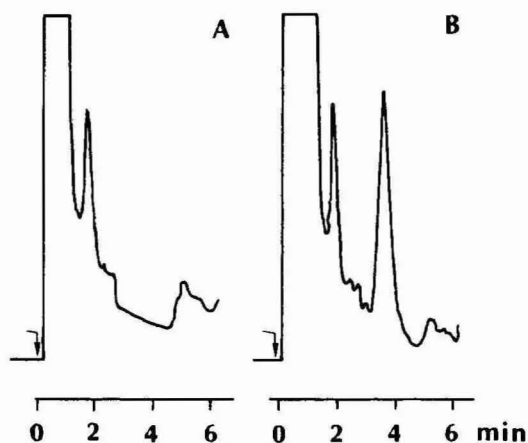
Fig. 1. Chromatograms of (A) urine sample derivatized using King's method[13]; (B) urine sample fortified with 1.5 ppm of PLTU and then derivatized.

same urine samples, to reveal the unmodified Propineb and all those metabolites, such as propylenethiourea disulphide, which can give carbon disulphide after acid hydrolysis. The results in Table I indicate that both the derivatization procedure and the analysis of biological fluids and tissues were successful.

As pointed out by King[13], the use of the S-(m-trifluoromethylbenzyl) derivative avoids a double reaction sequence and possibilities for loss of the sample; moreover, the gas–liquid chromatographic analysis is very rapid for the reduced retention time, and the sensitivity (0.1 ppm) is satisfactory for our purpose. Therefore, we can conclude that King's derivatization method is suitable for the determination of PLTU in biological materials. Our future studies will be extended to the development of methods for the analysis of all the propylenebis(dithiocarbamates) metabolites to provide detailed information on the toxicity of these pesticides.

ACKNOWLEDGEMENTS

TABLE I

URINE EXCRETION (mg) OF PLTU AND CS_2 IN THE RAT AFTER ADMINISTRATION OF PROPINEB p.o.

Propineb (g/kg, p.o.)*	24 h		48 h		72 h	
	PLTU	CS_2	PLTU	CS_2	PLTU	CS_2
1.063	5.3	0.08	5.2	N.D.**	1.50	N.D.
2.125	6.8	0.20	6.5	N.D.	0.95	N.D.
4.250	11.0	0.20	11.6	N.D.	2.50	N.D.

    * The doses listed are 1/8, 1/4 and 1/2 of the LD_{50}, respectively; each dose was given to three rats and the determinations were performed on the pooled urines.
    ** N.D. = Not detected.

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Note

Analysis of terpenes from *Ginkgo biloba* L. by high-performance liquid chromatography

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Ginkgo biloba L., used in the treatment of vascular diseases, occupies a unique position in botany and chemistry: as this "living fossil" has remained unchanged for 200 million years and is the sole surviving species of the Ginkgoales group, it shows many botanical originalities[1,2]. From the chemical point of view, it synthesizes terpenoid compounds called the "bitter principles of the ginkgo tree"[3], whose structures are very complex and unusual. Three diterpenes and one sesquiterpene are reported to be present. The ginkgolides (diterpenes) were first isolated from the root bark[4], and later from the leaves of the Ginkgo[5]. They contain three γ-lactone rings and a *tert.*-butyl group. They differ from each other in the number and the position of the hydroxyl groups (Fig. 1). Bilobalide (a sesquiterpene) was isolated only from the leaves[6,7]. Its structure is very similar to those of the ginkgolides, as shown in Fig. 1.

Several workers have achieved, with much difficulty the separation of these terpenes by liquid (LC) and thin-layer chromatography (TLC). These techniques are still limited and unsatisfactory, especially the latter, which suffers from a lack of sensitivity and specificity because of detection problems.

This paper describes the high-performance liquid chromatographic (HPLC) separation of a mixture of these terpenes and the detection of small amounts of three of them in a purified extract prepared from *Ginkgo biloba* L. leaves.

EXPERIMENTAL

Chemicals

The solvents used for the HPLC analysis (acetonitrile, tetrahydrofuran and methanol) were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) and filtered with a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). Fresh deionized, glass-distilled water, filtered with a 0.45 μm Millipore filter, was used.

Other solvents (cyclohexane, chloroform, ethyl acetate, diethyl ether and ethanol) and chemicals (hydrochloric acid, acetic anhydride, anhydrous sodium sulphate) used for the standard mixture and the Ginkgo leaf extract preparations were of analytical-reagent grade.

Apparatus

Analytical liquid chromatographic separations were conducted with a Perkin-

Diterpenes:
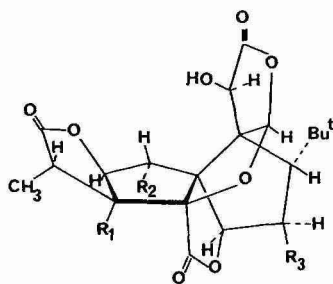
GINKGOLIDES

	R_1	R_2	R_3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH

Sesquiterpene:
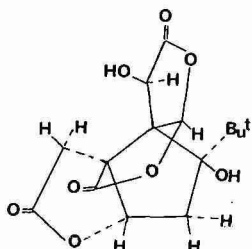
BILOBALIDE

Fig. 1. Structures of terpenes from *Ginkgo biloba* L.

Elmer Model 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne Model 7125 injection valve and a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). Detection was carried out with an LC-75 variable-wavelength spectrophotometric detector connected to an LC-75 Autocontrol module (Perkin-Elmer). A Hibar column (25 × 0.4 cm I.D.) containing LiChrosorb RP-18 (10 μm) (Merck) was used for separations.

Preparative LC purifications were conducted with a Chromatospac Prep 10 (Jobin-Yvon, Longjumeau, France) with a silica stationary phase [250 g of silica H 60, 0.063 0.2 mm (Merck) in a 40 mm I.D. column].

Preparation of standard mixture

A purified mixture of ginkgolides and bilobalide was prepared by extraction from an already purified *Ginkgo biloba* L. leaf extract, prepared according to reported techniques[8]. Dried, powdered, purified *Ginkgo biloba* L. leaf extract (120 g) was treated with boiling ethyl acetate (7 × 600 ml). The absence of terpenes in the last ethyl acetate extract was controlled by TLC examinations. It was chromatographed over aluminium sheets coated with silica gel 60F 254 (20 × 20 cm, layer thickness 0.2 mm; Merck) using cyclohexane–ethyl acetate (50:50) as the solvent. The developed chromatograms were air-dried, sprayed with acetic anhydride reagent and heated at

150°C for 20–30 min. Under these conditions, ginkgolides and bilobalide show a faint orange fluorescence at 365 nm.

The combined ethyl acetate extracts were concentrated to 300 ml, treated with the same volume of chloroform, cooled overnight, decanted and finally filtered. The filtrate was evaporated to dryness under vacuum and the residue was extracted with several successive portions of diethyl ether until free from terpenes (TLC examination). The combined diethyl ether extracts were dried over anhydrous sodium sulphate, then distilled under reduced pressure to a dark syrupy liquid (21.5 g). The latter was chromatographed on a silica gel column (250 g) with a preparative apparatus (Chromatospac Prep-10) using cyclohexane–ethyl acetate (40:60) as eluting solvent. Fractions of 100 ml were collected, monitored by TLC as mentioned above, then the terpene-rich fractions were concentrated. It was found that further purification of ginkgolides and bilobalide can be obtained by successive precipitations with chloroform, diethyl ether and methanol.

Separation and identification of terpenes

The identity of each terpene of this pure mixture was established by TLC and HPLC examinations, in comparison with pure authentic reference compounds:

TLC examinations of the standard mixture were carried out as described above and revealed the presence of four spots (R_F = 0.46, 0.34, 0.30 and 0.15), corresponding to the reference compounds (bilobalide and ginkgolides A, B and C, respectively).

HPLC analyses were carried out at room temperature with the following isocratic systems:

System A: water–methanol (70:30), flow-rate 1 ml/min;

System B: water–acetonitrile (80:20), flow-rate 1 ml/min;

System C: water–tetrahydrofuran (80:20), flow-rate 1 ml/min;

System D: water–methanol–tetrahydrofuran (75:5:15), flow-rate 1.5 ml/min.

Detection was effected at a wavelength of 220 nm (range 0.02 a.u.). A volume of 15 μl of the standard methanolic solutions was injected with a Hamilton syringe.

Preparation of the Ginkgo biloba L. leaf extract for HPLC

Leaves of *Ginkgo biloba* L. were collected in October 1980 from the botanical

TABLE I

HPLC CHARACTERISTICS OF THE TERPENES FROM *GINKGO BILOBA* L.

Compound	Peak	Elution system*							
		A		B		C		D	
		t_R**	S***	t_R	S	t_R	S	t_R	S
Ginkgolide A	2	21.4	1.08	27.0	1.80	23.2	1.50	11.9	1.00
Ginkgolide B	3	24.2	1.12	27.0	1.80	37.4	1.25	19.3	1.20
Ginkgolide C	1	10.6	1.00	13.6	1.00	17.2	1.50	9.2	1.00
Bilobalide	4	8.8	1.17	10.0	1.00	24.8	1.06	13.6	1.14

* See *Separation and identification of terpenes*.

