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CONTENTS

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D.C. Turnell and J.D.H. Cooper, J. Chromatogr., 395 (1987) 613-621.
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AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY

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The rapid development of new packings for aqueous size-exclusion chromatography has revolutionized this field. High resolution non-adsorptive columns now make possible the efficient separation of proteins and the rapid and precise determination of the molecular weight distribution of synthetic polymers. This technology is also being applied to the separation of small ions, the characterization of associating systems, and the measurement of branching. At the same time, fundamental studies are elucidating the mechanisms of the various chromatographic processes.

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Journal of Chromatography, 466 (1989) V–VI
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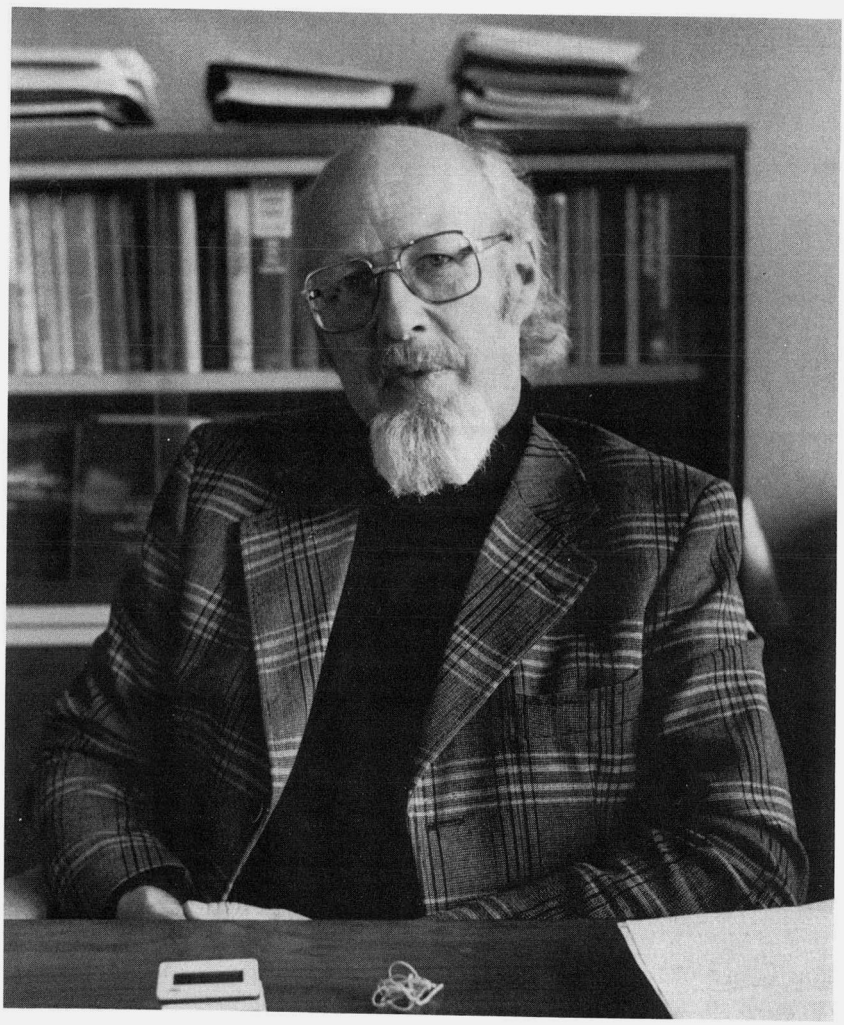
CHROM. 21 427

Obituary

Roland W. Frei (1936–1989)



On Sunday 29 January 1989, Professor Roland Frei died after an illness of several months in which he courageously fought to recover his health. The high hopes which he, his family and friends still had during the Christmas holidays, were dramatically shattered in the early weeks of January. He died peacefully in his hometown, Allschwil, in Switzerland, where many came to lay him to rest three days later.

Roland Frei arrived in the Netherlands in 1977 to take up a position as professor of Analytical Chemistry at the *Vrije Universiteit*, known to most of his and our colleagues as the Free University at Amsterdam. At that stage, he had already travelled widely: after studying in Switzerland, he was awarded his Ph.D. degree in Hawaii (U.S.A.), became an associate professor at Dalhousie University (Halifax, Canada) and then head of the analytical chemistry department of Sandoz (Basle, Switzerland). He had a wide-ranging interest in analytical chemistry but, in the middle seventies, it was high-performance liquid chromatography that primarily interested him. It was with this technique that he wanted to make a name for the *Vakgroep* of General and Analytical Chemistry at the Free University. Life in the Netherlands and, especially, in Amsterdam, where there is an informal atmosphere and an easy-going lifestyle, suited him well. He rapidly learnt to speak and lecture in Dutch, and often held open house in Amstelveen, the suburb of Amsterdam where he lived with his family.

With regard to his professional career, in the decade he worked in Amsterdam he succeeded in bringing his plans to fruition. Together with his colleagues and an ever increasing number of collaborators, he published a steady stream of scientific and review papers on on-line pre-column technology, post-column reaction detection, electrochemical detection and automated analytical systems. Good care was always taken that the practicability of the various techniques and systems was demonstrated in environmental or biomedical applications.

Roland Frei was an enthusiastic researcher who liked to combine well known principles in a novel way, to further develop and optimize them, and to test their applicability to real-life systems. He was a very industrious man. Besides being (co-)author of several hundred papers, he edited some ten books, was editor of the *International Journal of Environmental Analytical Chemistry* and served on the editorial board of most internationally known scientific chromatographic journals. Moreover, as president of the *International Association of Environmental Analytical Chemistry*, he laid the basis for several successful symposia series, such as the "Analytical chemistry of pollutants", "Sample handling" and "Liquid chromatography–mass spectrometry" series. In 1987, he was invited to lecture at the *École Supérieure de Physique et de Chimie* in Paris. About a year ago, he was awarded the prestigious Scheele medal by the Swedish Academy of Pharmaceutical Sciences.

Roland Frei loved life and his work in an uncomplicated way: he tried to stimulate people. He enjoyed his many trips abroad, liked to lecture on the research projects on which he was working, and was always happy to meet old friends and make new ones. Early in September 1988 one of us was standing next to him during a symposium of which he was a co-organiser. He said he wanted to leave a day early in order to finally find out what was wrong with his leg, which was giving him some trouble. After two difficult operations, during Christmas he still spoke of returning to the Free University in the early months of 1989. Four weeks later we had to bury him. This unexpected course of events made all of us feel very sad and humble. During his illness, Roland Frei repeatedly expressed his trust in the Lord. May He also comfort and support Marianne Frei-Häusler and her daughters.

Amsterdam (The Netherlands) U. A. Th. BRINKMAN
N. H. VELTHORST

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CHROM. 21 179

REVIEW

FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

G. GRASSINI-STRAZZA
Istituto di Cromatografia del CNR, Area della Ricerca di Roma, 00016 Monterotondo Stazione, C.P. 10, Rome (Italy)
and
V. CARUNCHIO* and A. M. GIRELLI
Dipartimento di Chimica, Università degli Studi "La Sapienza", P. le Aldo Moro 5, 00185 Rome (Italy)
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CONTENTS

1. INTRODUCTION

Thin-layer chromatography (TLC), a flat-bed liquid chromatographic technique first introduced in the early 1950s by Kirchner and Stahl, appeared to be one of

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the most promising separation methods for samples that are not amenable to analysis by gas chromatography (GC). The number of papers involving TLC has increased rapidly, especially since improvements in several respects (plate technology, sample throughput, detection limit, separation efficiency and precision) led to high-performance TLC (HPTLC). This technique is widely employed both in industry and in research work[1–4] and compares well with high-performance column liquid chromatography (HPLC)[2]. The advantages of TLC are technical simplicity, economy, rapidity of analysis and the possibility of applying and developing simultaneously numerous samples and standards on the same plate under identical experimental conditions. Moreover, the chromatographic parameters can be easily adjusted and the success of the separation rapidly evaluated.

Further improvements in the selectivity of flat-bed chromatography and broadening of its applicability were realized by modifying the chromatographic properties of the supports (paper or thin layers) by chemical reactions or physical methods. Chemical modifications of the support are widely used in TLC; the groups of interest are chemically bonded to the reactive groups of the packing material of the layers. In the last decade many types of precoated alkylsilica bonded plates have become commercially available and papers reporting their use in reversed-phase (RP) TLC are increasing[5].

The simplest physical method is to adsorb on the supports chemically selective reagents with special reactive groups (*e.g.*, complexing or ion-pairing agents and ion exchangers) or liquids of very low volatility and very low or very high polarity (*e.g.*, paraffin oil or formamide, respectively). In the latter instance, the impregnating agents behave as stationary phases and by choosing the appropriate mobile phase, a partition separation process is obtained[2]. Acids, bases or buffers, which modify the pH of the layers, can be also used as impregnating agents. This impregnation technique, used earlier in paper chromatography (PC)[6], has been successfully tried in TLC and it is still widely applied in flat-bed chromatography on both paper and thin layers.

The aim of this review is to draw the attention of users of liquid chromatography to the possibilities of analysis offered by PC and TLC techniques on coated supports by surveying the wide variety of impregnating agents tested, the possibility of obtaining relationships between the chromatographic behaviour and chemical structure of a compound and the many practical applications. The review covers the subject from 1978 to 1987 and includes our own experience and comments. Some works published in 1988 are also mentioned. The literature prior to 1978 was covered in books by Stahl[7] and Kirchner[8], and the extensive general review on TLC and PC by Zweig and Sherma[1], Sherma and Fried[2,3] and Sherma[4] proved valuable in compiling this review. In the last 10 years several reviews dealing with certain aspects of TLC have appeared[9–12] and more specific ones will be cited where appropriate.

Studies in which chemically modified precoated plates or papers have been used, the first subject of a review by Brinkman[5], are not reported here.

Several impregnation techniques are used[8], depending on whether the plates are laboratory-made or precoated commercially. In the former instance, a solution of a coating agent is added to a slurry, whereas commercial plates are dipped, sprayed or chromatographed in a solution of the impregnating agent. A more uniform layer is generally obtained by the dipping technique. In order to obtain reproducible results, the preparation of impregnated plates or papers requires care and the method should

be standardized. In addition, in chromatographic experiments with impregnated layers, care should be taken that the eluent does not wash away or interact with the coating agent.

2. COMPLEXATION

The use of complexing agents plays an important role in liquid chromatography because it allows the separation of a large number of different analytes (organic compounds, metal ions, enantiomers) by interaction of two or more molecules or ions. An equilibrium process occurs and the formation of complexes changes the chromatographic behaviour of the compounds under examination.

2.1. *Charge transfer*

The need to separate a wide variety of aromatic compounds in environmental and toxicológical analyses stimulated efforts to improve chromatographic techniques by incorporating electron acceptors or donors in the stationary phase. In this way a weak charge-transfer complex is formed by interaction between the analytes and the modified stationary phase. The complexation generally involves π-electrons, but σ- or n-orbitals and antibonding σ-orbitals can also play a role in the interactions.

In recent years, whereas silica materials chemically modified with electron donors or acceptors have been prepared and tested as packing materials for high-performance liquid chromatographic (HPLC) columns[13], impregnation techniques are still preferred in flat-bed chromatography. In the latter technique, silver salts and boric acid derivatives are the most frequently employed charge-transfer complexing agents.

2.1.1. *Silver salts.* Argentation chromatography, in which silver is used as a π-complexing metal, is widely used for the separation of organic compounds with electron-donor properties due to presence of unsaturated groups in the molecule. It has been hypothesized that the unsaturated group is attached to the metal by essentially a double bond, in which unsaturation electrons from the organic molecule form a σ-coordinate bond with the metal and the metal in turn donates a pair of d-electrons to the organic molecule through a π-bond. The stability of the π-complexes depends on the number, type, geometry and position of the double bonds in the molecule of the analytes.

Most studies using silver complexation employ TLC, because the instability of silver at elevated temperatures and problems with reproducibility and column lifetime impose severe limitations on GC and HPLC methods, respectively[14,15].

Silver nitrate as the impregnating agent and silica gel layers as supports are usually employed in argentation TLC. Recently several studies were made to test the effect of the anion of different silver salts on the R_F values. The anion can influence the stability of silver–olefin complexes; thus silver sulphamate proved to be very suitable for the separation of fatty acid cholesteryl esters[16]; silver benzenesulphonate allowed the critical separation of *cis*-monoenoic esters and *trans,trans*-dienoic esters (*e.g. cis*-oleic and *trans,trans*-linoleic acids) which could not be achieved with silver nitrate[17]; silver iodate, which is a stronger complexing agent than silver nitrate, gave a better resolution of some terpenoids in some instances and in others it was at least as good as silver nitrate[18]. Ammoniacal silver-impregnated plates gave a better

separation of fatty acid methyl esters than did impregnated plates prepared from aqueous silver solution[19].

The influence of silver nitrate concentration[20,21] and of the coating material on the thin layer[21,22] have been studied. A 2.5% concentration of silver nitrate reduced the resolution whereas plates treated 5 and 10% silver nitrate behave identically[20]. Generally a 5% concentration has been preferred by many workers[18,20,23–25].

As regards the thin layer, Breuer *et al.*[22], employing silver-impregnated alumina plates, separated fatty acid methyl esters (FAMEs) with different carbon chain lengths and fatty acid mixtures with a significant improvement in the time required over that using silica gel impregnated layers, whereas Inomata *et al.*[21] found HPTLC silica gel plates to be unsuitable for argentation TLC because of rapid darkening during the activation process.

Silver-impregnated silica gel layers are employed to separate analytes differing in geometry (*cis/trans*-Δ^{11}-octadecenol and *cis/trans*-Δ^{11}-octadecenic acid[25]; *cis/trans*-fatty acid monoenoic esters and *cis/trans*-dienoic esters[17]), the position of the double bond, as in pentacyclic triterpene monools and diols[26] and different numbers of double bonds[20,22,23,25–28]. Generally, additional double bonds in the side-chain lead to a decrease in the R_F values. The active adsorptive sites of the silica gel layers were found to interact also after silver impregnation[23]. In this study of the chromatographic behaviour of β-carotene, the R_F values were attributed to the combined effects of the two different type of interactions. In addition, the polarity of the solvent systems influenced the retention mechanism: interactions with silver ions prevailed with less polar solvents whereas interactions with the residual silanol groups prevailed with more polar solvents.