** t_R = retention time (min).

*** S = peak symmetry.

garden at Strasbourg. Air-dried, powdered leaves (30 g) were extracted with ethanol in a Soxhlet apparatus. The ethanol extract was filtered and then evaporated under vacuum to a thick residue, which was suspended in water (100 ml) and then extracted with cyclohexane (10 × 150 ml). The partially purified aqueous phase was acidified to pH 2 with hydrochloric acid (1 N) and extracted with ethyl acetate (10 × 150 ml). The organic phase was dried over anhydrous sodium sulphate and distilled under reduced pressure. The residue was dissolved in water (50 ml) and extracted with diethyl ether (15 × 50 ml). The combined ether extracts were dried over anhydrous
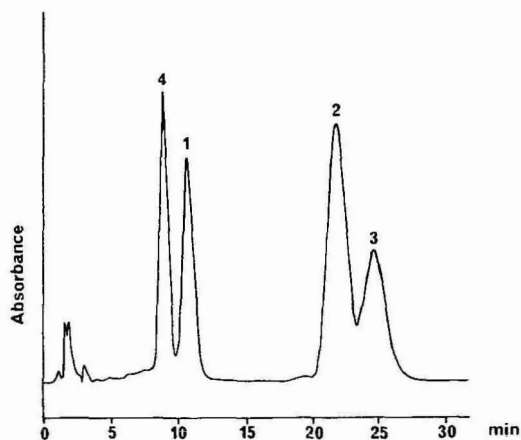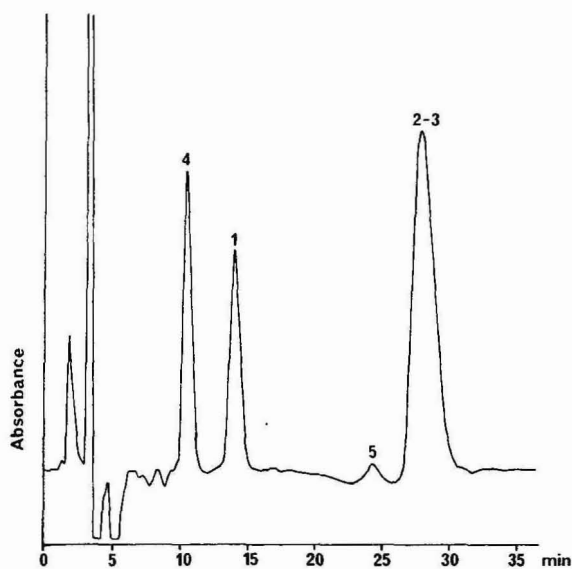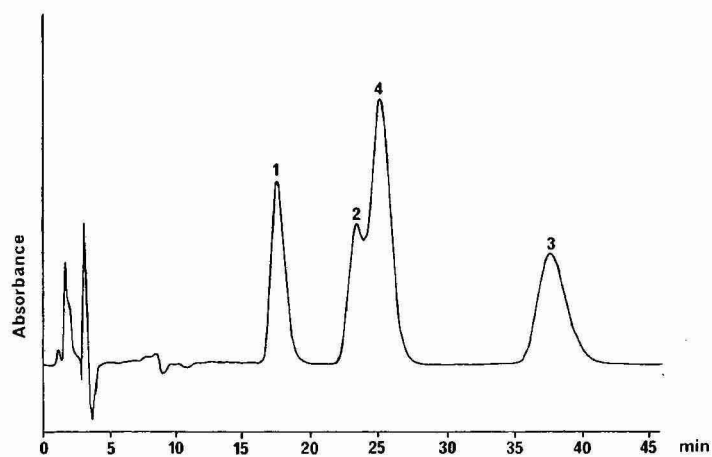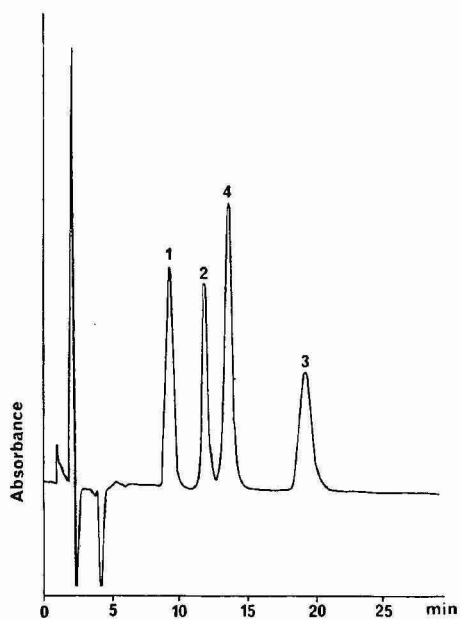
Fig. 2.

Fig. 2. HPLC traces of terpene mixture from *Ginkgo biloba* L. Conditions: (A) solvent system A; (B) system B; (C) system C; (D) system D. Peaks: 1 = ginkgolide C; 2 = ginkgolide A; 3 = ginkgolide B; 4 = bilobalide.

sodium sulphate and the solvent was evaporated to dryness under vacuum. The residue was dissolved in methanol (1 ml) and kept for HPLC analysis. Detection was effected at a wavelength of 220 nm (range 0.08 a.u.).

RESULTS AND DISCUSSION

Selectivity of the elution systems

Fig. 2 shows the HPLC resolution of a pure mixture of standards, in four isocratic sytems.

Table I lists the different HPLC characteristics of the Ginkgo terpenes.

System A. With these first elution conditions, all four peaks are symmetrical (Table I). The resolution factor of peaks 2 and 3 (0.82) is less satisfactory than those of peaks 1 and 4 (0.97). Several attempts made to improve the resolution limit of the ginkgolides A and B, using gradient elution or flow programmes, were unsuccessful.

Other isocratic systems were chosen according to Schoenmakers *et al.*[9]. The procedure permits one to establish, for the elution power of a particular water–methanol mixture, the equivalent compositions of water–acetonitrile or water–tetrahydrofuran mixtures. In our case, the theoretical optimal isocratic conditions were water–acetonitrile (80:20) = system B and water–tetrahydrofuran (80:20) = system C.

System B. With these second elution conditions, the peaks are also symmetrical (Table I). Peaks 1 and 4 are totally separated (resolution factor = 4), but peaks 2 and 3 overlap. Any gradient elution or flow programme ameliorated their separation. Therefore, this chromatographic system was eliminated.

Nevertheless, it is of interest to note that under these conditions, a minor peak was appeared (peak 5, retention time, t_R = 23.4 min). This peak could be either a Ginkgo terpene such as the ginkgolide M present in very small amounts in the Ginkgo leaves and previously described by Nakanishi[4], or a new di- or sesquiterpene. Its identification needs further investigation.

System C. All the peaks still show good symmetry (Table I). The resolution factors of peaks 2 and 3 (4.08) and peaks 1 and 4 (2.81) are much greater than in the two last elution systems, but those of peaks 2 and 4 (0.56) are now unsatisfactory. For this reason, this chromatographic system was also eliminated.

Nevertheless, these conditions could be useful for the separation of a mixture of ginkgolides A and B; the separation of these two diterpenes usually needs a 10–15-step fractional crystallization procedure and is complicated by a strong tendency of ginkgolide A to exhibit polymorphism[4]. Therefore, this new chromatographic system could provide more satisfactory results for the resolution of this particular problem.

System D. According to Roggendorf and Spatz's results on the selectivity benefits of a ternary mobile phase containing tetrahydrofuran in reversed-phase liquid chromatography[10], a fourth elution system was tried with a ternary mixture: water–methanol–tetrahydrofuran. The best results were obtained with the proportions 75:5:15 (system D).

These chromatographic conditions allowed satisfactory separations, as the four constituents of the standard mixture gave perfectly symmetrical and well resolved peaks (Table I). The resolution factors of peaks 1 and 2, 2 and 4, and 4 and 3 are 1.60, 1.06 and 2.32, respectively. Moreover, the analysis time is reduced (20 min instead of 25, 27 or 38 min for the systems A, B and C, respectively). For these reasons, this elution system was the system of choice for the analysis of a purified extract of *Ginkgo biloba* L. leaves.

HPLC of a purified extract of Ginkgo biloba L. leaves

The ginkgolides and bilobalide are present in very small amounts in *Ginkgo biloba* L. At the wavelength of analysis, they have low UV absorption coefficients; their minimum detectable amounts are approximately 30 μg. Therefore, it is impossible to detect them in a crude extract of Ginkgo leaves.

Accordingly, several attempts were made to purify this crude extract prior to HPLC examination. The major impurities were found to be waxes, chlorophylls and polyphenolic compounds. The elimination of waxes and chlorophylls was first tried with different chromatographic procedures using several minicolumns (alumina, Florisil, silica gel, silica gel bounded C_{18}, Sephadex), without satisfactory results. Treatment of the crude ethanol extract suspended in water with sodium hydroxide or lead acetate[11] can remove major polyphenolic impurities but results in a partial loss of terpenes.

Finally, good purification of the crude ethanolic extract suspended in water was obtained by extraction with cyclohexane, followed by ethyl acetate after acidification, and finally with diethyl ether in neutral medium. The diethyl ether extract was then evaporated to dryness and the residue dissolved in 1 ml of methanol prior to injection.

The HPLC trace of this partially purified *Ginkgo biloba* L. leaf extract is shown in Fig. 3. It shows the three main terpenes of the Ginkgo leaves previously recorded in the literature, *i.e.*, ginkgolides A and B and bilobalide. Ginkgolide C is not detectable under these chromatographic conditions.
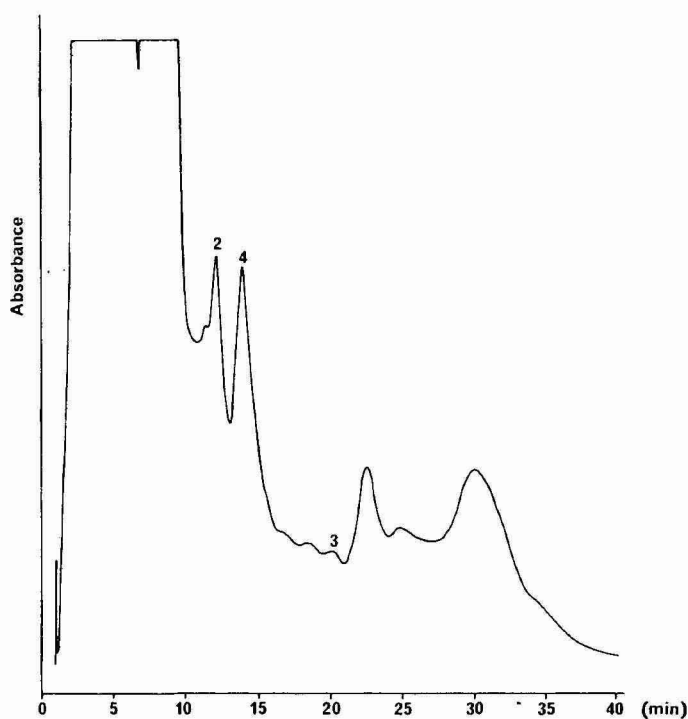
Fig. 3. HPLC trace of a partially purified *Ginkgo biloba* L. leaf extract. Conditions: solvent system D.

CONCLUSION

HPLC is a useful technique for the separation of terpenes from *Ginkgo biloba* L. Their detection was found to be impossible in a crude extract, but can be achieved with a partially purified extract. The purification process needs further amelioration in order to make possible their qualitative and quantitative determination in *Ginkgo biloba* L. extracts.

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CHROM. 15,957

Note

High-performance liquid chromatography of isoflavonoid phytoalexins in French Bean (*Phaseolus vulgaris*)

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Phytoalexins are low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms[1]. Therefore, they are believed to play a rôle in disease resistance in plants[2]. All phytoalexins accumulated in beans or other legumes are isoflavonoids. Especially in *Phaseolus vulgaris* L., a wide range of isoflavonoid phytoalexins is produced, *e.g.*, phaseollin, phaseollidin, phaseollinisoflavan, licoisoflavone A, kievitone and coumestrol (Fig. 1).

The relative amounts of these compounds depend on the type of micro-organism used as inducing agent[3], as well as on the incubation conditions during the accumulation period[4].

Up to now their isolation and quantification has been carried out by gas–liquid chromatography of the acetates[5] or by thin-layer chromatography (TLC). High-performance liquid chromatography (HPLC) has already been used succesfully for the separation of isoflavonoids from *Glycine max*[6–8] and *Cicer arietinum*[9]. Our work on the rôle of plant hormones in the phytoalexin interrelationship in *Phaseolus* required a rapid method of analysis of these compounds in different tissues. This report describes a method for rapid purification and quantification of isoflavonoid phytoalexins by HPLC, using a normal phase and gradient elution with isohydric solvents.
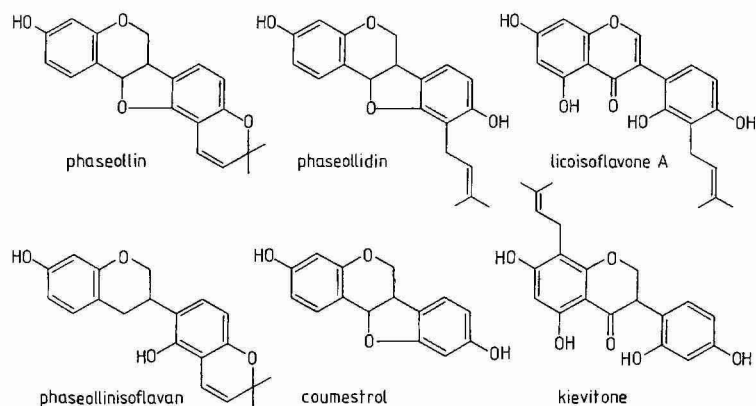
Fig. 1. Isoflavonoid phytoalexins in *Phaseolus vulgaris*.