Two-dimensional TLC was employed for the complete separation of analytes with different chain lengths and degree of unsaturation as labelled fatty acid methyl esters[29] and 5-*n*-alk(en)ylresorcinol homologues[27]. In particular, silica gel plates impregnated side-by-side with silver nitrate and paraffin oil or decane, forming coupled two-phase layers, were used. In this way different retention mechanisms dictated the chromatographic behaviour of the analytes: in one direction the separation of compounds with different degrees of unsaturation and in the other direction with different chain length is achieved on silver-impregnated silica gel and reversed-phase layers.

It is interesting that argentation chromatography may be used most effectively in conjunction with other chromatographic techniques. The fact that it affects separations according to the degree and type of unsaturation, with little if any separation of compounds with different chain lengths, makes it particularly suitable for combination with other partition methods (GC and HPLC). Breuer *et al.*[22] described a method of argentation TLC combined with GC for analysing complex mixtures of positional and geometric isomers of fatty acid methyl esters. Combination with HPLC has been used for the quantitative analysis of subclasses (alk-1-enylacyl, alkylacyl and diacyl types) and molecular species within each subclass of glycerophosphatides[30] and for the study of the metabolism of species of a given lysophospholipid class[24].

As regards analytical applications, in addition to those mentioned above, the separation of prostaglandins[31,32] and *p*-nitrobenzyl esters of giberellins[28], the isolation of *trans*-hexadecenoic and *trans*-octadecenoic fatty acid methyl esters from

lipid extracts[33] and the determination of triazine and chlorophenoxy acid herbicides in natural waters[34] and cholesterol in egg yolk[35] have been reported.

In conclusion, silver-impregnated TLC is now being successfully employed for analytical purposes and there are relatively few papers devoted to the study of parameters that can improve the separation process.

2.1.2. *Boric acid and borates.* Flat-bed chromatography using boric acid and its derivatives as impregnating agents for paper or silica gel layers is generally employed for the separation of polyhydroxyl compounds (carbohydrates, fatty acids, polyalcohols). Vicinal hydroxyl groups in a position and conformation favourable for the formation of a cyclic boric acid derivative and a polarity of the derivative different from that of the free diol are required of the analytes. The position of the active pair of hydroxyl groups influences the stability of the cyclic boric acid derivative and the retention of the analytes. As an example, the relative mobilities of cardenolides containing active hydroxyl groups only in the carbohydrate chain are reduced by boric acid, whereas the mobility of cardenolides with active hydroxyl groups in the genin part is considerably increased in the presence of boric acid[36]. This difference is probably related to the tetrahedral state of the boron atom in the cyclic sugar ester compared with a trigonal state in the genin ester.

Megges *et al.* studied the influence of phenylboronic and diphenylborinic acids[37] and of some borates[38] on the PC mobilities of cardenolides and bufadienolides and concluded that there are two requirements for the interaction of *cis*-1,2- or *cis*-1,3-diol groups with these acids: the capability of the diol to reach an O–O distance like that in phenylboronic acid esters or diphenylborinic acid complexes and the absence of considerable steric hindrance from a substituent near the reactive diol group.

Boric acid-impregnated plates have been used for the TLC resolution of various lipids[39–42], for the detection of lactulose in milk[43] and for the assay of ribonucleotide reductase[44].

2.1.3. *Other agents.* Several attempts have been made to employ other electron acceptors and donors in TLC with the formation of charge-transfer complexes. Weak electron donors may be small unsaturated or aromatic hydrocarbons with or without an electron-releasing substituent such as an alkyl, alkoxy or amino group. Weak electron acceptors may be aromatic or unsaturated compounds containing electron-withdrawing substituents such as NO_2, Cl or CN groups.

As a weak electron donor, Jain and Agarwal[45] employed *p*-toluidine as an impregnating agent of silica gel layers for the identification of trace amounts of 1-(2,4-dinitrophenyl)-3,5-dimethyl- or -diphenyl-4-arylazopyrazoles.

For the separation and identification of some hydroxyacetophenones, impregnation of silica gel with chlorobenzene rather than nitrobenzene was preferred[46]. Chlorobenzene impregnation also improved the separation of lichen acids of the pulvinic acid series compared with unimpregnated plates[47].

Slifkin and co-workers[48–50] studied the complexing effect of amino acids, nucleic acid bases and other organic acceptors on different analytes in charge-transfer TLC. They measured the interaction between the analytes and the impregnant by a binding constant B, defined as

$$B = \frac{R_F - R'_F}{R_F} \cdot 100$$

where R_F is the value obtained in the absence and R'_F that in the presence of the impregnant. In this way, they determined the effect of the structure and concentration of the impregnant on charge-transfer complex formation.

In conclusion the papers discussed reveal that in flat-bed chromatography over the last decade silver salts and boric acid are the most extensively employed charge-transfer impregnating agents. In contrast to HPLC, where silica and organic polymers modified with covalently bound electron donors or acceptors are still widely tested[13], relatively little work has been published on other possible charge-transfer complexing agents in TLC.

2.2. *Coordination bonds*

The formation of complexes between a metal ion and an electron-donor molecule with at least one free pair of electrons can be employed for the separation of organic compounds or metal ions. The thin layer or the paper is impregnated with the appropriate metal salts[51–70] or with the ligands[71–81], respectively. In both instances the resolution of a complex mixture is correlated with the complex stability constant, which in turn depends on the structure of the compounds and on the polarity of the solvent.

2.2.1. *Metal ions as impregnating agents.* The impregnation of adsorbents with transition metal salts has been of great utility in the separation of different classes of compounds: alkaloids[51], sulpha drugs[52], carbamates[53], phenolic acids[54–56], amino acids[57–59], PTH-amino acids[60–62], dyes[63–65], barbiturates[66], carbohydrates[67,68] and organic acids[69].

Most of the reported systems improved the resolution of analytes[51–69] and spots with reduced tailing[51–53,60,64,66] compared with those on bare thin-layer plates.

In TLC, the complexation of phenolic acids or aldehydes[54–56] with impregnated Fe^{III} ions, depending on the eluent system, caused a decrease in retention of the analytes respect to that on plain silica gel plates, where hydrogen bonding is the predominant interaction.

Bhushan and co-workers[57–62] studied the resolution of amino acids and their PTH derivatives on silica gel plates impregnated with different transition metal ions at various concentrations in different eluent systems. In all instances the time required for developing the impregnated layers is less than that for plain silica gel. Zn^{II}-impregnated silica gel plates showed a greater ability to form complexes with amino acids[57] and their PTH derivatives[60] than Cd^{II}- and Hg^{II}-impregnated plates, whereas Fe^{II} and Ni^{II} were the best impregnants for the resolution of PTH-amino acids[61] when compared with transition metals of the same period (Co^{II}, Mn^{II}, Cr^{III}, Cu^{II}, Zn^{II}). Alkaline earth metal-impregnated silica gel plates showed a similar chromatographic behaviour to bare silica gel with respect to amino acids[59].

The R_F values were also influenced by the nature of anions present in metal salt[52,53,63,65,66]; acetate anion generally gave the best results[52,53,63,65].

A rapid method of resolving mixtures of some commonly occurring carbohydrates on cellulose or paper impregnated with tungstate was reported by Briggs and co-workers[67,68], with comparable results on the two supports.

2.2.2. *Ligands as impregnating agents.* The TLC separation of different metal ions has been attempted by many workers by impregnating the layer with a complexing agent[70–87].

Various compounds with amino[70–73] or acid[74–77] groups, salts of penicillin G (PG) or penicillin V (PV)[78], 8-hydroxyquinoline[79], dibenzoylmethane[79] and diantipyrylmethane solutions containing potassium iodide or ammonium thiocyanate[80] are considered good complexing agents and capable of separating different metal ions. In addition, a sparingly soluble compound such as a Schiff base, forming a complex with copper(II) even less soluble than the impregnant itself, permitted the determination of microgram amounts of the test ions on impregnated paper[81].

Other complexing agents such as digitonin and EDTA have been employed for the separation of sterols[82] and tetracyclines[83–87], respectively. 3β-Hydroxysterols with or without a C-22 double bond were fractionated in accordance with the length of the side-chain; the analytes with longer side-chains complex faster than digitonin and move more slowly on the TLC plates[82]. Generally, tetracyclines show tailing on the bare silica gel layers because of their chelation with trace metals present as impurities in the silica gel. In order to avoid this, HPTLC plates are generally impregnated with Na_2EDTA and then activated. In this way, simple, rapid and sensitive methods for the separation of tetracyclines from their major degradation products were developed[83–86]. The separation and semiquantitative determination of tetracycline degradation products in tetracycline hydrochloride powders and capsules on cellulose impregnated with EDTA was reported[87].

2.3. *Ligand exchange*

Ligand-exchange chromatography (LEC) is a technique mainly employed for the separation of compounds according to their ability to enter inside the coordination sphere of the complex-forming ion. This mode differs from ion-exchange, adsorption and other types of chromatography in its basic process of interaction between the analyte and stationary phase. The functional groups of the packing material (ion-exchange resin, silica gel) enter into the coordination sphere of the metals acting as fixed ligands. In this way an analyte (A), acting as a mobile ligand, coordinates the stationary complex (RM) by an equilibrium reaction:

$$RM + A \rightleftharpoons RMA$$

The selectivity and resolution of the chromatographic process are related to the different stabilities of the complex species (RMA) according to the physical and/or chemical properties of the analytes.

LEC offers interesting possibilities for the separation of various classes of compounds[88–92]. Various amines were separated qualitatively on impregnated papers and quantitatively on columns of Cu^{II}-impregnated zinc silicate by Singh and Darbari[88]. Mixtures of carbohydrates were separated according to their complex stabilities by TLC employing copper ion and a mixture of silica gel and Ionex 25-SA[89]. For comparison purpose complexes with other metal ions were investigated but the extent of complex formation was greatest with copper(II) ions. A successfully application of LEC was reported by Antonelli *et al.*[92] for the analysis of a mixture of α-amino acids, their corresponding β-isomers and some dipeptides. Ni^{II} and Cu^{II} in the form of $M(NH_3)_x$ were the metal ions chosen because both formed sufficiently strong and appreciably different complex species with the considered ligands. The best exchanger was $Ni(NH_3)_6^{2+}$, which was able to separate the analytes according to the

stability of the corresponding coordination compounds in aqueous solution. Data obtained by TLC were advantageously translated to column chromatography and comparable results were obtained[90]. The separation and determination of sulphur compounds in high-boiling petroleum distillates was obtained on mercury(II) acetate-impregnated silica gel plates with *n*-hexane as developing solvent[91].

An important application of LEC is the separation of optically active substances without any previous conversion into diastereomers. In the last 10 years, many workers have used chiral stationary phases instead of chiral mobile phase additives for the direct separation of enantiomers by TLC and HPLC with reversed-phase systems. Davankov and Semechkin[93], Armstrong[94], Mehta[95] and Günther[96] have discussed in detail these chiral separations by liquid chromatography based on the formation of an enantioselective ternary complex formed by a chiral reagent of a definite steric configuraton (fixed ligand) adsorbed on the stationary phase (generally C_{18} bonded silica gel), a metal ion (*e.g.*, Cu^{II}) or one or another of the two enantiomeric analytes (mobile ligand). Therefore, the two mixed-ligand diastereomeric complexes formed possess different stabilities if the fixed ligand (resolving selector) recognizes the configuration of the mobile ligand. The chiral reagent employed was generally an amino acid with a pronounced hydrophilic character that displays the largest enantioselective recognition ability with Cu^{II} complexes. In order to obtain strong adsorption of the chiral selector on the stationary phase, a reversed-phase surface and a aqueous–organic eluent were employed.

A suitable chiral selector was (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)-proline. As regards chiral TLC investigations, Günther *et al.* [97] obtained chiral layers by treating an octadecyl-modified silica TLC plate with a solution of copper(II) acetate followed by a solution of the above chiral selector; a simple and rapid method of monitoring optical purity was obtained. These plates, now commercially available, allowed the separation of optical isomers of amino acids, N-methylamino acids, N-formylamino acids, dipeptides and N-carbamyltryptophan[98–103].

Another chiral selector employed for the separation of Dns-amino acids was N,N-di-*n*-propyl-L-alanine (DPA)[104,105]. Grinberg and Weinstein[105] introduced a two-dimensional RP-TLC technique in order to separate a large number of Dns-amino acids more effectively than in a previous study[104]. In the first direction, the separation of Dns-amino acids in a non-chiral mode was achieved with an elution gradient (aqueous sodium acetate buffer–acetonitrile). A temperature gradient was applied to the plates treated with the chiral copper(II) complexes of DPA during the development in the second dimension, and an enantiomeric separation was obtained. Another application of the two-dimensional separation of D,L-Dns-amino acids was described by Marchelli *et al.*[106]. They prepared different chiral ligands in which two L-amino acids were joined via an amide bond by ethylene (AA-NN-2) or trimethylene bridges (AA-NN-3). Small variations in the length of the *n*-alkyl chain and in the lipophilicity of the amino acid strongly influenced the stereoselectivity: ligands of the AA-NN-3 type gave poor or no resolution whereas those of the AA-NN-2 type gave satisfactory results. The best separation was achieved with the copper complex of Phe-NN-2.

A new chiral selector (poly-L-phenylalaninamide) was prepared easily and without tedious purification procedures by Sinibaldi *et al.*[107]. Poly-L-phenylalanin-amide–Cu^{II} complex-impregnated RP_{18} silica gel plates separated a large number of

Dns-amino acid enantiomers using water–acetonitrile mixtures as the eluent under isocratic conditions.