MATERIALS AND METHODS

Preparation of bean samples

Bean tissues were prepared and extracted as described elsewhere[4]. Prior to extraction, 100 μl vesidryl (2',4,4'-trimethoxychalcone, 1 mg/ml) were added to each sample as an internal standard. After extraction the residue was redissolved in chloroform and a 25-μl sample was analysed by HPLC.

Isoflavonoid standards

Phaseollin and kievitone were purified from extracts of *Rhizoctonia solani*-infected bean hypocotyls following the method of Smith *et al.*[10]. Phaseollidin and licoisoflavone A were kindly provided by M. D. Woodward (University of Colorado, Boulder, CO, U.S.A.); phaseollinisoflavan was a gift from J. A. Bailey (Long Ashton Research Station, Bristol, Great Britain). Coumestrol was purchased from Eastman (Rochester, NY, U.S.A.). The internal standard vesidryl was synthesized following the cold condensation method of Geissman and Clinton[11].

Chromatography

The equipment was supplied by Waters Assoc. (Milford, MA, U.S.A.) and consisted of a M6000A and M45A Solvent Delivery System, a M660 Solvent Programmer, a U6K Universal Injector and a M40 Absorbance Detector with a 280-nm filter. A Hewlett-Packard 3390A Integrator was used for measuring peak areas.

The column (Lichroma, 0.3 cm I.D. × 30 cm) was packed with Lichrospher SI100 (10 μm) and eluted at 20°C with a linear gradient over 20 min (starting 1 min after injection), from 100% hexane–chloroform (2:1) to 100% chloroform–methanol
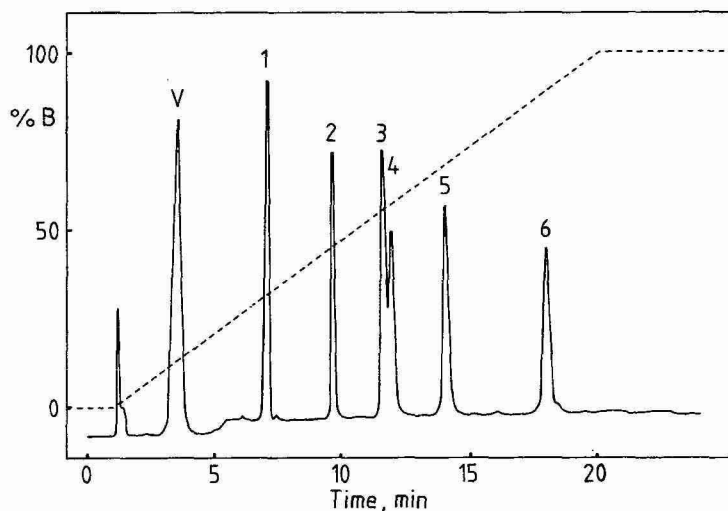
Fig. 2. Gradient elution of a mixture of isoflavonoid phytoalexin standards. Compounds: V = vesidryl; 1 = phaseollin; 2 = phaseollidin; 3 = phaseollinisoflavan; 4 = licoisoflavone; 5 = coumestrol; 6 = kievitone. Solvent A: hexane–chloroform (2:1). Solvent B: chloroform–methanol (100:8). The gradient as %B is represented by the broken line.

(100:8). The flow-rate was 2 ml/min. A guard-column (0.4 cm I.D. × 4 cm) packed with Spherisorb A (35–50 μm) was used to prevent deterioration of the main column.

Chloroform was purified by distillation (stabilized with 1% ethanol). Hexane was pre-washed with active charcoal to remove trace aromatics. Methanol was dried over a molecular sieve (Merck 5708) during 24 h, distilled and then 5.2% water was added to obtain an isohydric solvent[12].

RESULTS AND DISCUSSION

The chromatogram in Fig. 2 illustrates that in a gradient elution the six iso-flavonoid phytoalexins were satisfactorily resolved. Only phaseollinisoflavan and li-coisoflavone A were not completely separated. The position of vesidryl was of special importance with regard to its use as an internal standard. It eluted long enough after the solvent front and before the phytoalexins. Because of its chemical properties, similar to those of the phytoalexins, it could be used as internal standard to calculate extraction efficiencies (50–90% recovery). The time for one analysis was about 23 min, including re-equilibration to the initial conditions for 5 min.

This short re-equilibration time resulted from the use of isohydric methanol. By adding 5.2% water to dry methanol, the water content of the silica column remained constant during the gradient elution[12]. Therefore, the equilibration time between two runs and also retention time drift were greatly reduced. No water had to be added to the other solvents because of their already low water-holding capacity.
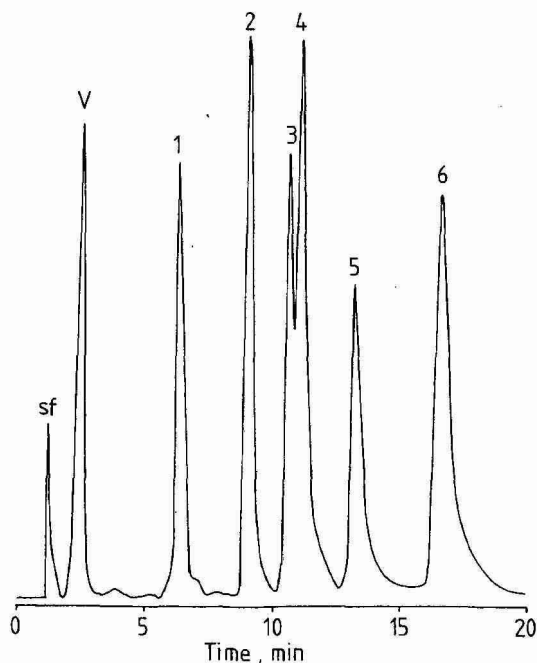
Fig. 3. Gradient elution profile of a mixture of isoflavonoid phytoalexin standards. Isohydric methanol in solvent B was substituted by dry methanol (0.1% water). Compounds as in Fig. 2. sf = Solvent front.
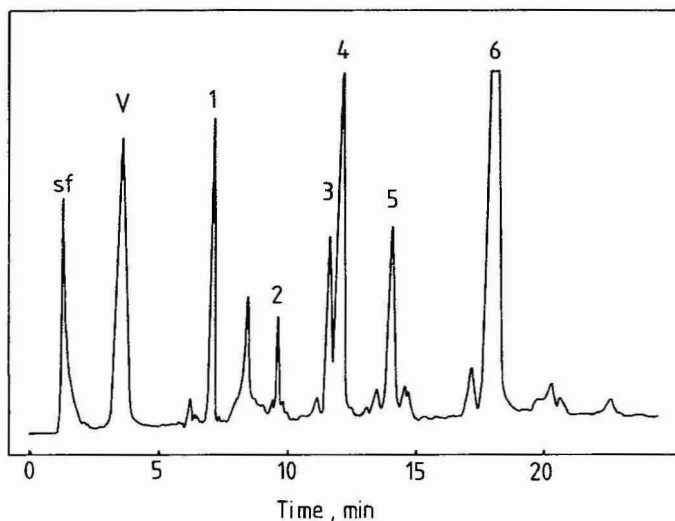
Fig. 4. Gradient elution of isoflavonoids in an ethanolic extract of bean cotyledons which received a phytoalexin-inducing treatment. Compounds as in Fig. 2. sf = Solvent front.

The use of isohydric methanol also improved peak shape (Fig. 3) and column life.

The elution pattern in Fig. 4 was obtained from an extract of bean cotyledons, which received a phytoalexin-inducing treatment. It shows that the six phytoalexins are present in this extract. This was confirmed by collecting the separate phytoalexins from several runs and identifying them by UV and mass spectra. The data obtained correspond to those reported previously[13,14].

This procedure makes large-scale TLC separations unnecessary. It is much faster and lowers the phytoalexin detection limit to 0.2 μg. With an internal standard, the method is accurate and useful for routine analysis.

ACKNOWLEDGEMENT

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Note

Separation of some tyrosine, tryptophan and phenylalanine derivatives by thin-layer chromatography

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Investigations on the pathways of synthesis of natural plant growth hormones from the aromatic amino acids tyrosine[1], tryptophan[2,3] and phenylalanine[1,4] have led to a need for the development of separation techniques for postulated intermediates of related chemical structure. These metabolites are extracted from the plant and fractionated into neutral, acidic or basic ether fractions[2]. From these organic fractions, some tryptophan and phenylalanine metabolites have previously been separated by paper chromatography[2–4] and thin-layer chromatography (TLC)[3,4].

This paper describes the separation by TLC of a more complete list of authentic aromatic metabolites which may be involved in auxin biosynthesis. We include the description of the resolution of the 2,4-dinitrophenylhydrazone derivatives of ketone- or aldehyde-containing compounds[3] which have been shown to be very labile to extraction procedures in the underivatized form[5].

EXPERIMENTAL

Authentic standards

Phenylacetic acid was purchased from Aldrich, cinnamic acid from Eastman, benzoic acid from Fisher, benzaldehyde and *p*-hydroxybenzaldehyde from ICN Pharmaceuticals, phenylacetaldehyde and *p*-coumaric acid from K & K Labs., 3-indolylacetic, 3-indolylpropionic, 3-indolylacrylic and 3-indolyllactic acids from Nutritional Biochemical Corp., phenylpropionic and *p*-hydroxyphenylpropionic acids from Pfalz and Bauer and tryptophol from Regis. 3-Indolylaldehyde, *p*-hydroxyphenylethanol and *p*-hydroxyphenylacetaldehyde were synthesized by M. T. Ceska, Department of Chemistry, Carleton University, Ottawa, Canada. All other standards were bought from Sigma.

Solvent systems

The solvent systems used for the separation of the authentic aromatic substances are listed in Table I.