The studies reported above indicated that several parameters influence the retention and enantioselectivity of the chromatographic system: (i) the Cu^{II} concentration necessary for the impregnation of the plate —the best concentration for the separation of D,L-Dns-amino acids is 3 or 4 mM[103–105,107]; (ii) the pH of the eluent— solutions are generally employed in the pH range 5–8 because the amino acids must act as bidentate ligands and therefore it is necessary to release the proton from the ammonium group[103–105,108]; (iii) temperature —a decrease in temperature generally results in improved separations[103,105]; and (iv) the water to organic modifier ratio in the eluent— the experimental conditions depend on the polarity of the analytes.

In addition, it is noteworthy that the L-enantiomer is generally more strongly retained than the D-enantiomer[97,103–105,107], depending on stereochemical interactions between the chiral selector and the analyte.

In conclusion, LEC could contribute to the solution of a number of problems, in particular the separation of optical isomers, which is of great interest in the design of novel pharmaceuticals, where isomers may have different effects on an organism; one isomer may be inactive or even harmful.

2.4. *Inclusion complexes*

The torus-shaped cyclic oligosaccharides α- and β-cyclodextrins (α- and β-CD), made up of six or seven α-1,4-linked D-glucopyranose units, respectively, can selectively include in their central cavity various inorganic and organic molecules and ions[109]. The fit of guest molecules in the CD (host) cavity determines the stability of the inclusion compounds and the selectivity of the complexation process. This property of CDs has been used to advantage in liquid chromatography in the last 10 years; in HPLC and in TLC the separation of various compounds and isomers through CD complexation was achieved using CDs as mobile phase components in RP systems[110,111]. Recently, chemically bonded CD–silica stationary phases have become commercially available as packing materials for HPLC columns[112].

Comparative studies on the application of α- and β-CD as mobile phase components for the separation of various isomers by RP-TLC and RP-HPLC on C_{18} layers and columns were recently reported[113]. In TLC experiments, before the usual chromatographic development, it was necessary to pretreat the C_{18} plates with the CD solution to be used as mobile phase. With both techniques the separation of *ortho*-, *meta*- and *para*-substituted benzenes was obtained. With 1- and 2-substituted naphthalenes only one peak was eluted (2-substituted naphthalenes) because isomers substituted in position 1 were irreversibly adsorbed on the RP-18 column. In such a case, only TLC can be used for evaluating the composition of mixtures of this type of isomer.

Studies on the stability of various CD complexes were also performed by using RP-TLC[105,114–120]. In these experiments, in order to obtain a reversed-phase chromatograpic system, the layers were coated with paraffin oil. Several hydrophilic β-CD derivatives are widely utilized for the stabilization and solubilization of drugs, pesticides, etc. [109]. The cyclodextrins are generally more hydrophilic than the guest compound and as a consequence the CD complexes should be more hydrophilic than the original guest compound. This lipophilicity difference could be measured by

RP-TLC by determining the variation in the R_F value of a compound in a chromatographic system in the presence and absence of β-CD soluble derivatives[114]. The larger the difference in R_F values, the higher is the stability of the complex. Changes in the guest molecule structure allow structure–complex stability correlations to be studied.

Cserháti and co-workers employed RP-TLC to study inclusion complexes formed by a water-soluble β-CD polymer (SCDP) and various compounds such as polymixyn (an antibiotic)[115], *sym*-triazine[116], triphenylmethane[117], nitrostyrene[118] and barbituric acid derivatives[119] and chlorophenols[108]. Paraffin oil-coated silica gel plates were employed as the stationary phase and ethanol– or methanol–water solutions of SCDP as the eluent. Experiments with the guest molecules dissolved in the eluent were also performed[120]. The effect on the stability of the complexes of the percentage of organic modifier in the eluent, salts differing in their charge and the radii of the cations and basic and acidic environments were investigated. An increasing proportion of organic modifier or an increasing concentration of the salts and/or cations with greater ionic radii reduced the stability of the SCDP–polymixyn complex[94]. With barbituric acid derivatives, the stability of the complex increased with branching or increasing chain length of the alkyl substituents[119]. Basic and acidic environments decreased[108] whereas sodium chloride had little effect on the stability of the complex[108,120]. The formation of an inclusion complex between SCDP and chlorophenols was confirmed by ^{1}H NMR spectroscopy.

TLC was also employed by Schneider *et al.*[121] to examine host–guest interactions between another macrocyclic molecule, an azacyclophane derivative immobilized on silica gel layers, and numerous azo dyes as guest molecules. As a result, only compounds with acidic groups, particularly SO_3H, were selectively bonded. According to the selectivity found in TLC experiments and spectroscopic investigations in solution, the essential factors in complexation are the lipophilic–hydrophobic binding in the cavity of the macrocycle and the electrostatic attraction between polar substituents in the substrate and the positive charges in the host molecule.

From the results obtained, it seems that RP-TLC on impregnated plates, a technique that has the advantages of being very easy to carry out and not requiring complicated instrumentation, is suitable for characterizing the stability of inclusion complexes.

3. ION EXCHANGE

Insoluble salts of polybasic acids with tetravalent metals (phosphates, arsenates, molybdate and tungstate of zirconium, titanium and cerium), ammonium salts of heteropoly acids (molybdophosphate, molybdoarsenate, molybdosilicate and tungstophosphate) and hydrous oxides have been extensively investigated as ion-exchange materials in column and flat-bed chromatography[122,123]. Zirconium phosphate-impregnated papers have been used for separations of inorganic ions[124], amino acids[125] and alkaloids[126].

The advantages of chromatography on papers coated with inorganic ion exchangers over partition chromatography on plain paper are the possibility of choosing an exchanger with a marked selectivity for a given compound and the use of aqueous solutions of inorganic salts as eluents. In selecting other ion-exchange systems for the separation of inorganic ions, Testa[127] showed that papers impregnated with

liquid ion exchangers, such as tri-*n*-octylamine (TOA), were good separation media. The suitability of these ion-exchange paper systems for the separation of a wide variety of compounds has been reviewed by Brinkman[128].

In recent years, as expected considering the decreasing interest in inorganic ion exchangers as column packing materials, few workers[129–139] have utilized this type of ion-exchange chromatography on paper or thin layers for separations of inorganic ions[129–132] or organic compounds[133–139]. In most of the studies[129–136,138,139], the effect of the ion exchanger was evaluated by comparing retentions on plain paper (or thin layer) and on the corresponding coated systems. For the separation of inorganic cations, aqueous acid–organic modifier mixtures [dimethyl sulphoxide (DMSO)[129], *n*-propanol[130,131] or benzene[132]] were tested as eluents. On tin(IV) arsenate paper, DMSO–aqueous nitric acid was found to be almost specific for In^{III} by Qureshi and Sharma[129], and the separation of In^{III} from Ga^{III} and Al^{III} was reported. Two-dimensional TLC on silica gel–pyridinium tungstate layers allowed the separation of numerous amino acids; a linear relationship between the R_M values of several amino acids and the number of carbon atoms was demonstrated by Srivastava *et al.*[134]. Separations of alkaloid mixtures containing up to five compounds were achieved within 15 min on zirconium antimonate paper[135], and zinc silicate papers were used for the separation of phenols[138] or amines[139] by Rawat and co-workers. In addition, papers impregnated with liquid anion exchangers have been used by several workers[140–144]. Tri-*n*-octylamine salts and Aliquat 336 were generally tested and the separation of anions by using mixtures of these liquid anion exchangers with aqueous inorganic[140] or organic[141] acids was reported. A comparison of the chromatographic behaviours of 25 steroidal glucosiduronic esters on normal- and reversed-phase systems was reported by Mattox and Litwiller[144]. Using the same liquid anion exchanger, the resolving properties of the two systems were markedly different, increasing the possibility of separation of a given mixture.

With synthetic insoluble inorganic exchangers, the ion-exchange papers are usually prepared by precipitation of the desired impregnating agents in the paper[124] and with liquid anion exchangers by dipping the papers in a solution of the exchanger in an organic solvent[127]. In earlier TLC experiments with ion exchangers, the material on the plates was simply the exchanger mixed with starch or plaster of Paris as binder[8]. More recently, better results and stable thin layers were obtained by coating the plates with a mixture of silica gel and the inorganic material[132–134]. For TLC experiments, a slurry of the exchanger and silica gel (1:50 or 1:100, w/w) in the appropriate solvent is sprayed on the glass plates and dried under different conditions[132–134].

In conclusion, on inorganic exchange papers, the frequently noted elongation of the spots allowed the separation of only two compounds in many instances. By using liquid anion exchangers as impregnating agents, PC results can be useful for predicting the anion-exchange behaviour of anions in liquid–liquid extraction processes[141].

4. ION PAIRING

Over the years, a variety of different approaches have been adopted in order to achieve for ionic or ionizable species, such as compounds of biological or pharmaceutical interest, the resolution, sensitivity and rapidity expected of new HPLC systems, as these properties were difficult to obtain with the traditional ion-exchange

packing materials. In HPLC on polar or non-polar columns, increases in retention, reduction in tailing and improvements in selectivity have been achieved by adding to the eluent counterions of the opposite charge with respect to the samples and generally containing hydrocarbon chains[145–148]. For the separation of anions quaternary alkylammonium ions were employed, whereas for the separation of cations hydrophobic (alkylsulphates or -sulphonates) or hydrophilic ion-pairing (IP) reagents, such as inorganic anions, were used. Ion-pair formation between the analyte ions and the oppositely charged counter ions is assumed to be responsible for the observed increase in retention. Obviously, an appropriate pH can be selected in order to ensure that the analytes and the ion-pairing agents are in a charged form. Many terms have been proposed for this chromatographic technique, *e.g.*, ion-pair chromatography[145], soap chromatography[146], solvent-generated (dynamic) ion-exchange chromatography[147], hetaeric chromatography[148] and chromatography on sorbed ionic sites[149], reflecting the uncertainty which exists regarding the retention mechanism. Detailed discussions on the various proposed models can be found in reviews[150–152].

In contrast to column chromatography (HPLC), there have been relatively few applications of IP techniques in flat-bed chromatography, after a report appeared in the mid-1960s on the usefulness of paper impregnated with surface-active agents for the separation of dyes[153]. In TLC the first investigations were carried out by Lepri and co-workers[154–169] using alkyl-bonded silica gel layers and anionic or cationic surface-active agents as pairing ions. The necessity to impregnate the plates with the IP reagent before TLC was demonstrated, because by dissolving the surfactant only in the eluent, as in paper[153] or column chromatography[146], no change in the chromatographic behaviour of the analytes was observed[154]. Precoating of the plates before use has been confirmed to be necessary if IP reagents with long-chain alkyl groups are employed[170–175]. Indeed, if they were dissolved only in the eluent, it appeared to "demix", all of the spots ran with or very near to the solvent front and no formation of ion pairs was observed. In some instances, two spots appeared[175]. The lack of the effect observed was related to the lower mobility of the IP reagent with respect to the solvent on the layer material, which caused the analytes to be eluted before the counter ions reached them. By using short-alkyl-chain IP reagents, precoating of the plates was generally believed to be more efficient for ion-pair formation, especially for data transfer from TLC to HPLC[170,175]. However, some results with IP reagents of the second type, dissolved only in the eluent, have been reported[176–178].

Table 1 lists the IP reagents, the type of thin layer, the eluents and the analytes or analyte classes used in IP-TLC. The papers cited are ordered on the basis of the chemical nature of the IP reagents, *viz.*, surface-active, alkyl sulphates and sulphonates, quaternary alkylammonium and inorganic anions. Most of the work reported in Table 1 was aimed mainly at improving the separation characteristics of chromatographic systems and at showing the applicability of IP-TLC as a versatile method for the separation of a wide range of compounds[154–169, 171–173,185]. In many of them, comparisons between IP-TLC and other TLC systems were reported[156,158,160,161,164,165,168,169,173,185]. In several instances, IP-TLC was utilized as a rapid and efficient method for data transfer to HPLC[170,174,181] or for correlating the chromatographic behaviour of organic species with their chemical characteristics[180].

TABLE 1
ION-PAIR CHROMATOGRAPHY ON IMPREGNATED LAYERS

Impregnating agent	*Thin-layer*[a]	*Eluents*	*Compounds or compound class*	*Ref.*
(a) Surface active:				
Anionic:				
(Triethanolamine) dodecylbenzensulphonate (DBS)	Silanized silica gel 60H (L)	Methanol–water–acetic acid at different pH	Primary aliphatic and aromatic amines	154,155,158
		(a) Methanol–water–acetic acid; (b) methanol–water	Sulphonamides	157
		(a) Methanol–aqueous buffers (pH 5.0–11.3); (b) ammonia solutions in 20% methanol	Phenols	161
Dodecylbenzenesulphonic acid (H-DBS)		Methanol–water–acetic acid at different pH (0.7–6.1)	Amino acids	159
		(a) Methanol–water–acetic acid at different pH (1.2–8.1)	Peptides and dipeptides	160,162
	Silica RP-2; RP-84; RP-18 (C)	Methanol–water–acetic acid at different pH	Amino acids; dipeptides	163
	Silica RP-18 (C)	Methanol–water–acetic acid at different pH and ionic strength	Di- and polypeptides; amino sugars	165
	Silica RP-18; SIL C_{18}-50; OPTI$_{12}$-UPC (C)	Methanol–aqueous buffers or ammonia at different pH	Chloro-, bromo- and alkyl phenols	166
	Silica RP-18; SIL C_{18}-50 (C)	Methanol–water–acetic acid at different pH and ionic strength	Primary aliphatic mono- and diamines; amino acids	165
	Silica SIL C_{18}-50 (C)	Methanol–water–acetic acid at different pH	Diastereomeric di- and tripeptides	168
	Silica SIL C_{18}-50 (C)	Methanol–water–acetic acid	Amino acids and derivatives	169
	Silica KC_{18} (C)	1 *M* acetic acid + 0.2 *M* HCl in methanol–water (7:3)	Amino acids	173
Sodium dodecylsulphate (SDS)	Silica $KC_{18}F_{254}$ (C)	Aqueous solution of NaCl (3%) and SDS (0.2%)–acetonitrile–methanol (50:10:10), apparent pH 2.5	Peptides	172
	Silica Si 60 F_{254} (C)	0.05 *M* ion-pairing agent in methanol–water (6:4)	Carboxylic and sulphonic acids; aliphatic and aromatic amines	177
Cationic:				
N-Dodecylpyridinium-chloride (N-DPC)	Silanized silica gel 60 HF (C)	(a) Methanol–water–acetic acid; (b) methanol–water–acetic and hydrochloric acid	Water-soluble food dyes	156

(Continued on p. 14)

TABLE 1 *(continued)*

Impregnating agent	*Thin-layer*[a]	*Eluents*	*Compounds or compound class*	*Ref.*
		Methanol–water at different pH (1.4–9.2) and ionic strength	Primary aromatic amines	157
	Silanized silica gel 60 HF (L); Silica RP-2 (C)	(a) Methanol–water–acetic acid; (b) methanol–water; (c) water	Polypeptides (MW 897–3495)	164
	Silica Sil C_{18}-50 (C)	0.5 *M* sodium acetate in water–methanol (20%)	Diastereomeric di- and tripeptides	168
	Silica Sil C_{18}-50 (C)	(a) Methanol–aqueous ammonia buffer at different concentration; (b) hexane–ethyl acetate–acetic acid (67:32:1)	Indole derivatives	167
(b) Alkylsulphonic acids and -sulphonates:				
Heptanesulphonic acid (HSA)	HPTLC silica RP-2; RP-8; RP-18 (C)	0.25–3% HSA in methanol–water–glacial acetic acid (80:15:5)	Phenothiazine bases and sulphoxides	176
	RP-8 F_{254} (N-15388); RP-18 F_{254} (N-15389) (usable at any water content)	0.2% HSA in methanol–water–glacial acetic acid in different proportions	Alkaloids	179
Sodium heptylmethyl-sulphonate	Silica Si F_{254} (C)	0.05 *M* ion pairing agent in methanol–water (6:4)	Aromatic amines	180
(c) Quaternary alkylammonium salts:				
Cetyltrimethylammonium bromide (CTMA)	Silica gel (C)	0.1 *M* CTMA in methanol–acetone (9:1) with or without 1% glacial acetic acid	Ionic food dyes (sulphonates)	171
Tetradecyltrimethyl-ammonium bromide (cetrimide, CTA)	Silica Sil G/UV_{254}; Silica 60 F_{254}; Silica RP-KC_{18} (C)	0.1 *M* CTA in phosphate buffer–methanol (or acetonitrile or tetrahydrofuran) (pH 5.0)	Carboxylic and sulphonic acids; phenols	174,181
Cetrimide; tetrabutyl (or hexyl, octyl, dodecyl)ammonium bromide	HPTLC silica RP-2; RP-8; RP-18 (C)	Methanol–water + 0.1–0.5% ion-pairing agent	Hydrophilic food dyes	170
Tetrabutyl (or methyl)ammonium halides	Silica Si F_{254} (C)	0.05 *M* ion-pairing agent in methanol–water (6:4)	Carboxylic and sulphonic acids	180
Tetramethyl (or propyl, *n*-butyl, heptyl)ammonium halide (TMA, TPA, TBA, THA) Cetrimide; Cetyl (or dodecyl)trimethyl-ammonium bromide (CTA, DTA)	Silica gel; paraffin-coated silica gel; silica RP-C_{18} (C)	Methanol–phosphate buffer (pH 2–11)	Hydroxybenzoic acids; aspirin and anti-pyrene metabolites	175,182

TABLE 1 *(continued)*

Impregnating agent	*Thin-layer*[a]	*Eluents*	*Compounds or compound class*	*Ref.*
Hexyl (or octyl, decyl)trimethylammonium halide; cetrimide 1,12-Bis(trimethylammonium)dodecyl bromide	Paraffin-coated silica gel (C)	Methanol–water at different pH	2,5- (or 2,6-)Dihydroxybenzoic acid; gentisic and salicylic acids	183
1,3-Bis(trimethylammonium)propyl bromide 1,12-Bis(trimethylammonium)dodecyl bromide Tetramethyl (or *n*-butyl)ammonium halide	Silica gel; paraffin-coated silica gel; silica RP-C_{18} (C)	Methanol–water; methanol–dichloromethane	2,4-(or 2,6-) dihydroxybenzoic acid; gentisic and salicylic acids	184
(d) Inorganic salts				
Chloride, bromide as alkali metal salts	Silica 60 F_{254} (L); silica 60 F_{254} (C)	(a) Methanol with or without ion-pairing agent; (b) chloroform–methanol (9:1)	Basic drugs	185

[a] (L) = laboratory-made; (C) = commercially available.

4.1. *Parameters affecting the retention of analytes*

A number of parameters affecting the retention of the analytes were investigated: thin-layer material, concentration and characteristics of the IP reagent (hydrophobicity related to the chain length of its alkyl groups), organic modifier to water ratio and apparent pH and ionic strength of the eluents[154,155,157–159,161–164,171,174,175,179,182,183,185].

4.1.1. *Layer material on the plates.* As regards the thin layers, commercial alkyl-bonded silica gel plates were generally used and were found to have many advantages over the previously employed laboratory-made plates (considerable decrease in the migration time, greater sensitivity, ability to separate a larger number of compounds in a single chromatographic run)[163,165]. The retention increased substantially from RP-C_2 to RP-C_8, but varied very little or remained constant on changing from RP-C_8 to RP-C_{18}[163,170,179]. By impregnating and using under the same experimental conditions bare silica gel commercial plates, obtained from various sources, differences in selectivity were found[174], as reported for unimpregnated silica gel layers[186], showing that the different adsorptive characteristics of silica gel can also affect the retention after impregnation.

4.1.2. *Ion-pairing reagents.* It can be seen from Table 1 that with respect to the inorganic IP reagents, organic counter ions differing in lipophilic character and structure are generally preferred. As regards the influence of the lipophilic character of IP agents, Gonnet *et al.*[170] found a drastic increase in the retention of some dyes with increase in the alkyl chain length of the counter ions tested ($R_F = 1$ with tetrabutylammonium and $R_F = 0$ with tetradodecylammonium). Accordingly, Lewis and Wilson[175] noted an increase in the retention of some hydroxybenzoic acids on RP plates impregnated with long-chain IP reagents such as cetrimide, CTA and DTA with

respect to that observed on layers impregnated with tetramethyl- tetrapropyl- or tetrabutylammonium halides. With inorganic counter ions, chloride, bromide, iodide and perchlorate were found to be efficient IP reagents, whereas sulphate, nitrate and acetate did not give adequate ion pairs. Therefore, differences in retention were obtained by using choride or bromide as counter ions[185].

The structure of the IP reagents must also be considered. Some primary aliphatic amines were retained more strongly on triethanolamine–dodecylbenzenesulphonate- than on sodium lauryl ether sulphate-impregnated RP layers, whereas the retention sequences were the same[154]. As in HPLC[150–152], increasing the concentration of the IP reagent in the plates increases retention until, generally, a limiting value is reached[154,170,174,175]. The optimal concentration of IP reagent solution was found to be 2–4%.

4.1.3. *Organic modifier.* In IP-TLC with RP systems, methanol was generally employed as the organic modifier. No significant change in selectivity was noted on replacing methanol with some other solvents such as ethanol, *n*-propanol, tetrahydrofuran or acetonitrile[160,175]. An increase in methanol concentration resulted in a decrease in the retention of the analytes[154–156,158,160,162,164,170,171,174,175,179], in agreement with that predicted from RP chromatography. Generally, the strongest resolving power of the layers was achieved with 35–50% methanol in the eluent.

A different behaviour was noted with two new bifunctional IP reagents 1,12-bis(trimethylammonium)dodecyl and 1,3-bis(trimethylammonium)propyl bromide. On bare or paraffin-coated silica gel plates impregnated with these reagents, a decrease in retention and a reversal of the retention sequence of the analytes was noted at high methanol concentrations (>80%). This effect might reflect a change in the retention mechanism from reversed to normal phase and can present a potentially useful property of these recently synthesized IP reagents[184].

4.1.4. *Eluent pH.* An important parameter in IP chromatography is the pH of the eluent, as maximum retention is obtained when analytes and IP reagents are completely ionized. When using strongly ionized pairing ions a pH change affects the retention by altering the ionization of the analytes. Compared with columns, where in order to avoid technical problem in IP-RP systems the pH is generally chosen in the range 2–8[152], thin layers are less affected by changes in the pH of the eluent and IP-RP-TLC in the pH range 0.7–11.3 has been reported[159,161,182]. In particular, attention was paid to the general problem of the eluent pH with respect to both the characteristics of the analytes[154,158–163,165,185] and the IP reagents[175,182,183]. As regards nitrogen compounds, on layers impregnated with anionic IP reagents, such as DBS or HDBS, the retention of primary aliphatic and aromatic amines generally decreased on using eluents containing increasing concentrations of hydrochloric acid[154,155,158,165]. With amino acids and peptides, on the basis of the acid–base characteristics of these compounds, species with different charges may exist in solution, depending on the apparent pH of the eluent. Different R_F values were found by Lepri *et al.*[159,160,162,163] for the anionic, zwitterionic and cationic forms of these compounds. For the amino acids investigated, on HDBS-impregnated layers the change in the apparent pH of the eluent was found to be useful for analytical purposes only in acidic media (pH 0.7–6.1); with basic eluents the amino acids were only slightly retained and tailed spots were observed[160].

For some polypeptides, by using plates impregnated with a cationic IP reagent

such as N-DPC, an increase in the apparent eluent pH resulted in a considerable decrease in their R_F values and, for several angiotensins, in a different order of retention. Therefore, an increase in the ionic strength of the eluent caused an increase in retention[164]. For numerous alkyl- and halogen-substituted phenols on N-DPC impregnated layers, as the apparent pH of the eluent was increased, a decrease and a levelling of their R_F values was observed, whereas on layers impregnated with DBS an increase in their R_F values was found with pH values increasing from 5.0 to 11.3[161].

Wilson and co-workers[175,182,183], using bare, paraffin-coated and C_{18}-bonded silica gel layers, studied the influence of the eluent pH on the chromatographic behaviour of some hydroxybenzoic acids in relation to the IP reagent employed. Quaternary ammonium halides [$^+NR_4$ and $^+N(CH_3)_3R$] differing in alkyl chain length were investigated. The IP reagents examined fell into two distinct classes: tetrabutyl-, tetraheptyl- and hexyltrimethylammonium halides did not show pH dependence effects, as no significant variations in the R_F values of the test compounds in the pH range 2–11 were noted; in contrast, IP reagents with long lipophilic alkyl chains, such as octyl-, decyl-, tetradecyl- and cetyltrimetylammonium halides, showed a clear pH dependence, with a general increase in the retention as the pH increased. The largest changes in the R_F values were noted in the pH range 2–4.

To establish whether a pH-dependent IP reagent would have only a long alkyl chain, or both hydrophobic and hydrophilic groups such as cetrimide, Troke and Wilson[183] synthesized a bifunctional IP reagent, 1,12-bis(trimethylammonium)dodecyl dibromide, having hydrophilic trimethylammonium groups at each end of the alkyl chain and no hydrophobic end. On paraffin-coated silica gel plates impregnated with this IP reagent, no difference in the retention of the test compounds was obtained over the pH range 2–9, indicating that in the chromatographic system investigated both hydrophilic and hydrophobic groups must be present in the molecule of a pH-dependent IP reagent[183].

In order to understand better the influence of eluent pH on IP liquid chromatography, control, after TLC, of the pH of the whole layer, as reported for other IP reagents[155], and further investigations by using other test compounds differing in pK values and chemical structure, alkyl-bonded silica gel layers instead of paraffin-coated plates and, if possible, comparison with HPLC results, would be useful.

As regards the mechanism operating in IP-TLC, at present the results are not sufficient to establish whether the models proposed for IP-HPLC can be applied to IP-TLC. With surface-active agents as counter ions, the charge of the surfactant ionized groups sorbed on the layers plays an important role in the separation process and an ion-exchange mechanism is hypothesized to be prevalent in many instances[155,158,163,165,168,173,187].

From the results reported in the papers listed in Table 1, some conclusions can be drawn about the usefulness of IP-TLC:

(a) In analytical applications, aliphatic monoamines[154,157], closely related peptides[160,162,172], diastereomeric di- and tripeptides[168], phenols[161,166] and food dyes[156,164,171] have been separated. The use of IP-TLC has been found to have great potential as a general screening method for basic drug analysis, in combination with a general basic development system[185].

(b) Data obtained in high-performance or over-pressurized TLC (HPTLC and

OPTLC, respectively) by using IP-reagent-impregnated layers, could be advantageously applied in HPLC. A good correlation between HPTLC or OPTLC retention values and the capacity factor obtained in HPLC was obtained[170,181]. Therefore, data transfer from HPTLC to HPLC was found to be easier in IP than in RP chromatography[170].

(c) Qualitative IP-TLC has been used for the identification of ionogenic groups in numerous aromatic and aliphatic compounds and it is believed to be a helpful technique in the identification of functional groups of unknown compounds[180].

In conclusion, the above results show that IP-TLC is a very promising, versatile technique and more attention should be paid to its possibilities in future, considering also the increasing use of IP systems in column HPLC.

5. PARTITION FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

Impregnation of paper or silica gel layers with reagents of low volatility and polarity, such as paraffin or silicone oil, and elution with aqueous–organic mixtures are commonly used in partition reversed-phase chromatography[7,8]. In spite of the great possibilities of chemically bonded silica gel precoated plates[5] in RP-TLC, impregnated layers are preferred by many workers. Normal-phase (NP) partition systems prepared by coating the support, generally cellulose, with polar compounds such as formamide, are also used. Both RP and NP chromatographic systems are utilized for investigations of the molecular lipophilicity of organic compounds and also for analytical purposes.