TABLE I

SOLVENT SYSTEMS USED FOR TLC SEPARATION

No.	Components	Development time (h)
S1	Isopropanol–57% ammonia solution–water (10:1:1)	6
S2	Chloroform–acetic acid (9:1)	3
S3	Benzene–1,4-dioxane–acetic acid (90:25:4)	3
S4	n-Butanol–ethanol–57% ammonia solution–water (4:4:1:1)	5
S5	Isopropanol–57% ammonia solution–water (85:15:10)	6
S6	Benzene–ethyl acetate–acetic acid (90:5:5)	1
S7	Benzene	1
S8	Methanol–57% ammonia solution–water (80:10:10)	6
S9	Ethanol–57% ammonia solution–water (100:18:10)	3
S10	Chloroform–acetic acid (95:5)	3
S11	Benzene–methanol–acetic acid (45:8:4)	2
S12	Benzene–methanol (75:25)	2
S13	Carbon tetrachloride–acetic acid (50:1)	2
S14	Carbon tetrachloride–acetic acid (9:1)	2
S15	Isopropanol–1-butanol–*tert.*-butanol–57% ammonia solution–water (40:20:20:10:10)	5
S16	Carbon tetrachloride–acetic acid (4:1)	2
S17	Chloroform–ethyl acetate–formic acid (105:100:25)	4
S18	Ethyl acetate–isopropanol–57% ammonia solution (45:35:20)	5
S19	Chloroform–carbon tetrachloride–methanol (2:1:1)	3
S20	Chloroform–ethanol (13:7)	4
S21	Ethyl acetate–isopropanol–water (65:24:1)	5
S22	Benzene–methanol–acetic acid (75:23:2)	2

Development

The TLC plates used were 20 × 20 cm silica gel 60 plastic-backed plates with F-254 fluorescent indicator, layer thickness of 0.2 mm (E. Merck, Darmstadt, F.R.G.). Each chromatogram was developed at 20°C over a distance of 15 cm in a 7 × 25 × 25 cm glass chamber lined with filter-paper and pre-saturated for 1 h. All solvent systems were freshly prepared and used for a maximum of two developments.

TABLE II

R_F VALUES OF AUTHENTIC STANDARDS FOR ACIDIC TYROSINE-DERIVED METABOLITES

The compounds were developed on silica gel 60 F_{254} pre-coated plastic plates 20 × 20 cm (Merck) over a distance of 15 cm, in glass chambers previously saturated for 1 h. STR means streaks.

Compound	Solvent				
	S1	S2	S3	S4	S5
p-Hydroxyphenyllactic acid	0.28	0.05	STR	0.14	0.26
p-Coumaric acid	0.33	0.50	0.44	0.17	0.32
p-Hydroxyphenylacetic acid	0.28	0.35	0.40	0.12	0.28
p-Hydroxybenzoic acid	0.28	0.43	0.48	0.10	0.28
p-Hydroxyphenylpropionic acid	0.35	0.47	0.50	0.08	0.35

TABLE III

R_F VALUES OF AUTHENTIC STANDARDS FOR ACIDIC TRYPTOPHAN-DERIVED METABOLITES

Conditions as in Table II.

Compound	Solvent								
	S1	S10	S12	S17	S18	S19	S20	S21	S22
3-Indolyllactic acid	0.35	0.04	0.08	0.40	0.33	STR	STR	0.25	–
3-Indolylacrylic acid	0.40	0.36	0.38	0.70	0.36	0.57	0.78	0.78	0.55
3-Indolylacetic acid	0.35	0.39	0.32	0.72	0.32	0.50	0.75	0.75	0.53
3-Indolylcarboxylic acid	0.32	0.33	0.42	0.73	0.30	0.57	0.75	0.82	0.50
3-Indolylpropionic acid	–	0.42	0.36	–	–	–	–	–	0.37

TABLE IV

R_F VALUES OF AUTHENTIC STANDARDS FOR PHENYLALANINE-DERIVED METABOLITES

Conditions as in Table II. DEC means decomposes.

Compound	Solvent												
	S3	S4	S5	S6	S8	S9	S10	S11	S12	S13	S14	S15	S16
Phenyllactic acid	0.19	0.27	0.49	0.00	0.73	0.77	0.65	0.18	0.05	0.00	0.39	0.28	0.04
Cinnamic acid	0.52	0.24	STR	DEC	0.73	STR	0.50	0.69	0.28	0.08	0.37	0.25	0.35
Phenylacetic acid	0.51	0.23	0.30	0.27	0.69	0.68	0.46	DEC	0.36	0.60	0.33	0.22	0.32
Benzoic acid	0.53	0.23	0.34	DEC	0.72	0.72	0.50	0.68	0.36	0.10	0.40	0.24	0.41
Phenylpropionic acid	0.56	0.25	0.35	0.33	0.68	0.70	0.55	–	0.42	0.09	0.39	0.25	0.35
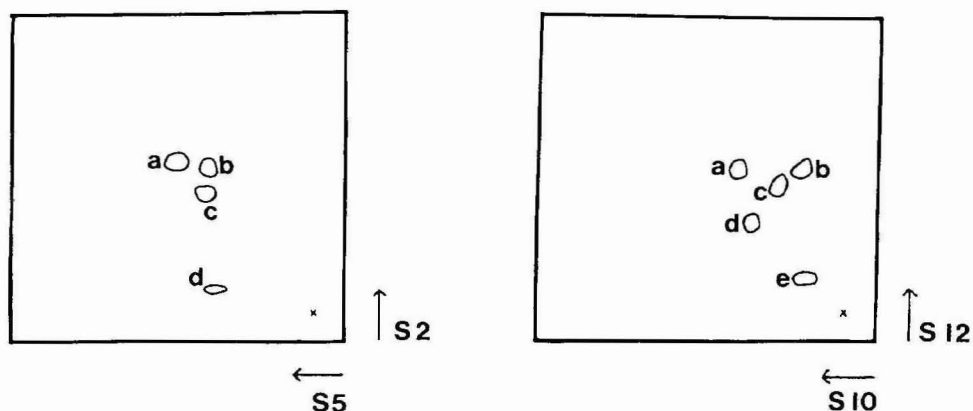
Fig. 1. Description of the location of *p*-hydroxyphenyl acids after two-dimensional chromatography, first in S2, then in S5. (a) Combined location of *p*-coumaric and *p*-hydroxyphenylpropionic acids; (b) *p*-hydroxybenzoic acid; (c) *p*-hydroxyphenylacetic acid; (d) *p*-hydroxyphenyllactic acid.

Fig. 2. Description of the location of indolic acids after two-dimensional chromatography, first in S12, then in S10. (a) 3-Indolylpropionic acid; (b) 3-indolylcarboxylic acid; (c) 3-indolylacrylic acid; (d) 3-indolylacetic acid; (e) 3-indolyllactic acid.

TABLE V

R_F VALUES FOR BASIC AND NEUTRAL AUTHENTIC SUBSTANCES

Conditions as in Table II.

Type	Compound	Solvent		
		S1	S3	S5
Basic	p-Hydroxyphenylethylamine	0.42	0.00	0.40
	Phenylethylamine	–	0.05	0.48
	Tryptamine	0.58	–	0.52
Neutral	p-Hydroxyphenylethanol	–	0.52	–
	Phenylethanol	–	0.65	–
	Tryptophol	0.70	0.25	–

Detection

For visual detection of indole compounds on the TLC plates, the modified Van Urk–Salkowski reagent[6] was used and for p-hydroxyphenyl substances the plates were sprayed with Folin–Ciocalteu reagent[7]. Phenyl compounds were detected by examination under UV light (254 nm). The dinitrophenylhydrazone derivatives were easily seen under normal light owing to their yellow colour.

RESULTS AND DISCUSSION

Tables II–IV summarize the results obtained with solvent systems found to give partial separation of the authentic standards of the acidic derivatives of tyrosine, tryptophan and phenylalanine. It was observed that the use of a second dimensional

TABLE VI

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONE DERIVATIVES OF AUTHENTIC SUBSTANCES

All chromatograms were run with three successive developments in the same dimension.

Compound	Solvent	
	S6	S7
p-Hydroxyphenylpyruvic acid	0.17	0.34
	0.28	0.50
p-Hydroxybenzaldehyde	0.46	0.10
p-Hydroxyphenylacetaldehyde	0.80	0.34
Phenylpyruvic acid	0.38	0.34
	0.42	0.46
Benzaldehyde	0.87	0.57
Phenylacetaldehyde	0.68	0.52
	0.77	
3-Indolylpyruvic acid	0.13	0.33
	0.18	0.46
3-Indolylaldehyde	0.52	0.04
3-Indolylacetaldehyde	0.08	0.78

development was more appropriate for optimal resolution of tyrosine and trypto-phane metabolites (Figs. 1 and 2). Tyrosine metabolites were separated by develop-ment in the first dimension with S2 and in the second dimension with S5. Complete resolution of *p*-coumaric and *p*-hydroxyphenylpropionic acids could not be achieved (Fig. 1). The five homologous compounds derived from tryptophan were, however, resolved using S12 first, followed by S10 in the second dimension (Fig. 2). Kaldewey[8] reported the separation of these indole acids in S10 using one development; however, we found the resolution of 3-indolylacetic acid from 3-indolylacrylic acid to be op-timized by using first S12, then S10 as a second-dimensional solvent system. Partial resolution of the phenylalanine metabolites was obtained with S16 in one dimension; we could not achieve a complete separation of cinnamic and phenylpropionic acids using a second dimension development.

Table V illustrates the separation of basic metabolites from the three aromatic amino acids which was obtained using S5, and the separation of the neutral metab-olites resolved by S3. The R_F values of ketone- or aldehyde-containing compounds derivatized to their 2,4-dinitrophenylhydrazones[3] appear in Table VI; after three suc-cessive developments in the same dimension, all pyruvic acids present two distinct bands corresponding most probably to their *syn-* and *anti-*isomers. The double band observed for phenylacetaldehyde in S6 is hypothesized to be due to the separation of a monomeric and polymeric form of the molecule[9].

CONCLUSIONS

The chromatographic systems described allow a rapid separation of many au-thentic derivatives of tyrosine, tryptophan and phenylalanine. These techniques were used with plant extracts[1,5] and shown, as checked by gas–liquid chromatography, to give a satisfactory separation of the endogenous substances as well.

ACKNOWLEDGEMENTS

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Note

Thin-layer chromatography of disperse dyes on polyamide sheets

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Disperse dyes are being increasingly used in the man-made fibre industry, particularly with polyester fibres. The finished commercial products are usually blends of two or more dye components resulting either from side reactions during the synthesis of the dyes, or introduced into the ready dye as shading components. A knowledge of the heterogeneity of the finished commercial products is essential both for their industrial application and for comparing dyes of different manufacturers and of different trade names. Analytical control carried out at considerable speed and high sensitivity has proved to be an effective means for the control of industrial dye synthesis.

The possibilities of using different chromatographic methods for a better solution of the analytical control of dyes, including disperse dyes, have been investigated extensively in recent years. The best results were achieved by using thin-layer chromatography (TLC) on silica gel plates with different solvent systems, was discussed by Dousheva *et al.*[1]. TLC methods have since developed considerably and achieved exceptional acceleration and sensitivity by using polyamide sheets as the carrier. There are no data, however, on the use of this carrier in the dye industry.

The application of TLC with micropolyamide sheets for control of the heterogeneity of disperse dyes was studied in this work. Tests were made with different solvent systems on 18 dyes produced by four companies.

EXPERIMENTAL

TLC was performed with appropriate attention to the necessary conditions for reproducibility of the chromatograms using micropolyamide sheets produced by Schleicher & Schüll (Dassel, F.R.G.), cut to 3 × 3 cm in size. The sheets were used without activation; after use they were washed with acetone–85% formic acid (9:1) and dried with warm air. When cleaned in this way, the sheets can be used at least ten times.

The following organic solvents were tested both singly and in combination: *n*-heptane, *n*-hexane, light petroleum, toluene, benzene, chlorobenzene, chloroform, dichloroethane, diethylether, ethyl acetate, 1,4-dioxane, acetone, ethanol, methanol and formamide.

The following solvents were used in the systems showing optimal separation after being purified as described. Benzene (purum) was shaken continuously with concentrated sulphuric acid (10:1) for 30 min. After washing with water, 2 M sodium carbonate solution and water, the benzene was dried over calcium chloride, followed by fractional distillation on a short column. The fraction of b.p. 80–81°C was used. Acetone (puriss) was boiled for several hours on a reflux condenser over sodium carbonate and then subjected to repeated distillation. The fraction of b.p. 56–57°C was used as a dye solvent. Light petroleum (purum) was subjected to fractional distillation and the fraction of b.p. 64–66°C was used. Chloroform (puriss) was washed with 10% sodium hydrogen carbonate solution and water, then dried over calcium chloride and subjected to fractional distillation. The fraction of b.p. 57–58°C was used. Glacial acetic acid was boiled for 4 h on a reflux condenser over 3% CrO_3 and then subjected to distillation on a short column. The fraction of b.p. 115–116°C was used. The remaining solvents were purified after distillation of the analytical-reagent grade products.

The specimens investigated were applied on the starting line as a 0.1% solution in acetone according to Rettie and Haynes[2]. The chromatography was carried out to the edges of the sheet, taking about 3 min.

The best solvent systems proved to be the following: (S1) benzene–light petroleum–methanol–glacial acetic acid (3:9.5:1:0.1); (S2) light petroleum–chloroform–methanol–glacial acetic acid (6:1:0.3:0.2); (S3) chloroform–n-hexane–methanol–glacial acetic acid (5:30:2:0.1); and (S4) light petroleum–benzene–methanol (5:11:2).

TABLE I

TLC OF DISPERSE DYES USING SOLVENT SYSTEMS S1–S4 ON POLYAMIDE SHEETS

No.	Dye*	Colour of spots	R_F value**			
			S1	S2	S3	S4
1	Terasil brillant yellow	Lemon yellow 6G	0.35	0.24	0.22	0.32
2	Terasilmarineblau BGL	Yellow-brown	—	—	0.00	
		Blue	0.42	0.34	0.30	
		Blue	0.68	0.48	0.48	
		Blue	0.90	0.68	0.68	
3	Terasilrot 3BL	Red	0.05	0.00	0.00	0.02
		Purple	0.18	0.08	0.08	0.11
		Red	0.54	0.37	0.32	0.46
4	Terasilbraun 3R	Brown	0.19	0.08	0.08	
5	Terasilbrillantviolet BL	Blue	0.30t	0.12t	0.12	
		Violet	0.58	0.36	0.30	
6	Terasilschwarz BL	Grey	0.00	0.00	0.00	
		Brown	0.20	0.09	0.08	
		Light brown	0.22	0.12	0.11	
		Light brown	0.30	0.20	0.18	
		Blue	0.43	0.35	0.29	
		Grey	0.68	0.48	0.46	
		Blue	0.91	0.69	0.67	

No.	Dye[*]	Colour of spots	R_F value[**]			
			S1	S2	S3	S4
7	Dispersol blue D-2R	Blue	0.00	0.00	0.00	0.00
		Blue	—	—	—	0.21t
		Blue	0.16t	0.10t	0.06t	0.75t
8	Dispersol red C-3B	Pink	0.00	0.00	0.00	
		Pink	0.13	0.08	0.07	0.11
		Red	0.56	0.42	0.36	0.46
9	Dispersol yellow C-42	Orange-yellow	0.00	0.00	0.00	0.22t
10	Resolinmarineblau GLS 200%	Blue	0.69	0.52	0.47	
11	Resolinrot BBL 200%	Pink	0.00	0.00	0.00	—
		Pink	0.12	0.08	0.07	0.10
		Red	0.56	0.42	0.36	0.46
12	Resolinorange 3GL	Yellow	0.00	0.00	0.00	0.24d
13	Syntengelb P-5G	Yellow	0.48	0.24t	0.26t	0.38t
14	Syntenblau P-BGL	Blue	0.04t	0.00	0.00	0.00
		Blue	0.28t	0.12	0.14	0.75
15	Syntenrubin P-3B	Ruby	0.16	0.05	0.08	
16	Syntenblau GRL	Grey	0.00			
		Blue	0.10	0.05dt	0.05dt	0–0.45 dt
		Pink	0.84	0.24	0.24	
17	Syntenbraun P-RBL	Grey	0.02dt		0.02	
		Pink	0.12	0.04	0.05	
		Light brown	0.16		0.09	
		Blue	0.32	0.16	0.17	
		Orange	0.53	0.28	0.27	
		Yellow-brown	0.68	0.37	0.36	
		Blue	0.84	0.51	0.53	
18	Syntenschwarz P-2BL	Grey	0.02dt		0.02	
		Pink	0.12	0.04	0.05	
		Light brown	0.16		0.09	
		Blue	0.32	0.16	0.17	
		Orange	0.53	0.28	0.27	
		Yellow-brown	0.68	0.37	0.36	
		Blue	0.84	0.51	0.53	

[*] Producers of dyes: Terasil, Ciba-Geigy; Dispersol, ICI; Resolin, Bayer; Synten, Ciech.

[**] d = Diffuse spot; t = tailing.

The dye specimens were selected so as to contain individual synthetic dyes from four manufacturers; some of them could be the same chemical products as well as dyes representing a complex composition of several components, say black and brown. The names of these dyes and manufacturers are given in Table I.

RESULTS AND DISCUSSION

The heterogeneity of the investigated dyes obtained by TLC on micropolyamide sheets is given in Table I. Figs. 1–4 indicate the quality of the separation process achieved with the use of the above four solvent systems.
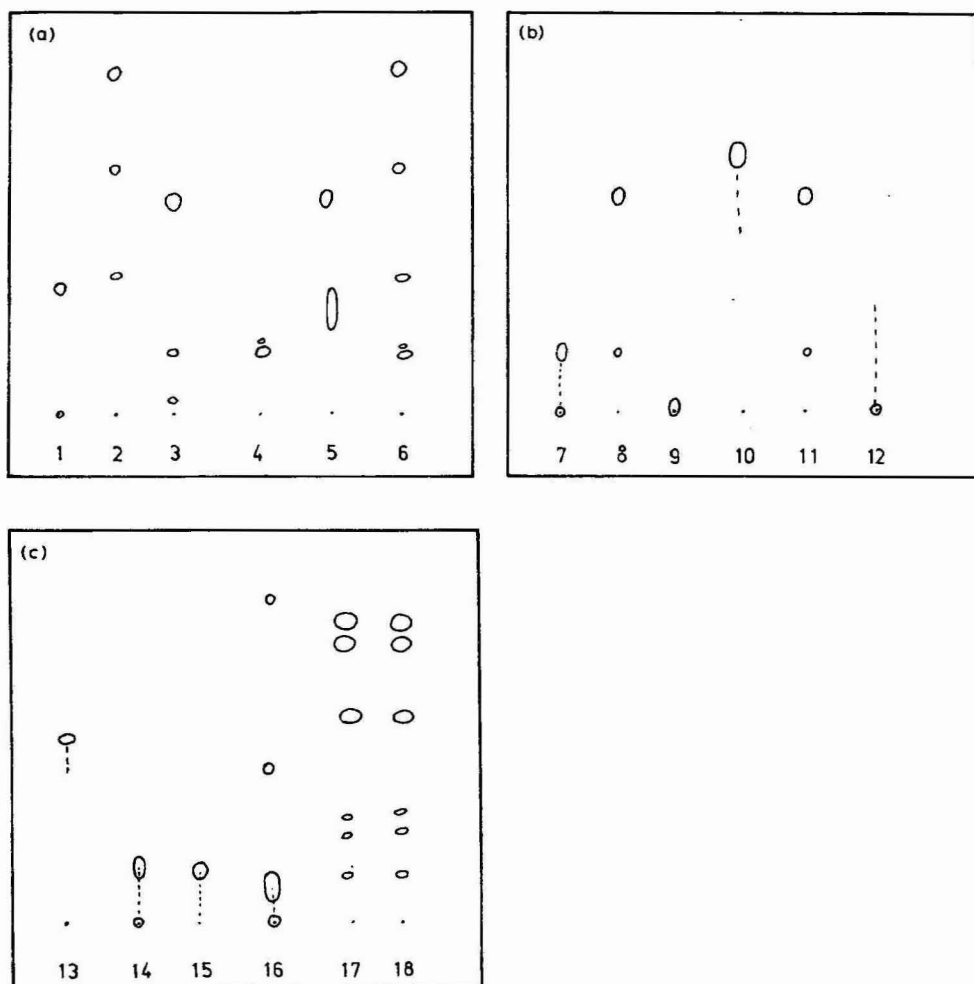
Fig. 1. TLC of dyes in benzene–light petroleum–methanol–glacial acetic acid (3:9.5:1:0.1) (system S1).

Solvent system S4 allows the migration of the dyes that showed complete immobility in the other systems. In this system the other dyes move very fast and therefore the information obtained refers to the heterogeneity only of the dyes which in the other two systems remain at the start.

As can be seen from the results, although the R_F values of the dyes were higher in S1, the separation effect was equal for S1, S2 and S3. The separation of the dyes investigated in this work showed considerably comparable heterogeneity to that obtained by TLC on silica gel. The particularly high sensitivity of the present method is apparent in the separation of the complex mixture Syntenschwarz P-2BL, where six components were separated, whereas Dousheva et al.[1] found only four components. The compactness of the chromatographic spots obtained on polyamide is an advantage. On the basis of the results, we conclude that Terasilmarineblau BGL is included in the combination of Terasilschwarz BL, and that Syntenbraun P-RBL
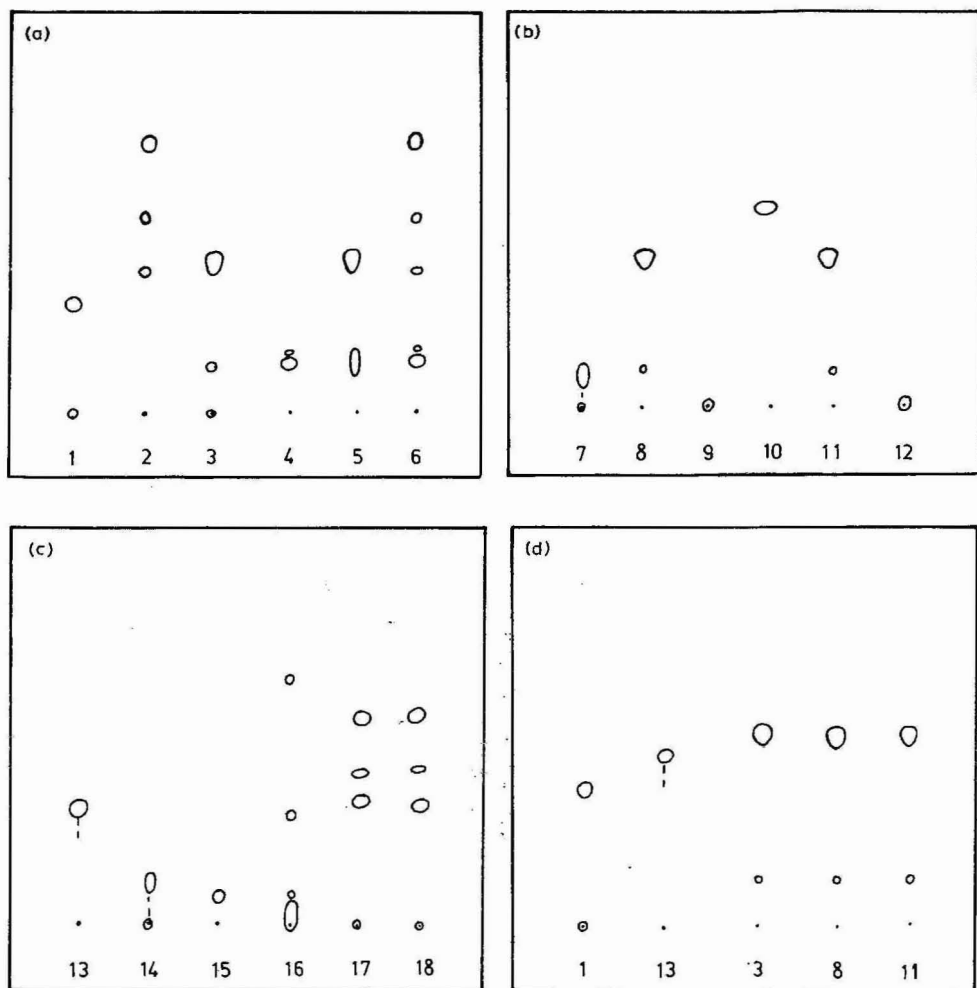
Fig. 2. TLC of dyes in light petroleum–chloroform–methanol–glacial acetic acid (6:1:0.3:0.2) (system S2).

and Syntenschwarz P-2BL are also mixtures of the same components in different proportions.