5.1. *Molecular lipophilicity determination: correlation with chemical structure and biological activity*

Lipophilicity, that is the tendency for a species to be readily soluble in most non-polar solvents but only sparingly soluble in water[188], is a useful and important physico-chemical parameter for studies of distribution processes of organic compounds in aqueous media, *e.g.*, penetration through membranes of living cells, bio-concentration in aquatic animals and soil sorption phenomena[189] and more recently to investigate the stability of inclusion complexes[93–101]. Hydrophobicity can be measured in many ways, *e.g.*, by partition between an immiscible polar and non-polar solvent pair (log P values)[190–192], by partition flat-bed chromatography (R_M values)[193,194] and by RP-HPLC (log k' values)[195]. The partition coefficient, P, is generally measured in the reference system *n*-octanol–water and it is frequently used to interpret quantitative structure–activity relationships of drugs (QSAR studies). QSAR methods are very useful and widely used in the design of new and biologically effective molecules. The determination of log P values by the usual shake-flask methods is time consuming and often difficult in comparison with the simpler and faster chromatographic techniques. In particular, flat-bed chromatography allows the analysis of compounds available only in small amounts, containing impurities and liable to decomposition. The theoretical basis of the correlation between the chemical structure of a compound and its R_M value obtained from flat-bed liquid–liquid partition chromatography was elaborated by Martin and co-workers[196,197] and experimentally established by Bate-Smith and Westall[198], who introduced the term $R_M = \log [(1/R_F - 1]$, and by Boyce and Milborrow[194]. The latter workers showed that the R_M values

could be used as hydrophobic parameters and that the change in the R_M values for a substituent group (ΔR_M) is a free-energy constant equivalent to the substituent constant π obtained from partition data used by Hansch and Leo[199] in QSAR studies. The equation mainly used for the transfer of TLC data:

$$\log P = a R_M + b$$

is an extension of the Collander[190] equation:

$$\log P_1 = a \log P_2 + b$$

where P_1 and P_2 are partition coefficients in solvent systems 1 and 2, respectively. Naturally, the correlation between log P and R_M values is valid for chromatographic determinations in systems (RP or NP) where partition is either the sole process taking place or predominates over others. In addition, in order to obtain sufficient accuracy, the chromatographic system must give R_F values between 0.2 and 0.8[200,201]. With compounds with large differences in lipophilicity, two or three eluent mixtures, with different organic modifiers, might provide this range of R_F values for all the compounds. R_F values smaller or greater than 0.5 correspond to positive or negative R_M values, respectively. Higher and/or positive R_M values indicate compounds more lipophilic than those with lower and/or negative R_M values. A detailed discussion of the measurement of chromatographic parameters and of the correlations of R_M values with biological and biochemical systems was given in Tomlinson's review[200] and recent achievements in the field can be found in the extensive reviews by Kaliszan[201,202].

Although investigations on chemical structure–chromatographic behaviour relationships[203–211] or QSAR studies[212–222], using the R_M values obtained from partition TLC on plates impregnated with non-polar or polar compounds to express the lipophilic character of the molecules, have been carried out by many workers, in recent years attention has mainly been focused on the chromatographic parameters that could influence the determination of R_M values, aiming always to increase the accuracy of investigations in QSAR studies.

Hydrophobicity can be expressed in terms of solvent partition values either from experiments or by calculation from fragmental constants (Hansch and Leo[199] or Rekker methods). Rekker and co-workers[223,224] reported data obtained from a series of benzophenones using RP-TLC on silica gel plates coated with non-polar compounds. The R_M values correlated well with Σf, where Σf is the sum of the hydrophobic fragmental values of the constituent parts of the benzophenone concerned.

As regards the correlations of chemical structure with chromatographic behaviour in RP-TLC systems, a linear relationship between the R_M values and the number of carbon atoms was found by Prandi[203] for numerous aliphatic amines, by Horna *et al.*[206] for alkyl acrylates and methacrylates and by Zemanová and Zeman[204] for N-mono- and N,N-dialkyl-substituted di- and trinitroanilines, in agreement with Boyce and Milborrow's results[194]. For amines, with an increase in the number of functional groups (NH_2 or OH) the R_M values increased, whereas a decrease occurred on replacing the amino hydrogen atoms with either alkyl or hydroxyalkyl groups. Śliwiok and co-workers[208,209], using adsorption and partition TLC, compared the

hydrophobic properties of selected isomeric α and β derivatives of naphthalene and *cis–trans* geometric isomers (oleic and elaidic acid, respectively, and their methyl esters). Under the experimental conditions tested, adsorption chromatography seemed to be the most efficient method for describing semi-quantitatively the hydrophobic properties of the isomers. The influence of five structural features of alkylphenoxyalkanoic acids on the chromatographic behaviour was investigated by Davídková and Gasparič[211] and the relationhips between chromatographic properties, partition data and chemical structure of a series of O-alkyl-O-arylphenylphosphonothioates were reported by Steurbaut *et al.* [205].

In QSAR studies, Biagi *et al.*[212], one of the first research groups in the field, found a very good correlation between the extrapolated R_M values of a series of alkyl-2-naphthols, naphthols and acetophenones and their antibacterial or haemolytic activity. It should be noted that they used extrapolated R_M values[225]. By extrapolation from the linear part of the graph obtained by plotting the R_M values *versus* percentage of organic modifier in the eluent, the theoretical R_M value (R_{M_0}) for each compound at 0% of organic modifier could be calculated. The R_{M_0} value, which should be related to the partitioning of the compounds between water and silicone or paraffin oil, is theoretically independent of the mobile phase composition and R_M or ΔR_M values obtained in different eluents[225] could be compared. Relationships between R_M and log P values for a series of benzodiazepines[213], 5-nitroimidazoles[214] and xanthone derivatives[216], between R_M or log k' (from HPLC data) and π values for a series of potentially mutagenic nitroimidazo[2,1-*b*]thiazoles[215] were reported. In addition, molecular lipophilicity–activity correlations have been formulated for the activity of benzodiazepines in rats[213] and of xanthone derivatives in mice[216]. A linear relationship between R_M values and log P or π values was found by Bachratá *et al.*[220] and by Dadáková *et al.*[221] in QSAR investigations on basic esters of substituted carbanilic acids[220] and on crotolactones[221], compounds with potential local anaesthetic and antituberculotic activity, respectively. A correlation between the R_M values and the pharmacological characteristic, log U, was established only for the carbanilic acid derivatives. However, the antituberculotic activity of crotolactones is related to a greater number of molecular parameters than simply the hydrophobic properties of the molecules[221].

5.2. *Parameters affecting the determination of R_M values*

Many parameters can influence the determination of R_M values, *e.g.*, the layer material, the impregnating agent used as the stationary phase and its concentration, the composition of the mobile phase and the chemical structure of the compounds.

The layer material may partially retain its original adsorptive characteristics even after impregnation, as reported by many workers[211,214–216,221–231]. Gasparič[226], in a study on the use of RP-TLC to determine molecular lipophilicity, investigated the chromatographic behaviour of compounds differing in polarity. Cellulose or silica gel plates and liquid paraffin or 1-octanol as impregnating agents were tested. The hydrophilic compounds behaved as if the layers were not impregnated, whereas the strongly lipophilic species followed the RP partition mechanism. A comparison of the adsorptive behaviour in RP-TLC of Kieselguhr, cellulose, Kieselgel and silanized Kieselguhr plates, all impregnated with oleyl alcohol, was reported by Van der Giesen and Janssen[227]. Kieselguhr and cellulose showed

adsorption, whereas Kieselgel is not a good support, probably because of its large specific surface area. Silanized Kieselguhr gave a support without adsorptive sites; the R_M values obtained correlated very well with log P values. By using paraffin oil-coated silica or alumina plates, different R_M values and different dependences of retention on the percentage of organic modifier in the eluent were obtained by Cserháti[228] for some 3,5-dinitrobenzoic acid esters. Silica, alumina and celluose plates coated with paraffin oil were compared by Cserháti *et al.*[229] for the determination of the lipophilicity of some neutral, acidic and basic compounds. In the RP-TLC experiments, basic compounds showed higher R_M values on silica and acidic compounds on alumina, indicating that with polar compounds the surface pH of the support, also after impregnation, influenced the determination of lipophilicity. In measurements of the lipophilicity of benzophenones, Bijloo and Rekker[224] investigated the influence of modifications of the stationary phase by using different layer materials (silica gel or silica gel–Kieselguhr mixtures) and different coating agents (paraffin or silicone oil). The replacement of paraffin with silicone oil caused significant changes in the retention of compounds with particular functional groups and made it difficult to explain the investigated structures in terms of lipophilicity. The addition of Kieselguhr to the silica influenced only the behaviour of the silicone oil-coated plates.

Silicone and paraffin oil are widely used as non-polar stationary phases and a concentration range of 5–10% generally permits the required separation. However, lower impregnation (<2%) improved the separation of organophosphate esters by RP-TLC in both chromatographic systems tested by Gandhe *et al.*[232]. In undecane, squalane or liquid paraffin systems, the R_M values of 5-nitroimidazoles where higher than in a silicone oil system[214]. Differences were found between silicone oil and 1-octanol[213] and between paraffin oil and a series of C_{16}, C_{18} and C_{20} fatty acids or their esters as stationary phases[218]. By using secondary amide derivatives, better reproducibility of the R_F values was observed by Churáček[233] on formamide- than on dimethylformamide-coated layers, and for some triazine herbicides a linear relationship between the R_M values determined by using silanized plates coated with two different stationary phases (diethylene glycol and formamide) was found by Ogierman and Silowiecki[234].

The effect of the composition of the eluent on the determination of molecular lipophilicity was also investigated. With compounds containing dissociable substituent groups, the eluent pH[235] and salt concentration can modify the lipophilicity[236,237]. Cserháti and Gasparič[238] showed that when buffer solutions are used as the mobile phase in both NP- and RP-TLC systems in QSAR studies of ionizable compounds, false results could be obtained if the possible change in the support pH, due to eluent demixing and/or to the buffering effect, is not considered. From a comparison of the buffer capacities of sodium phosphate, sodium acetate and sodium diethylbarbiturate (veronal buffer), all at pH 8.5, on paraffin oil–silica gel-coated plates and with methanol–water mixtures as eluents, the movement of the alkaline front decreased with increasing methanol concentration and with increasing extent of coating. The buffering capacity increased in the order veronal (highest R_F value, 0.12) < phosphate (up to $R_F = 0.50$) < acetate. Only the acetate produced a buffering effect that was evenly distributed along the plates.

As regards the effect of different organic modifiers on the determination of molecular lipophilicity[207,210,239–245], aqueous mixtures of acetone, acetonitrile,

TABLE 2
ANALYTICAL SEPARATIONS BY USING PARTITION CHROMATOGRAPHY ON IMPREGNATED LAYERS

Compounds or class of compounds	*Thin layer*[a]	*Impregnating agent*	*Eluent*	*Remarks*	*Ref.*
Aliphatic amines	Kieselguhr (C)	5% paraffin oil in acetone	Acetone–17% ammonia (55:45 or 70:30)	Good separation of fatty amines	203
Barbituric acid derivatives	Silica gel 60H (L)	5% paraffin oil in benzene–acetone (1:1)	Water–acetonitrile, acetone, isopropanol, methanol in various proportions	Best eluent: water–isopropanol	207
Alkylphenoxyalkanoic acids	Silica gel and cellulose sheets (C)	(a) 10% liquid paraffin in *n*-hexane; (b) 20% formamide in methanol or 50% DMF in acetone	Various	On formamide layers higher ΔR_M values for CH_2 group of all the homologues	211
Diethylphenylphosphates	Silica gel G (L)	(a) 1.5% *n*-octanol in hexane; (b) 1.5% silicone oil in ethanol	Acetone–water (40:60)	Separation of positional isomers with both the RP-TLC systems	232
Triazine herbicides	Silica gel G (L)	(a) 20% diethylene glycol in acetone; (b) 20% formamide in acetone	(a) *n*-Hexane–benzene–THF (4:1:1); (b) *n*-hexane–chloroform–diethyl ether	Separation of most herbicides in both TLC systems	234
Aliphatic C_2–C_{18} alkyl esters of acrylic and methacrylic acids	Silica gel (L)	5 or 10% paraffin oil in light petroleum	(a) DMF–methanol–water (9:5:1); (b) DMF–water in various ratios	No derivatizion of the compounds before TLC. Poor separation of some neighbouring members in each series	206,247

C_2–C_{18} alkyl esters of acrylic acid	Silica gel (C) and cellulose (C)	(a) 5% paraffin oil in light petroleum; (b) 40% DMF in ethanol	(a) DMF–methanol–water (2:1:1); (b) cyclohexane–benzene	Derivatization with diazomethane prior to TLC. In the RP-TLC system (a), complete separation of the whole homologous series	248
C_{19} steroids	Cellulose (C)	1,2-Propanediol in methanol	Benzene–cyclohexane (50:50)	Separation of various 3-hydroxy epimers of C_{19} steroids, such as androsterone and epiandrosterone	246
Hydroxycinnamic acids	Silica gel 60 H (L)	5% paraffin oil in benzene–acetone (1:1)	Different buffers at various pH (2.9–10.1)	Separation of *cis–trans* isomers in the pH range 4.5–6.5	249
Organophosphorus insecticides	Silica and alumina sheets (C)	60% DMF in methanol	DMF saturated with *n*-hexane	Separation of Phoxim from Baytex from human cadaver organs	252
Ecdysteroids (insect-moulting hormones)	Silica gel (C)	7.5% Nujol in dichloromethane	Methanol–water (1:1)	For the separation of this class of compounds, both NP- and RP-TLC systems can be used	255
Dothistromin (metabolite produced by *Dothistroma pini*)	Silica gel GF_{254} (L)	5% paraffin oil in hexane	Methanol–water (2:1) containing 4% formic acid	Quantitative analysis after separation from chlorophylls and other compounds	250
Reserpine and ajmaline in *Rauwolfia vomitoria*	Silica gel (C)	20% formamide in acetone	Methyl ethyl ketone–*n*-heptane (1:1) in ammonia atmosphere	Determination of both alkaloids	251
Steroidal glucosiduronic esters	Paper	Formamide	Various liquid ion exchangers in cloroform	Resolving characteristics of the various systems compared in terms of R_M values	144

[a] (L) = laboratory made; (C) = commercially available.

methanol and tetrahydrofuran[210] or isopropanol[207] were investigated. For phenolic[210] and barbituric acid[207] and aniline derivatives[243], linear relationships between R_M values and the percentage of the organic modifier were observed for most of the compounds investigated. However, the R_M values of the aniline derivatives were lowest in acetonitrile and highest in methanol. The solvent strength was acetonitrile > acetone > methanol[243]. Using a spectral mapping technique, Cserháti and Bordás[240] evaluated the relative strengths of 27 organic solvents in RP-TLC on impregnated layers. In determinations of the lipophilicity of *n*-alkyl phenyl ketone homologues, acetone appeared to be less suitable than methanol or N,N-dimethylformamide, probably because of acetone-induced perturbations of the stationary phase[241]. The use of a hydrophobic parameter on the basis of R_M values, but independent of the composition of the mobile phase, was suggested by Draffehn *et al.*[242]. A comparison of extrapolated R_M values (R_{M_0}) with water, aqueous methanol or aqueous acetone as eluents was reported by Barbaro and co-workers. Silica gel plates coated with silicone oil as stationary phase and dermophin-related oligopeptides[244] or a series of prostaglandines[245] as test compounds were examined. It was pointed out that the relationship between R_M values and the percentage of organic modifier can generally be described by an S-shaped curve. They suggested calculating the R_{M_0} values from the linear part of the curve, which corresponds to a narrow range of organic solvent concentration. In this way, very similar R_{M_0} values were obtained from both the water–acetone and water–methanol systems, confirming that the extrapolated R_{M_0} values are independent of the nature of the organic modifier[225]. For some of the test compounds, the R_{M_0} values were very similar to the experimental R_M values obtained with water as the eluent[244]. The data seem to provide a further contribution to the use of RP-TLC as a standard system for molecular lipophilicity determinations.