The proposed method can rapidly solve the question of the interchangeability of the dyes from different producers. Thus, from Fig. 4 it can be seen that Dispersol blue D-2R and Syntenblau P-BGL are similar chemical products. From Fig. 2d and from the other figures it is clear that Terasilrot 3BL, Dispersol red C-3B and Resolinrot BBL 200% are identical products. It can be assumed that the presence of the two admixed red components with low mobility in the three dyes explains their technological origin. In fact, the difference between these three products proved to be the different amounts of the admixed components. Although composed of only one component with similar chromatographic behaviour (Fig. 1b), the identity of Dispersol yellow C-4R (No. 9) and Resolinorange 3GL (No. 12) is apparent in Fig. 4. Although system S4 leads to considerable tailing for some dyes, it proved useful in solving the
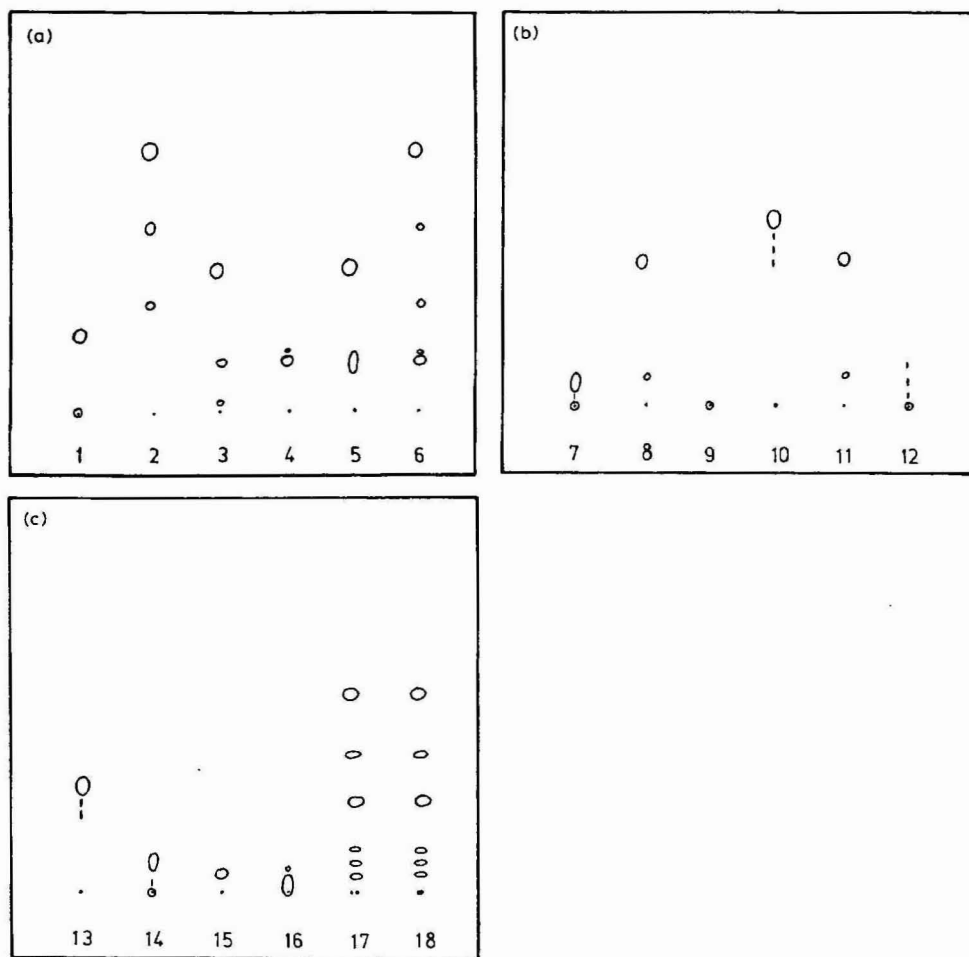
Fig. 3. TLC of dyes in chloroform–n-hexane–methanol–glacial acetic acid (5:30:2:0.1) (system S3).
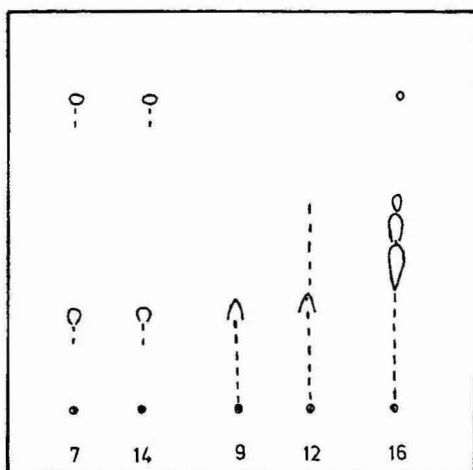
Fig. 4. TLC of dyes in light petroleum–benzene–methanol (5:11:2) (system S4).

problems of homogeneity and chromatographic comparability. The individuality of the two yellow dyes, Nos. 1 and 13 (Fig. 2d), is proved in the same manner.

These results show the advantage of having more than one solvent system and the possibility of solving some difficult questions without applying other methods. They also show that the proposed chromatographic systems make it possible to investigate disperse dyes rapidly and with sufficient reliability as regards their heterogeneity and identification. Naturally, in the latter instance it is necessary to extract the different fractions from the thin-layer plate with a solvent (acetone) and to compare the absorption spectra of the dyes in the visible and UV regions. Because of its rapid application and high sensitivity, the proposed method is very useful for the industrial control of the synthesis of disperse dyes and for toning finished commercial products.

REFERENCES

1 M. Dousheva, A. Arsov, V. Kostova and B. Mesrob, *J. Chromatogr.*, 121 (1976) 131.
2 G. H. Rettie and C. G. Haynes, *J. Soc. Dyers Colour.*, 80 (1964) 628.

Note

Determination of nitrate, chloride and sulphate in drinking water by capillary free-zone electrophoresis*

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(Received May 2nd, 1983)

Zone electrophoresis in stabilizing media has been a frequently used analytical techniques from its inception[1] to the present time[2]. Zone electrophoresis in free solutions still lacks wide practical utilization, despite its high separation potential shown with the use of both rotating tubes[3] and capillary columns with model mixtures[4,5].

The aim of this paper is to demonstrate the practical application of free zone electrophoresis in an isotachophoretic device using a capillary with on-line potential gradient detection for the rapid and sensitive determination of nitrate, chloride and sulphate in drinking water, which is an analytical problem of permanent interest[6].

EXPERIMENTAL

The experiments were carried out in an isotachophoretic column[7] consisting of a block of Perspex with electrode chambers, injection port, control valves and sensing electrodes of the potential gradient detector. The separations were performed in a capillary of rectangular cross-section (1.0×0.2 mm), 200 mm long, created by a groove in the organic glass block and covered with PTFE foil pressed on the block with a thermostated metal plate. A high-voltage constant-current supply controllable up to 400 μA at a maximum of 16 kV was used.

Potential gradients were detected by a device[8] consisting of a voltmeter with a high input resistance. The baseline signal corresponding to the background electrolyte (approximately 3 V) was electrically compensated on the output side of the voltmeter. The changes in the output signal (*ca.* 100 mV) were recorded by means of a Servogor RE 571 line recorder (Goertz, Vienna, Austria). In order to decrease the detection limit of the anions being analysed, the noise of the signal was suppressed by a low-pass filter (band width d.c. to 0.1 Hz) on the input of the voltmeter. In this way, the useful signal-to-noise ratio was increased approximately three-fold.

For selectively influencing the effective mobilities of the separated anions, complex formation with Cd^{2+} as the counter ion[9] was employed. A solution of cadmium acetate served as the background electrolyte; it was generated in the separation

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* Dedicated to Clark Hamilton, the founder of the Scientific Exchange Agreement, supporting research in chromatography and related methods in Europe.

capillary by an anionic isotachophoretic run with 0.004 M cadmium nitrate as the leading electrolyte and 0.01 M acetic acid as the terminator. After the isotachophoretic boundary had passed the detector, zone electrophoresis was performed in the adjusted terminator. The driving current was 120 μA in all experiments.

The chemicals used were of analytical-reagent grade (Lachema, Brno, Czechoslovakia). Solutions of nitrate, chloride, sulphate and nitrite (10^{-3} M) were sampled by means of a 1-μl microsyringe (Hamilton, Whittier, CA, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1a shows the record of the analysis of a model mixture containing $5 \cdot 10^{-10}$ mol of nitrate, chloride, sulphate and nitrite. It can be seen that complete resolution and detection were achieved within 5 min. Fig. 1b shows the record of the analysis of a real sample of drinking water, containing 22.9, 30.4 and 62.2 mg/l of nitrate, chloride and sulphate, respectively.

For quantitation, peak areas of the potential-gradient detection signal were used. The calibration graph for sulphate, nitrate and chloride in Fig. 2 shows a linear dependence of the peak area on the amount sampled in the range 0.1–0.7 nmol. The relative standard deviation was 2% for nitrate at the 0.5 nmol level (five analyses).