5.3. *Analytical separations*

In Table 2 the compounds or classes of compounds investigated for analytical purposes by partition TLC on coated plates, the chromatographic systems used and the results obtained are reported. A wide variety of compounds have been analysed with good results, which in many instances cannot be obtained on bare silica gel plates[206,207,232,246]. As can be seen, both RP[203,206,207,211,242,247–250] and NP[211,234,246,248,251,252] partition TLC were employed. A systematic collection of data on the chromatographic behaviour of a large number of aliphatic amines, with particular emphasis on eluents, R_F values and detection reagents, was reported by Prandi[203]. From these data, relationships between chemical structure, chromatographic behaviour and physical properties of the amines were obtained, which may be useful for identification purposes.

Some quantitative determinations have been reported[250,251]. With dothistromin, the sole metabolite identified in extracts of diseased pine foliage, the time required for sample application, TLC and analysis was 2 h. The method proposed may also be useful for the examination of foliage polyphenols and phytoalexins, where interference from chlorophylls may be a problem[250].

5.4. *Chemically modified and impregnated plates: comparison for reversed-phase thin-layer chromatographic use*

RP-TLC on alkyl-bonded silica gel plates has become widely used in the last 10 years[5] and has superseded the earlier use of paraffin- and silicone-oil-coated silica gel layers. A comparison between the two types of RP-TLC plates may help in the choice of the best system in relation to economy, convenience or saving of time.

In a series of studies[253–255], Wilson and co-workers compared C_8, C_{12} and C_{18} materials bonded with analytical and preparative paraffin-coated silica gel plates using a wide range of organic test compounds. In order to obtain comparable results, coated and bonded types were chromatographed simultaneously in the same TLC tank. On both paraffin-treated (analytical and preparative) and OPTI-UP-C_{12} (ref. 253) or on non-hydrophobic bonded C_{18}[255] a similar order of retention of ecdysteroids was found. As regards quantitative determination, comparable results were obtained on all three types of layers for amounts of material of the order of 200 ng, whereas below 100 ng per plate C_{12} gave better results[242]. In all instances, the C_{18} layers required a higher concentration of methanol in the eluent to achieve the same R_F values as on the coated plates. Like the non-hydrophobic C_{18} bonded plates, the paraffin-coated plates could be used with solvent systems containing from 0 to 100% of water[253–255].

Correlation plots of log k' (from HPLC results on C_2 bonded silica gel) *versus* the R_M values obtained on silanized and paraffin-impregnated silica gel layers were reported for some phenolic acid derivatives by Grodzińska-Zachwieja *et al.*[210]. The correlation was better for the silanized plates. However, the identical retention sequences of the test compounds on both bonded and coated plates compared with those obtained on a column indicated a similar separation mechanism and proved the usefulness of RP-TLC systems. As reported in Table 2, plates coated with formamide and liquid paraffin and RP-2 and RP-18 precoated HPTLC plates were compared for separations of alkylphenoxyalkanoic acids by Davídková and Gasparič[211]. RP-HPTLC on precoated plates and NP-TLC on formamide-coated silica gel layers seemed to be the most efficient methods.

A simple method of two-dimensional TLC with different separation mechanisms in the two directions was proposed by Wilson[256]. Normally, for this type of two-dimensional TLC commercially available RP-18 plates, which have a narrow strip of bare silica gel, are used. As an alternative, a section of a silica gel TLC plate may be silanized in the laboratory. The method proposed by Wilson is based on the incorporation of paraffin in the normal-phase solvent system used to obtain the initial separation. During the development in the first direction, a concomitant coating of the layer is obtained, and after the evaporation of the solvent a TLC plate ready for subsequent RP-TLC in the second direction is obtained. Of the numerous compounds tested, only with ecdysteroids was it not possible to add paraffin to the first solvent system. In such instances it may be possible to coat the TLC plates after the normal-phase separation.

In conclusion, partition TLC on plates coated with very low or very high polarity materials has been succesfully used in molecular lipophilicity determinations and for analytical purposes. For most applications, with appropriate modifications to the eluent paraffin-coated and non-hydrophobic C_{18} bonded silica gel plates may be interchangeable[255] and could be used for the optimization of the separation conditions in column HPLC[210]. In addition, whereas paraffin-coated plates can be used with any

proportion of water in the eluent, many types of commercially available silanized plates are hydrophobic, with a consequent limitation on the use of water in the eluent and in the direct application of biological samples to the layers[5]. However, in spite of the suggestion by Tomlinson[200] that chromatographically obtained parameters should have wider applicability in structure–activity relationships, papers dealing with the determination of lipophilicity by using TLC on coated or bonded plates are not very numerous. In addition, a comment is needed on some of the reported papers. In some instances the linear relationships reported by plotting R_M values *versus* the percentage of organic modifier in the eluent were obtained from few and/or not always chromatographically significant R_F values (<0.2 and >0.8). Indeed, for the most retained compounds, a small difference in R_F values (*e.g.*, $R_F = 0.04$ or 0.06) corresponds to a significant difference in R_M values (1.38 and 1.19, respectively). For compounds with $R_F > 0.8$, the uncertainty in the eluent front determination, due to probable solvent demixing of the mobile phase, can cause erroneous results.

6. pH CHANGES OF THE LAYERS

In liquid chromatography, the pH of the system exerts a marked effect on the chromatographic behaviour of ionizable compounds. Indeed, as reported by many workers, the retention of the same analyte in the protonated or deprotonated forms may differ considerably. In HPLC, reversed-phase columns can be made to retain weakly acidic or basic samples by buffering the eluents in the pH range 2–5 or 7–8, respectively, that is, by a shift of the chemical equilibria to undissociated forms. This technique has been termed "ion suppression" and a discussion of the current thinking may be found, *e.g.*, in a review by Bidlingmeyer[152]. In flat-bed chromatography, a convenient variation of this approach is to modify the pH of the layer. This treatment before the chromatographic development may be performed with the vapour phase of aqueous acids or bases[257–259] or by dipping the paper[260] or the plates[261–264] in aqueous acids[260,262] or bases[263] or in buffer solutions[261]. By an appropriate change in the pH of the layer, the separation of compounds of similar structure, such as benzoic acid or aniline derivatives[257], polar aromatic compounds[259], coumarin anticoagulants[261], amino acids[262] and barbiturates[263], was improved. Very sharp separations of six noble metals, Au^{III}–Os^{IV}–Pt^{IV}–Pd^{II}–Cu^{II}–Ag^{I}, and inorganic or organic mercury analysis[258] were also performed by Przeszlakowski and Flieger[260] and by Bruno *et al.* [258], respectively, by conditioning the layers prior to elution.

The advantage of the use of pH gradient layers was presented by Stahl and Müller[264] and confirmed by Quirin[265], when tested on crude phospholipid extracts of animal and vegetable origin or on a model mixture of ten components.

7. ELECTROLYTE-IMPREGNATED LAYERS

By addition of electrolytes to a chromatographic system, the ionic equilibrium may change and an improvement in the separation process is obtained. In column chromatography, sharp separations have been obtained by adding various electrolytes to the eluents[266], whereas in flat-bed chromatography, salt-impregnated paper or plates are commonly used to improve the separation of a wide range of compounds[8].

Many investigations on the separation of sugar mixtures by means of TLC on electrolyte-impregnated plates have been carried out and were reviewed by Ghebregzabher *et al.*[267]. Silica gel layers impregnated with mono- or dihydrogenphosphates were found to give the best results by Ovodov *et al.*[268]. As the solubility of sugars in inorganic salt solutions is known to increase, within certain limits, with an increase in salt concentration, a higher solubility of the sugar in the stationary phase and a decrease in the R_F values were obtained, with consequently better separations[268]. In recent years, some attempts have been made to improve the TLC separation of carbohydrates using phosphates as impregnating agents[269–272]. The determination of glucose, fructose and sucrose in molasses by HPTLC[270] and the application of sintered silica gel plates[271] or of aminopropyl-bonded silica gel plates[272] instead of plain silica gel plates have been reported. Potassium dihydrogenphosphate-impregnated silica gel plates were also employed by Touchstone *et al.*[273] for the separation of bile acids from diluted specimens of human bile. Potassium oxalate is another salt commonly employed as an impregnating agent. Numerous studies on the metabolism of phosphoinositides in biological membranes by using TLC on potassium oxalate–silica gel plates have been reported[274–280]. Calcium oxalate-impregnated silica gel plates were employed for the separation and identification of closely related sulpha drugs by Srivastava *et al.*[281] whereas in a study of the TLC separation of aromatic amines, the importance of the anion and not of the cation impregnation was indicated[282]. Two-dimensional TLC of polar lipids on ammonium sulphate-impregnated silica gel plates was developed by Jain and Subrahmanyam[283] and various salts were found to be good impregnating agents in the TLC separation of metal ions by Ajmal *et al.*[284].

In conclusion, pH gradient plates, with three different run directions, have not been used as expected, whereas potassium oxalate-impregnated plates are widely employed as sequestrating agents for any calcium that might be present when chromatographing phosphoinositides[8] and in their separation in studies of their metabolism in biological membranes.

8. CONCLUSION

As shown in Table 3, many compounds can be analysed by flat-bed chromatography on impregnated layers, achieving the separation by very different retention mechanisms. In most instances, separations not achieved on plain paper or silica gel plates were obtained on impregnated layers and quantitative results were reported. The advantages of this flat-bed technique over HPLC and GC are economy, technical simplicity and the possibility of testing, relatively rapidly, a large number of impregnating agents as possible packing materials for TLC and HPLC. As regards the future, the very promising ion-pair and ligand-exchange techniques, widely used in HPLC, deserve major attention in TLC on impregnated plates. In addition, more investigations on the chromatographic parameters, as performed by only a few research groups, could clarify the mechanisms of the separation processes and make possible further advances in TLC.

TABLE 3

COMPOUNDS OR CLASSES OF COMPOUNDS ANALYSED BY FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

Compounds or classes of compounds	*Ref.*	*Compounds or classes of compounds*	*Ref.*
Acetophenones	212	β-Cyclodextrins	120
Hydroxy-	46	Dothistromin	250
Acrylic and methacrylic acids		Drugs	55,136
(aliphatic C_2–C_{18}-alkyl esters)	206,247,248	Anti-inflammatory	254,256
Ajmaline	251	Basic	185
Aliphatic acids (aryl)	222	Sulpha	52
Alkaloids	51,135,179	Dyes	63,65
Amines	88,92,139	Azo	121,220
Aliphatic	154,158,165, 180,203	Fluorescent	264
		Food	156,170,171
Aromatic	155,157,180, 285, 286, 290	Synthetic	64
		Enantiomers	96–107
Amino acids	50,57–62,90,92, 134,159,163,165, 169,173,231,235, 237,262	Fatty acids	
		Cholesteryl esters	16
		Isomers	22–25
		Labelled methyl esters	29
		Polyunsaturated methyl esters	19,21
ω-Amino acid derivatives	218	*trans*-Hexadecenoic and	
Amino sugars	165	*trans*-Octadecenoic methyl esters	33
Aniline derivatives	204,257	Gibberellins (*p*-nitrobenzyl esters)	28
Ring-substituted	236,243	Glycerides	30
Anions	140,141	Hydrocarbons (polycyclic aromatic)	49
Antibiotics		Imidazoles (5-nitro)	214
4-Epi-meclocycline	86	Indole derivatives	167
Polymyxin	115	Inorganic ions	70–79,129–133,292
Tetracyclines	83–85, 87, 291	Isomers, positional	113
Barbituric acid derivatives	66,119,207,263	Ketones (*n*-alkylphenyl)	241
Benzodiazepines	213	Lactulose	43
Benzophenones	223,224	Lichen acids	47
Benzoic acid		Lysophospholipids	24
Derivatives	257,259	Mercaptans	91
Esters	228	Mercury (inorganic and organic)	258
Hydroxy-	254	Naphthalene (α and β derivatives)	208
Bile acids (dihydroxy conjugated)	273	Naphthols	212
Bufadienolides	37	Noble metals	260
Cannabinoids	293	Nucleic acids	49,50
Carbamates	53	Peptides	160,165,169,172,235
Carbanilic acid derivatives		Dipeptides	162,163,168
Basic esters	220	Oligopeptides	244
Carbohydrates	68,89,271	(dermophin related)	
Cardenolides	36–38	Polypeptides	164
Chelating agents (sulphonated)	142	Pesticides (organophosphorous)	288,289
Cholesterol	335	Phenolic acids	56
Cinnamic acid derivatives		Polyhydroxy derivatives	67
Hydroxy-	210	Phenols	138,161,174,287
cis- and *trans*-hydroxy-	249	Acid and aldehydes	54
Copper	81	Amino	254,256,290
Coumarins	227,261	Chloro	99
Crotonolactones	221	Chloro, bromo, alkyl	166
Crown ether derivatives	230		

TABLE 3 *(continued)*

Compounds or classes of compounds	*Ref.*	*Compounds or classes of compounds*	*Ref.*
Nitro, cyano, halogeno	229	Androstanes, androstenes	246
Phenothiazine bases and sulphoxides	176	Ecdysteroids	253–256
Phenoxyalkanoic acids (alkyl, substituted)	211	Steroidal glucosiduronic esters	144
Phenylhydrazones (2,4-dinitro-)	229	Styrene (nitro-)	118
Phosphates (diethylphenyl)	232	Sugars	269,270,272
Phosphatides	266	Sulphonamides	157,217,219,226
Phosphoionositides	40,274–280	Sulphonic acids	174,180
Phospholipids	20,30,39,41	Terpenoids	18
Phosphonothioates (phenyl)	205	Thiazole derivatives	215
Prenyllipids	23	Triazines	
Prostaglandins	31,32,245	17-Substituted symmetric	240
Purine derivatives	48	Trisubstituted symmetric	229
Pyrimidine derivatives	48	*s*-Triazine herbicides	34,116,234
Pyrazole derivatives	45	Triphenyl methane	117
Rare earths	143	Triterpene alcohols (trihydroxy pentacyclic)	26
Resorcinol derivatives	27	Uranium	80
Ribonucleotide reductase	44	Xanthone derivatives	216
Steroids	82,239,242		

9. SUMMARY

It is shown that improvements in paper and TLC analysis can be obtained by impregnating the layers with compounds differing in chromatographic behaviour. The results obtained by using complexing, ion-exchanging and ion-pairing agents are reviewed. Particular attention has been paid to the determination of molecular lipophilicity, as this parameter is widely used in studies in correlations with chemical structure and with biological activity (QSAR). For these studies, layers impregnated with compounds of very low or very high polarity and the appropriate eluents were used. The comparison between chemically modified and impregnated silica gel plates is discussed. Table 3 shows all the compounds or classes of compounds analysed by flat-bed chromatography on impregnated layers.

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REVIEW

APPLICATION OF LIQUID CRYSTALS IN CHROMATOGRAPHY[a]

ZYGFRYD WITKIEWICZ
Institute of Chemistry, Military Technical Academy, 01 489 Warsaw 49 (Poland)
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CONTENTS

1. INTRODUCTION

This review constitutes the second part of a survey of liquid crystalline stationary phases for gas chromatography, the first part of which dealt with the properties and applications of liquid crystals as stationary phases in gas chromatography[1]. When the first part was written, it was not expected that it would have a continuation. In recent years, however, many new papers have been published on the application of liquid crystals in chromatography. Not only have studies been continued in those

[a] This work is dedicated to Professor Hans Kelker who was the first to introduce liquid crystals in chromatography.

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countries and centres in which they were started earlier, but also research now is being undertaken elsewhere. Particularly interesting is the rapid development of studies on liquid crystal stationary phases in China.

Recent studies have concentrated on new analytical applications of liquid crystal stationary phases and the use of new liquid crystal phases, not only rod-like but also disc-like. Apart from the still most common applications of liquid crystals as stationary phases in gas chromatography, they are also finding application in supercritical fluid chromatography and liquid chromatography. In the latter instance, liquid crystals are not only useful as stationary phases in chromatographic columns but may also be used for visualizing thin-layer chromatograms.

In the recent years our knowledge of the properties of liquid crystal stationary phases has increased considerably, and so has the range of their practical, often routine, applications. In Poland, for instance, there are laboratories in which liquid crystals have been used for some time for the separation and determination of the isomers of polynuclear hydrocarbons. In addition, our knowledge of other possible applications of liquid crystals in chromatography and elsewhere has also expanded. For instance, the experience from chromatography has been utilized in piezoelectric detectors[2–4]. It has been shown that a quartz resonator coated with a liquid crystal layer reacts in different ways to isomers of substances differing in molecular structure and detects them with varying sensitivity.

Research on liquid crystal has been developing very rapidly both as regards the synthesis of new substances and the knowledge of their properties and applications, as indicated in the recent years in general monographs and reviews[5–12] and in those devoted to chromatographic applications[13–18]. In one of the reviews the separation properties of cyclodextrins and liquid chrystals as stationary phases in gas chromatography are compared[19]. About 3.6% of recent publications concerned with gas chromatography are devoted to liquid crystal stationary phases[20].

In future research on liquid crystals as chromatographic materials the efforts of organic chemists synthesizing liquid crystals should be combined more closely with those of analysts, chromatographers, physical chemists and physicists studying the properties of liquid crystals. Such cooperation might contribute to a better knowledge of the relationships between the structure of the liquid crystal molecule and its separation properties, and hence to the development of new materials with the required, optimum features. The lack of such cooperation was clear at the 12th International Liquid Crystal Conference (Freiburg, 1988), where the application of liquid crystals in chromatography was not even mentioned[21]. However, several papers on the application of liquid crystals were presented at the 17th International Symposium on Chromatography (Vienna, 1988)[22].

This review encompasses work that was published or became available after the first review was written[1]. A few of these were, in fact, included in the first review at the last moment, but it was not possible at that time to give them more detailed consideration. The fundamental properties of liquid crystals are not discussed here as they were considered in the first review.

2. NEW LIQUID CRYSTALS USEFUL IN CHROMATOGRAPHY

The number of new liquid crystals synthesized is very large but most of them are

designed for use in displays, although some of them may be applied succesfully in chromatography[23]. So far, however, only a small number of the known liquid crystals have been used in this field.

In recent years a range of liquid crystal polymers have been obtained[24]. Among them, siloxane polymers are gaining particular significance in gas chromatography. Many of the latter have been synthesized intentionally for that purpose.

2.1. Polysiloxanes

Siloxane polymers are among the best known stationary phases for gas chromatography[25–27], and the production of liquid crystal siloxane polymers is a logical step in the development of liquid crystal stationary phases and of stationary phases in general. Today several kinds of liquid crystal siloxane polymers are known. Most often they are obtained by adding to the siloxane polymer backbone mesomorphic groups that impart liquid crystal properties to the polymer. Such groups are bonded to the backbone by means of flexible aliphatic chains (spacers). However, mesomorphic compounds when bonded to the backbone do not always yield a liquid crystal polymer, and it is also possible that a liquid crystal is obtained after non-mesomorphic groups are bonded to the siloxane backbone. The properties of the liquid crystal siloxane polymer are influenced by the length of the polysiloxane chain, the number of the mesomorphic substituents, the kind of mesomorphic group and the length of the spacer bonding the mesomorphic groups with the backbone[28]. Alkane or alkene chains are used as spacers[29].

When synthesizing liquid crystal polymers from polymethylsiloxanes it was found that if the initial polymers used have different molecular weights but the mesomorphic compounds are the same, the resulting liquid crystal polymers have different melting points but the same mesophase to isotropic liquid transition temperature[30]. The phase transition temperatures depend on the number of methyl groups bonded to the siloxane backbone; the greater the number of these groups, the lower is the nematic–isotropic liquid transition point. It is expected that by selecting a suitable length of the siloxane backbone, mesomorphic groups, spacers and numbers of non-mesomorphic groups, it will be possible to obtain polysiloxanes with melting points close to ambient temperature and clearing points above 300°C.

Biphenylcarboxylic esters of polysiloxanes constitute a large group of liquid crystal polymers[29,31,32] and several procedures for their synthesis for chromatographic applications have been described[28,30–32]. Not only nematic but also smectic–nematic and smectic polymers are suitable as stationary phases[29,32,33]. Polymers with the cholesteric mesophase are also known[29], although to a much smaller extent.

Polymers with mesogenic groups in the side-chains are usually non-crystallizing. The lower limit of their liquid crystal state is the glassy to mesophase transition temperature above which a segment mobility appears owing to the lability of the particular fragments of the macromolecule. In the temperature range from the glassy to mesophase transition temperature to the clearing point the polymer is in the liquid crystal state. In the temperature range of the mesophase one can distinguish the flow temperature, below which the polymer is an elastomer, in which state it is unsuitable as a stationary phase. The non-crystallizing polymers do not freeze on cooling but at a characteristic temperature (for each of them) they become glassy, preserving their mesophase structure in the course of further cooling. The liquid crystal polymers

usually reveal a wider range of the mesophase than the corresponding monomeric liquid crystals.

The polymers have a high thermal resistance and show good behaviour in the column; further improvement in their properties is possible by cross-linking in the column[28]. Azo-*tert.*-butane has been succesfully used as the cross-linking agent. The cross-linked stationary phases are more stable, but the efficiency of the columns decreases by as much as 30%.

Some formulae of liquid crystal polysiloxanes are given in Fig. 1 as examples.

Among the siloxane polymers, attention should be drawn to the phthalocyanine derivatives of siloxane polymers that have not so far been tested in gas chromatography[34]. These compounds show exceptionally high chemical and thermal stability, a wide range of the mesophase and very low viscosity in some temperature ranges, which makes it probable that they will make excellent stationary phases.

2.2. Polyacrylates

These polymers are obtained from the corresponding acrylates by radical polymerization with the use of 2,2′-azoisobutyronitrile (AIBN) as initiator[35]. The scheme of this reaction is shown in Fig. 2.

From monomeric acrylates, which provide smectic and nematic mesophases, liquid crystal polymers are obtained with only the nematic phase, but wider and occurring at higher temperatures. The mesophase range of some of these polymers exceeds 200°C.

2.3. Isothiocyanates

The opinion seems to prevail that liquid polymers are, in general, better stationary phases than are monomeric liquid crystals. In addition to the advantages already mentioned, the better arrangement of the liquid crystal polymer molecules on the surface of the capillary column walls compared with monomeric liquid crystals is emphasized. However, this is not so in every instance, and the practical importance of some monomers is still significant, *e.g.*, azo- and azoxyarylethanes[36].

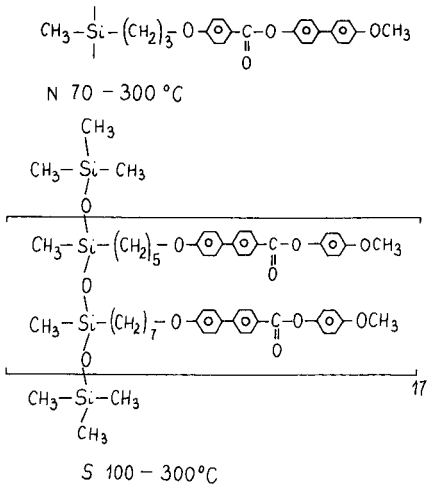

Fig. 1. Structure of liquid crystalline polysiloxanes.

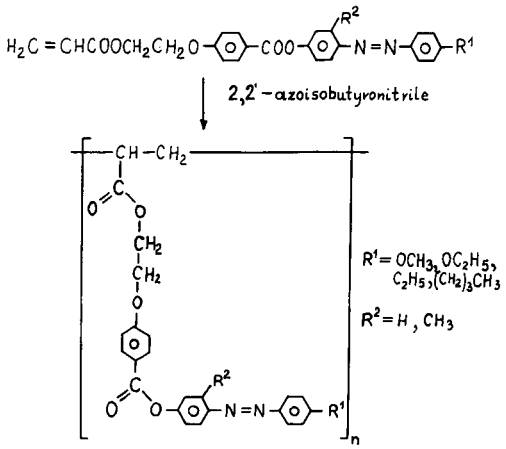

Fig. 2. Scheme of synthesis and structure of polyacrylates[35].

Great hopes can be set on the recently obtained liquid crystals belonging to the isothiocyanate group[37,38]. Some compounds belonging to this group reveal very wide ranges of the mesophase, comparable to those of the polymers. These compounds are chemically and thermally stable, have low viscosities and show good behaviour in capillary columns. Their advantage is that they are obtained more easily than the siloxane polymers. It can therefore be expected that some monomeric liquid crystal stationary phases will be competetive with polymeric liquid crystals.

Fig. 3 shows the formulae of several liquid crystal isothiocyanates.

2.4. Disc-like (discotic) liquid crystals

The study of disc-like liquid crystals as stationary phases is very interesting not only from the theoretical point of view but also increasingly for purely practical reasons. These liquid crystals have found application in chromatography only recently, although they have been known since 1977[39]. Today it is known that the rod-like structure of the molecule is not a necessary condition for a compound to be a liquid crystal; the molecule may be flat or disc-shaped[12]. Disc-like liquid crystals are known

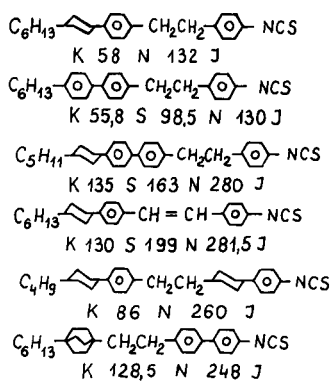

Fig. 3. Formulae and temperatures of phase transitions of liquid crystalline isothiocyanates.

which are derivatives of benzene[40,41], triphenylene[42,43], truxene[44,45], rufigallol[46,47], tetraphenylpyrylidenepyran and tetraphenylpyrylidenethiopyran[48,49], naphtalene[50], phthalocyanines[51,52] and other compounds[53,54]. Polymeric disc-like liquid crystals have been reported[55]. The disc-like liquid crystals reveal ranges of the mesophase narrower by several tens of degrees than those of the rod-like type. The discotic liquid crystals studied so far as stationary phases have very narrow ranges of the mesophase[56,57].

Fig. 4 shows a general scheme of molecules of discotic liquid crystals and the structures of the mesophase. The structure may be nematic, twisted nematic (cholesteric) or columnar[58–60]. The columnar structure, which may be considered as corresponding to the smectic structure, has several modifications.

A large number of known liquid crystals, their characteristic molecular structures and their fundamental properties have been surveyed by Demus and Zaschke[61].