In conclusion, it can be said that currently used equipment for analytical capillary isotachophoresis can be used successfully for free zone electrophoresis. It allows the simple and rapid determination of common anions in various aqueous
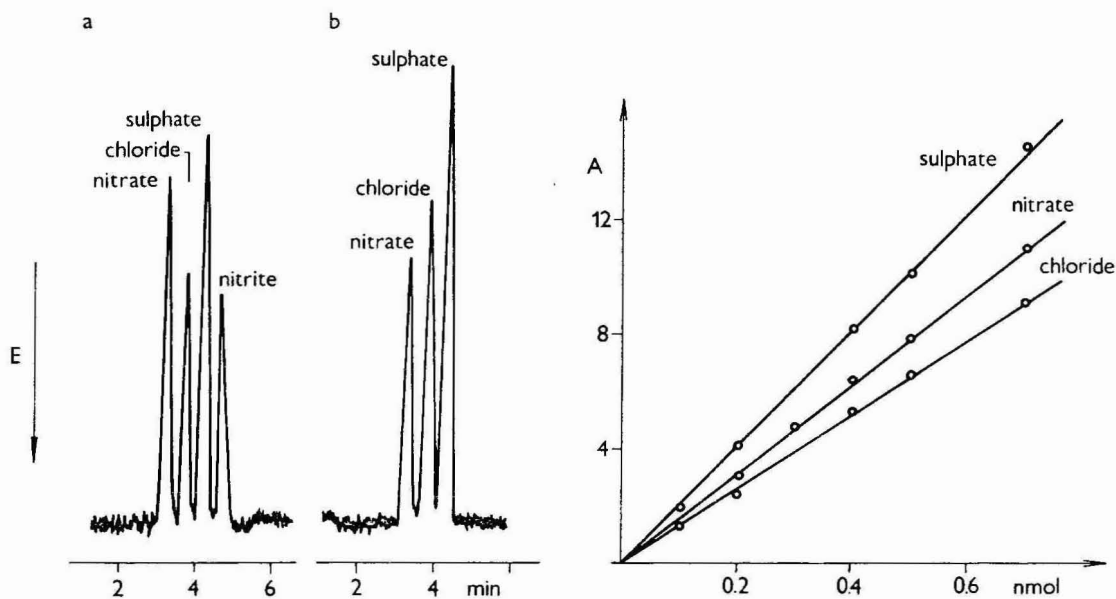
Fig. 1. Potential gradient records of zone-electrophoretic analyses of anions. Sample: (a) 0.50 μl of 10^{-3} M nitrate, chloride, sulphate and nitrite; (b) 1.00 μl of drinking water.

Fig. 2. Calibration graphs [peak area in arbitrary units (A) versus amount sampled] for sulphate, nitrate and chloride.

samples, the detection limits being at the 10 pmol level for each of the anions. A recent paper[6] on non-suppressed ion chromatography shows lower detection limits, but the zone electrophoretic analysis described here is much faster, with analysis times of less than 5 min.

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2 Z. Deyl (Editor), *Electrophoresis, Parts A and B*, Elsevier, Amsterdam, Oxford, New York, 1979 and 1983.
3 S. Hjertén, *Chromatogr. Rev.*, 9 (1967) 122.
4 F. E. P. Mikkers, F. M. Everaerts and Th. P. E. M. Verheggen, *J. Chromatogr.*, 169 (1979) 11.
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6 A. E. Buchholz, C. I. Verplough and J. L. Smith, *J. Chromatogr. Sci.*, 20 (1982) 499.
7 P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 106 (1975) 283.
8 M. Deml, P. Boček and J. Janák, *J. Chromatogr.*, 109 (1975) 49.
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CHROM. 16,114

Audio Course Review

Column selection in gas chromatography, by H. M. McNair, American Chemical Society, Washington, DC, four audiotape cassettes (4.2 h playing time) and a 140-page manual, price US$ 265.00 (U.S.A.), US$ 350.00 (rest of world).

This is an audio course consisting of four cassette tapes, each side between 17 and 43 min in length. The cassettes are accompanied by a 140-page course book.

It is soon apparent that the course should rather carry a heading like "Basic GC course", since a large part of the course deals with general aspects of GC. Naturally a sizable portion is devoted to column selection and column treatment, as it still holds true that the column is the heart of a GC system. The audio part of the course is not very easy to stick to. Due to the rather basic level of the course, those looking for a solution to their GC problems may find themselves frustrated by the longish explanations of simple facts.

As mentioned before, the course could be suitable for group or class work of a basic GC course. Then the course could be digestible if carried out in sections with intervals of practical work. This type of use might also justify the rather high price.

Author Index

Errata

J. Chromatogr., 246 (1982) 37–50.

Pages 49 and 50, the legends to Figs. 4 and 5 have erroneously been interchanged.

J. Chromatogr., 261 (1983) 189–212.

Pages 204 and 207, Figs. 5b and 8b, the peak numbers in the chromatograms are incorrect: the sequence of eluted compounds is the same as in Figs. 5a and 8a, respectively.

Page 212, ref. 25 should read: "H. Poppe and J. C. Kraak, *J. Chromatogr.*, 255 (1983) 395."

journal of
chromatography news section

NEW BOOKS

High-performance liquid chromatography —
advances and perspectives, Vol. 3, edited by
Cs. Horváth, Academic Press, New York,
London, 1983, XVI + 230 pp., price
US$ 36.00, ISBN 0-12-312203-1.

Drogenanalyse. Dünnschichtchromatographische
Analyse von Arzneidrogen, by H. Wagner,
S. Bladt and E.-M. Zgainski, Springer, Berlin,
Heidelberg, New York, 1983, *ca.* 316 pp.,
price DM 148.00, *ca.* US$ 61.70,
ISBN 3-540-11867-5.

Polymer characterization: spectroscopic, chro-
matographic, and physical instrumental meth-
ods, edited by C.D. Craver, American Chemical
Society, Washington, DC, 1983, 791 pp., price
US$ 69.95 (U.S.A. and Canada), US$ 83.95
(rest of world), ISBN 0-8412-0700-3.

The interpretation of analytical chemical data
by the use of cluster analysis (Chemical Analysis
Series, Vol. 65), by D.L. Massart and L. Kauf-
man, Wiley, New York, Chichester, 1983,
XII + 237 pp., price £ 42.75,
ISBN 0-471-07861-1.

Analytiker-Taschenbuch, Band 3, edited by
R. Bock, W. Fresenius, W. Huber and G. Tölg,
Springer, Berlin, Heidelberg, New York, 1983,
VIII + 410 pp., price DM 78.00, *ca.* US$32.60,
ISBN 3-540-11773-3.

Solid phase biochemistry: analytical and syn-
thetic aspects, edited by W.H. Scouten, Wiley,
Chichester, New York, 1983, *ca.* 784 pp., price
ca. US$ 99.75, £ 66.75, ISBN 0-471-08585-5.

Adsorption, by J. Oscik, Ellis Horwood, Chi-
chester, 1983, 222 pp., price £ 22.50,
US$ 40.00, ISBN 0-85312-166-4.

Handbook of polycyclic aromatic hydrocar-
bons, edited by A. Bjørseth, Marcel Dekker,
New York, Basel, 1983, 744 pp., price
Sfr. 332.00, ISBN 0-8247-1845-3.

Trichothecenes — chemical, biological and toxi-
cological aspects (Developments in Food
Science, Volume 4), edited by Y. Ueno, Else-
vier, Amsterdam, Oxford, New York, and
Kodansha, Tokyo, 1983, XIV + 313 pp.,
price US$ 85.00 (U.S.A. and Canada),
Dfl. 200.00 (rest of world),
ISBN 0-444-99661-3, 4-06-200341-4 (Japan).

Downstream processing (Advances in biochem-
ical engineering/biotechnology, Vol. 26),
Springer, Berlin, Heidelberg, New York, 1983,
ca. 220 pp., price DM 88.00, *ca.* US$ 35.20,
ISBN 3-540-12096-3.

MEETING ANNOUNCEMENT

INTERNATIONAL SYMPOSIUM ON ANALYTICAL PROBLEMS AND METHODS IN
BIOTECHNOLOGY

An International Symposium on Analytical Problems and Methods in Biotechnology will be held
in Noordwijkerhout, The Netherlands, on April 17–19, 1984. The Symposium is organized under the
auspices of the Analytical Division of the Royal Netherlands Chemical Society (KNCV) and the
Netherlands Biotechnological Society (NBV).

The development of analytical methods for biotechnological applications is an area of growing importance. Analytical methods currently available are now being adapted for practical use in biotechnological research, development and industrial production. A large gap remains to be bridged between experts in analytical methodology and experts in biotechnology. It is the purpose of this Symposium to outline the problems faced in this field and to describe the rapid developments taking place. The Symposium is aimed at an interdisciplinary audience of those involved in industrial and academic biotechnology, as well as at analytical chemists themselves. Analytical tools will be presented for process control in industrial biotechnology, for environmental biotechnology and for fundamental research. Current research activity on all aspects of analytical chemistry related to biotechnology will be described.

Topics covered will include: *analytical strategies* (on-line versus discontinuous analysis, on-line sampling, on-line process analysis); *analytical techniques* for both gas-phase and liquid-phase analysis; and *process control* (computerized data evaluation and process control, biochips, process control strategies).

The scientific programme will consist of invited plenary lectures, invited and submitted research papers (both oral and poster presentations) and discussion sessions. The Symposium language will be in English, and the papers presented will be refereed for publication in a special issue of *Analytica Chimica Acta*. A selection of review papers will be considered for publication in *TrAC–Trends in Analytical Chemistry*.

A special Symposium package, including registration fee, accommodation for two nights, all meals and a copy of the Proceedings will be available at Dfl. 460 (approx. US$ 180).

Further information about the Symposium may be obtained from: W.A. Scheffers, Symposium Analytical Methods and Problems in Biotechnology, Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft, The Netherlands. Tel: (015) 782411.

COURSE ON SEPARATION METHODS

COURSE ON BIOCHEMICAL SEPARATION METHODS, UPPSALA, SWEDEN, MARCH 20–JUNE 6, 1984

The above-mentioned course will be held at the Uppsala Separation School of the Institute of Biochemistry at the University of Uppsala, and is being organized by Professors S. Hjertén and P. Roos. The course is centered around modern analytical and preparative methods for the separation of cells, virus, proteins and nucleic acids, and their characterization. It consists of lectures and laboratory work dealing with the following methods: moving boundary electrophoresis, free zone electrophoresis, zone electrophoresis in both sieving and non-sieving anti-convection media, two-dimensional polyacrylamide gel electrophoresis (O'Farrell-technique), isoelectric focusing, displacement electrophoresis (isotachophoresis), molecular-sieve chromatography, hydroxyapatite chromatography, hydrophobic interaction chromatography, covalent chromatography, bioaffinity chromatography, GC, HPLC, counter-current distribution (liquid phase partition), analytical and preparative centrifugation methods (centrifugation in different kinds of density gradients, determination of sedimentation coefficients and of molecular weights), immuno-diffusion, rocket immunoelectrophoresis, crossed immunoelectrophoresis, determination of diffusion coefficients, light scattering, spectrofluorometry, radioimmunoassay and radio receptor assay.

Knowledge of biochemistry and mathematics, corresponding to a basic university degree, is required. Good knowledge of English is necessary. The number of participants is limited to 12, 6 from Sweden and 6 from abroad. The course fee is U.S. $450. Living expenses to cover food and accommodation in student rooms will be a minimum of US $1700. No fellowships are available through the organizers. The closing date for applications is January 15, 1984. Application forms can be obtained from: Secretary Eva Linder, Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden.

SHORT CONFERENCE REPORT

BUDAPEST CHROMATOGRAPHY CONFERENCE, JUNE 1–3, 1983

The Budapest Chromatography Conference was organized by the Hungarian Pharmacological Society and the Department of Enzymology of the Biological Research Center of the Hungarian Academy of Sciences.

The Conference was held in the Center of Research and Teaching Departments, Semmelweis University of Medicine, Nagyvárad tér 4, Budapest, Hungary, on June 1–3, 1983.

The Conference was opened by remarks from Professor J. Knoll, who outlined the importance of chromatography in the research of biologically active natural products, in biochemistry and in pharmacology.

Lectures were delivered on various topics: Professor Cs. Horváth summarized the perspectives of high-performance liquid chromatography (Quo Vadis HPLC), Professor E. Stahl gave a retrospective talk on thin-layer chromatography, while Professor E. Pungor discussed the importance and potential of electrochemical detection in chromatography. Several talks were

Cs. Hórvath, E. Stahl and H.J. Issaq.

J. Nagy, H. Kalász, N. Seiler, Zs. Bardócz and an unidentified participant.

given on the chromatography of polymers, amines, amino acids, peptides, proteins and nucleic acids. Numerous lectures during the Panel Discussion and the Poster Presentations dealt with the biological, chemical, pharmacological and industrial applications of chromatography. Other lectures and separate discussions were devoted to: "Gas Chromatography", "Stationary Phases for GC and HPLC", "Optimization in Chromatography and the Use of QSAR", and "Biologically Active Substances".

Hungarian contributions to chromatography were evidenced not only by the participation

of several Hungarian born scientists (Professors Horváth, Ettre, Kováts, Seiler, Varga and Molnár) who live abroad, but also by lectures on "Forced Flow Thin-Layer Chromatography" and "Ion-Exchange Thin-Layer Chromatography".

The Budapest Chromatography Conference provided an opportunity for the 200 participants, from 20 different countries, to exchange knowledge and to partake in scientific discussions with other chromatographers involved in chemistry, biochemistry, pharmacology, medicine, industry, instrumentation and theory.

UPDATED BIBLIOGRAPHY

IMPORTANT NOTICE TO ALL STEROID BIOCHEMISTS AND STEROID CHROMATOGRAPHERS

The third annual update of the "Comprehensive, fully indexed, bibliography on the methods for the analysis of oestrogens in biological materials" has now been completed. This publication is available, in microfiche form, from Dr. Oliver at the University of Salford. The number of references listed, classified and indexed is now 982, an increase of 257 over those forming the first issue of this bibliography (1980), which indicates the continuing interest in this assay by clinical chemists. Because of the cuts in U.K. Government Support Grants to British universities this publication is now priced at £10.00 ($15.00) and cheques (drawn in London in Sterling) should be sent with the order made payable to Dr. R.W.A. Oliver (Research Account).

SYMPOSIUM PROGRAM

CAPILLARY CHROMATOGRAPHY '83

An International Symposium

The 2nd International Symposium on Capillary Chromatography will be held October 10–12, 1983, at the Westchester Marriott Hotel in Tarrytown, NY, U.S.A. The symposium will consist of invited and submitted papers on all aspects of capillary chromatography given by leading authorities from throughout the world. Informal discussions will permit the free exchange of ideas on various current questions related to capillary chromatographic techniques and their applications. There will also be an exhibition of chromatographic instrumentation.

The complete program, registration, and hotel reservation forms may be obtained from:

Dr. A. Zlatkis
Chemistry Department
University of Houston
Houston, TX 77004, U.S.A.
Tel.: (713) 749-2623

Details of the program follow:

MONDAY, OCTOBER 10

Monday Morning

L.S. Ettre, presiding

9:00   E. Jellum (Institute of Clinical Biochemistry, Rikshospitalet, Oslo, Norway) — Towards ultra-high resolution: combined use of capillary GC–MS–computer, HPLC and two-dimensional electrophoresis in biomedical research.

9:30   R. Leshem, S. Lam and A. Karmen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) — Change of chromatographic properties of GLC columns with addition of organic vapor to carrier gas.

10:00   M. Novotny, S. Olesik and S. French (Indiana University, Bloomington, IN, U.S.A.) — Supercritical fluid chromatography/Fourier transform IR spectroscopy.

10:30   Intermission

11:00   R.P.W. Scott (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.) — The fully optimized capillary column system.

11:30   C.F. Poole, S.C. Dhanesar and F. Pacholec (Wayne State University, Detroit, MI, U.S.A.) — Organic molten salt and perfluorocarbon phases for gas chromatography.

2:00 J.A. Rijks, J. Curvers, Th. Noy and C. Cramers (Eindhoven University of Technology, Eindhoven, The Netherlands) – Applicability of steam-distillation/extraction and closed loop stripping for trace analysis with capillary gas chromatography.

2:30 J. Buijten, L. Blomberg, K. Markides and T. Wännmann (Department of Analytical Chemistry, University of Stockholm, Stockholm, Sweden) – Cross-linked methyltolyl and methylphenyl-silicones as stationary phases for fused-silica capillary gas chromatography.

3:00 S.R. Lipsky and W.J. McMurray (Yale University School of Medicine, New Haven, CT, U.S.A.) – Recent advances in the development of fused-silica glass capillary columns coated with a special variety of cross-linked non-polar and highly polar films.

3:30 Intermission

S. Trestianu, presiding

4:00 C.A. Cramers, E.A. Vermeer, J.A. Rijks, G.J. Scherpenzeel and C.P.M. Schutjes (Eindhoven University of Technology, Eindhoven, The Netherlands) – Fast high resolution gas chromatography on 50 μm I.D. capillary columns: an evaluation.

4:30 P. Sandra (Laboratory of Organic Chemistry, University of Ghent, Ghent, Belgium) – On-line selective sample introduction in capillary gas chromatography.

TUESDAY, OCTOBER 11

J.A. Rijks, presiding

9:00 P. Kucera (Hoffmann-La Roche Inc., Nutley, NJ, U.S.A.) – Open-tubular capillary liquid chromatography – The concept of multicapillary columns.

9:30 R. Meyer, P.B. Champlin and R.A. Hartwick (Rutgers University, New Brunswick, NJ, U.S.A.) – Multiple parallel capillaries as a practical approach to open tubular capillary HPLC.

10:00 R.C. Kong and M.L. Lee (Brigham Young University, Provo, UT, U.S.A.) – Preparation of small diameter capillary columns for gas and supercritical fluid chromatography.

10:30 Intermission

C.A. Cramers, presiding

11:00 R.G. Mathews, J. Torres and R.D. Schwartz (Pennzoil United, Inc., Shreveport, LA, U.S.A.) – Preparation and evaluation of polyphenylether sulfone glass capillary columns.

11:30 L.S. Ettre (Chromatography Division, The Perkin Elmer Corporation, Norwalk, CT, U.S.A.) and B. Kolb (Bodenseewerk Perkin-Elmer & Co. GmbH, Überlingen, F.R.G.) – Quantitative headspace gas chromatography with capillary columns.

12:00 J.A. Hubball and P.R. DiMauro (Foxboro, Analabs, North Haven, CT, U.S.A.) and E.F. Barry (University of Lowell, Lowell, MA, U.S.A.) – The performance of irradiated fused-silica open tubular (IFSOT) columns for capillary GC.

Tuesday Afternoon

2:00–5:00 Discussion Sessions

Wednesday Morning

C.F. Poole, presiding

9:00   R.E. Kaiser and R.I. Rieder (Institut für Chromatography, Bad Durkheim, F.R.G.) and P.K. Warme (Interactive Microware, Inc., State College, PA, U.S.A.) — Computer control of selectivity in capillary GC.

9:30   S. Trestianu, F. Munari and C. Saravalle (Carlo Erba Strumentazione, Milan, Italy) — Peak splitting phenomena in capillary gas chromatography.

10:00 D.W. Later, C.W. Wright, B.W. Wright, R.B. Lucke and W.C. Weimer (Pacific Northwest Laboratory, Richland, WA, U.S.A.) — Applied capillary column gas chromatography for the analyses of biologically active polycyclic aromatic compounds in coal liquefaction process materials.

10:30 Intermission

P. Sandra, presiding

11:00 P.H. Silvis, L.M. Sidisky, W.F. Fatula, N.H. Mosesman (Supelco, Inc., Bellefonte, PA, U.S.A.) — Reappraisal of wide bore (0.75 mm I.D.) glass capillary columns for GC.

11:30 J.V. Hinshaw, Jr. (Varian Instrument Group, Walnut Creek, CA, U.S.A.) — Classification and comparison of capillary inlet modes: non-vaporizing, temperature-programmed and vaporizing inlets.

12:00 F.F. Andrawes (American Cyanamid Company, Stamford, CT, U.S.A.) — Analysis of liquid samples by capillary gas chromatography and helium ionization detector — trace water analysis.

12:30 H. Brötell (KabiVitrum Ab, Stockholm, Sweden), H. Ehrsson (Karolinska Pharmacy, Stockholm, Sweden) and N-O. Ahnfelt (Pharmacia AB, Uppsala, Sweden) — Bioanalysis of drugs using heart cut GC–ECD with isothermal splitless injection.

1:00   Closing of symposium: L.S. Ettre

PUBLICATION SCHEDULE FOR 1983

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 2, pp. 491–494. A free reprint can be obtained by application to the publisher.)

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), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of 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. (Short communications and Notes 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.

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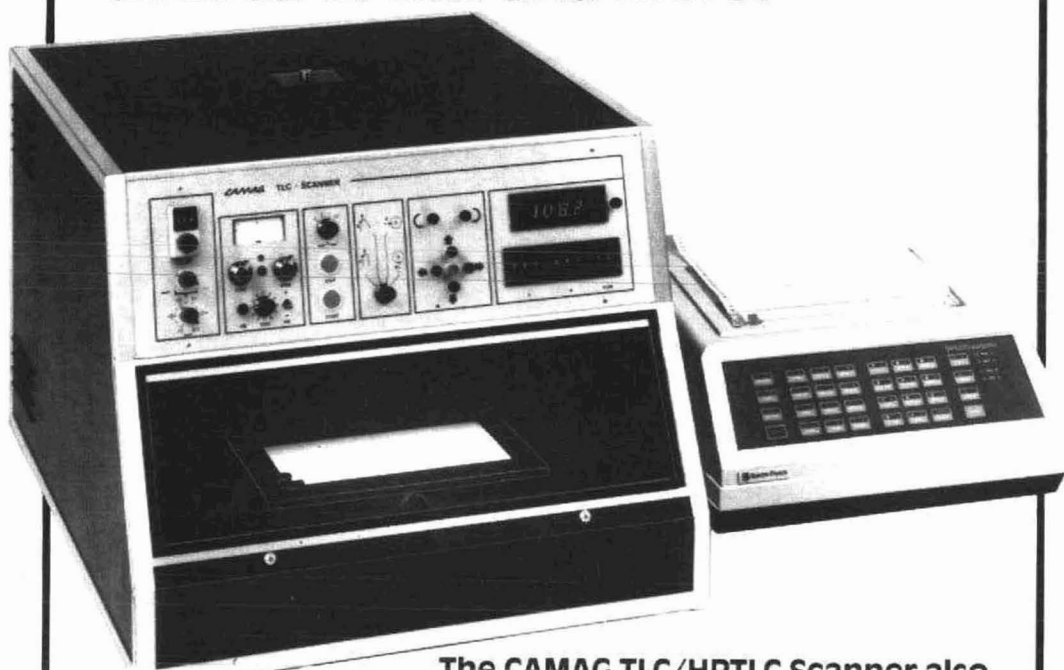
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