3. COLUMNS WITH LIQUID CRYSTAL STATIONARY PHASES

In recent years there has been a strong trend toward the use of liquid crystals in capillary columns. This is in accordance with the general growth in importance and increasing application of capillary columns in gas chromatography[62–65]. An additional reason for the spread of capillary columns is that the efficiency of normal analytical columns filled with liquid crystals is usually lower than that of columns filled with conventional stationary phases. This lower efficiency is due to the high viscosity of the liquid crystal stationary phases. To improve the efficiency of columns filled with liquid crystals, the latter are mixed with conventional isotropic stationary

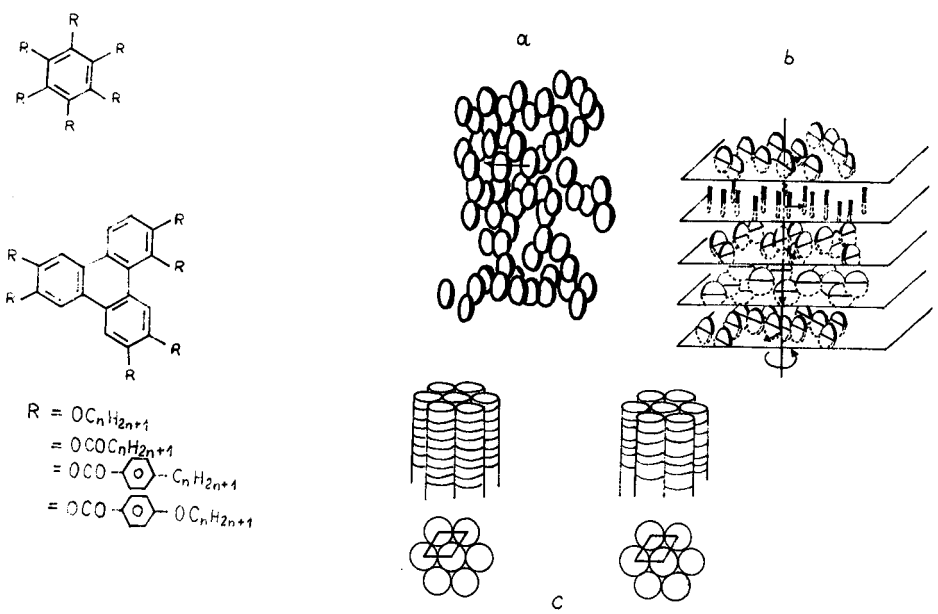

Fig. 4. Example of formulae and structures of the disc-like liquid crystal mesophase: (a) nematic; (b) cholesteric; (c) columnar.

phases, *e.g.*, SE-30, SE-52, SE-54 or OV-17[66–69]. Mixing of two liquid crystal stationary phases does not lead to a notable improvement of the column efficiency, which remains intermediate between the values obtained for the individual components of the mixture[70].

When the liquid crystal is deposited in a capillary column, the efficiency of the system becomes sufficiently high to allow, very good separations of complex mixtures to be achieved, considering the high selectivity of the liquid crystal stationary phases. The high selectivity of liquid crystals even makes it possible to use much shorter columns than those filled with conventional stationary phases. This is becoming easier because with liquid crystals of low viscosity, which are becoming increasingly available, the efficiency of the columns obtained is comparable to that of columns filled with conventional stationary phases.

However, despite this general trend towards capillary columns, in many instances liquid crystals continue to be used in conventional analytical columns.

3.1. Conventional analytical columns

In most instances Chromosorbs or Chromatons, usually silanized or acid washed, have been used as supports. Less frequently silica gels, *e.g.*, Silochrom 80[71], or glass beads[72,73] were applied. The liquid crystals (low or medium temperature) are applied to the conventional supports usually in amounts of 10–15% and seldom more than 20%, *e.g.*, 24.5%[74]. High-temperature liquid crystal stationary phases are applied to the conventional supports in an amount of 5%. This is related to the volatility of these substances; if the high-temperature phases, applied at about 300°C, were used in larger amounts bleeding of the phase from the columns might render the analysis impossible. This is particularly important when the substances to be analysed are presented in the sample in trace amounts, *e.g.*, in the analysis of polycyclic hydrocarbons. In the analysis of these hydrocarbons the liquid crystal phases may be deposited on glass beads[72,73], which were sometimes pretreated with sodium dodecylbenzenesulphonate[72]. The amount of the stationary phase (*e.g.*, BMBT) deposited on the beads was small (up to 0.025%)[73].

In most instances the liquid crystals are deposited on the supports from solutions in commonly used volatile solvents, but also in formic acid[75]. If active supports of high surface area and high adsorption potential such as Silochrom 80 are used, a special procedure for applying the liquid crystal is recommended, in which a few percent (*e.g.*, 6%) of the liquid crystal is deposited several times from solutions of increasing concentration[71]. This concentration should be adjusted depending on the properties of the adsorbent used as the support. The columns with a filling prepared in this way have a higher efficiencies than those with the filling prepared in the conventional manner. In addition, the efficiency may be higher at temperatures corresponding to the solid state than at those of the smectic or nematic. With columns with fillings prepared by the multi-step procedure the retention times of the chromatographed hydrocarbons (heptane, toluene) are shorter and the peaks are narrower and more symmetrical than with columns with fillings prepared in a one-step operation.

Some high-molecular-weight, high-temperature liquid crystals are poorly soluble and were deposited on the supports from suspensions in a solvent. Sometimes also the powdered stationary phase was mixed with the support, *e.g.*, for 48 h[76,77]. During conditioning of the column (at temperatures above the melting point) the liquid

crystal was distributed on the surface of the support, and the properties of the column did not differ from those with a filling prepared by deposition of the liquid crystal from a real solution.

By applying various liquid crystals in different systems, the optimum heights of the columns equivalent to a theoretical plate obtained were, *e.g.*, 0.6–1.8 mm[78,79]. When the columns were filled with a mixture of a liquid crystal and a conventional isotropic phase (*e.g.*, BBBT + SE-30), their efficiency depended on the quantitative composition of the mixture and on the column preparation procedure applied[66]. The height equivalent to a theoretical plate then varied, in various columns, from 0.9 to 1.6 mm.

The columns used had similar diameters (2–4 mm) and lengths (up to several metres) to those with conventional stationary phases. Sometimes, however, they were much shorter, *e.g.*, 0.7 m[72].

3.2. Capillary columns

Capillary columns make it possible to combine the high efficiency of such columns with the high selectivity of liquid crystals. The wide use in chromatographic practice of capillary columns filled with liquid crystals is envisaged, although it is realized that the general requirements regarding the reproducibility, thermal stability and wettability of the walls is greater with capillary columns than packed columns[80].

Liquid crystal stationary phases are deposited on the capillary walls from solutions by the generally used dynamic or static methods in layers 0.1–0.3 μm thick[64,65]. When applying the liquid crystals by the dynamic method, 20%[81] or 10%[82] solutions were used, and in the static method less concentrated solutions, *e.g.*, 0.25%[83].

The superdynamic method of Berezkin and Korolev[62] developed for conventional phases in which stationary phases are deposited on capillary walls, might be successful with liquid crystal phases also. This method makes it possible to obtain columns with good reproducibility and properties. The columns have 3500–5500 theoretical plates per metre and capacity factors of 2.2–2.4.

If the liquid crystal is poorly soluble but easy to produce, then its synthesis is conducted directly *in situ*. This method was applied, for instance, with liquid crystal Schiff bases (BPhBT)[84]. The solutions of the substrates, in this instance of an aldehyde and an amine, were mixed in suitable proportions and the mixture was introduced into the column, where it was heated to the required temperature at which the reaction took place. The resulting product was deposited on the column walls, whereas the unreacted substrates and solvent were removed from the column.

The efficiency of the column and its separation ability depend on the kind of liquid crystal used, its molecular structure and the related polarity, and also on the way in which the capillary walls have been pretreated and on the amount of the liquid crystal deposited on their surface[85–88]. The walls of glass columns were preliminary etched with hydrogen chloride and coated with barium carbonate. Often additional deactivation was achieved by applying a Carbowax layer, which improved the column efficiency. When liquid crystals obtained from azo or azoxy compounds were deposited on the walls of columns deactivated with Carbowax, their efficiency exceeded 3000 theoretical plates per metre and their stability was higher. The selectivities of the columns treated and untreated with Carbowax were, however, similar.[85]

Matišova *et al.*[87] studied the dependence of the properties of a liquid crystal

stationary phase on the thickness of the film on the column walls. They measured the phase transition temperatures of the liquid crystals, the column capacity factors, relative retentions and retention indices of the chromatographed substances for columns only etched with gaseous hydrogen chloride for 2 h at 330°C and for columns additionally deactivated with Carbowax 20M. The column efficiency depended considerably on the kind of reagent used for deactivating the column walls. Good columns, with 900–1000 theoretical plates per metre (as measured for anthracene at 230°C), were obtained by depositing BBBT on column walls deactivated with triphenylsilylamine[89]. When diphenyltetramethyldisilazane was used, the number of theoretical plates reduced to 500 per metre.

The column efficiency also depends on the phase in which the liquid crystal is used. For instance, for a brass column at temperatures corresponding to the mesophase and an isotropic liquid, 900–1300 theoretical plates per metre were obtained. whereas in the temperature range of the solid, the number of theoretical plates was only 100–150 per metre[81].

The column efficiency is strongly affected by the composition of the deposited mixed phase[68]. An increase in the proportion of the liquid crystal (BBBT) in the common isotropic phase (SE-52) lowered the column efficiency (in terms of the number of theoretical plates per metre) as follows: SE-52, 3381; BBBT – SE-52 (20:80), 1710; and BBBT – SE-52 (50:50), 858. When 20% of BMBT or 20% of BMxBT was added to SE-52, the column efficiency was 1600 or 1100 theoretical plates per metre, respectively.

Mainly glass capillary columns are used, although recently fused-silica columns have also been applied. Metal columns (steel, copper or brass)[81,82,90,91] are rarely used. The length of the columns varied for 4 m[81] to about 100 m.

When considering the choice of a chromatographic column, it should be realized that the separation of the components of a mixture depends much more on the kind of mesophase used and its molecular structure and range than on the kind and length of the column. Examples of the characteristics of capillary columns filled with liquid crystal stationary phases are given in Table 1.

4. FACTORS AFFECTING THE SEPARATION OF COMPONENTS OF MIXTURES ON LIQUID CRYSTAL STATIONARY PHASES

4.1. Kind of mesophase of the liquid crystal

In recent years, the mechanisms of chromatographic separations on liquid crystal stationary phases have seldom been studied, mainly because the general theoretical principles of such separations are known. However, it is not easy to describe the phenomena that occur at the molecular level and this aspect has not yet been completely elucidated. This task was undertaken and largely solved by Martire[102], who gave an exhaustive treatment of the problems connected with the mechanisms of the separation of substances of different molecular shape on liquid crystal stationary phases with various molecular structures. He proposed a theory based on the crystal lattice model in which statistical mechanics are utilized. Account was taken of molecules of the chromatographed substances of various shapes: thin and thick rods, plates, grains and semi-flexible chains. The liquid crystals considered were those whose molecular structure makes them low- or high-temperature types. These were

TABLE I

EXAMPLES OF CHARACTERISTICS OF CAPILLARY COLUMNS FILLED WITH LIQUID CRYSTAL STATIONARY PHASES

Liquid crystal stationary phase	*Temperatures of phase transitions[a] (°C)*	*Column (m × mm I.D.)*	*Method of filling, solution concentration (%)*	*Wall surface treatment*	*Test substance*	*Temperature of column testing (°C)*	*Number of theoretical plates (N)*	*Capacity factor of test substance (K)*	*Ref.*
MEPSIL IVB	C139N319I	Glass, 15 × 0.25	Static, 1.6 mg/cm^3	20% HCl, HMDS	Chrysene	230	2450/m		30
Polysiloxane	C118S300I	Fused silica 10 × 0.32	Static	N_2 at 250°C, 2h			4000/m	15	31
PMMS	C70N300I	Fused silica 19 × 0.32	Static, 0.3		Triphenylene	220	2200/m		67
CABHC	C119N232I	Glass, 24 × 0.26	Static	$BaCO_3$, Carbowax			75 000		85
PBO	C63N94I	Glass, 62 × 0.25	Dynamic, 2.5	HCl, N_2, 160°C	*trans*-2-Tetradecene	74	180 000 (160 000 eff.)	16.2	86
BBBT	C188N303I	Glass, 10 × 0.22	Static 2 mg/cm^3		Anthracene	230	900–1000/m		89
MEAB	C95.8N148I	Glass, 23 × 0.25			3-Phenyltridecane	140	60 000 (54 000 eff.)	17.4	92
MEAB	C95.8N148I	Glass, 20 × 0.25	Dynamic	HCl(g), N_2 150°C	*trans*-2-Tridecene	95	230 000 (170 000 eff.)	6.2	93

MEAB	C95.8N148I	Glass, 80 × 0.25	Dynamic, 3				215 000	5	94
MEAB	C95.8N148I	Glass, 63 × 0.25	Dynamic, 5				130 000	5	94
EBO	C97N112I	Glass	Dynamic, 5			50	106 000	2,1	95
EBO	C97N112I	Glass	Dynamic, 5			40	25 000	3,2	95
PBO	C63N94I	Glass, 100 × 0.25	Dynamic	HCl, 360°C			350 000	5	96
PBO	C63N94I	Glass, 100 × 0.25	Dynamic		1-Undecene	56	430 000 (360 000 eff.)	10.2	97
PBO	C63N94I	Glass, 100 × 0.25			*n*-Nonane	40	290 000 (170 000 eff.)	3.3	98
MEPSIL–SE-30		Glass, 60 × 0.25	Static		benz[*a*]anthracene	240	3210/m		99
MEPSIL		Glass, 60 × 0.25	Static		Chrysene	230	2450/m		99
HCFITFE	C58N132I	Glass, 15.5 × 0.3	Static	18% HCl, $BaCO_3$, Carbowax		61	700/m real		100
HBITFE	C55.8S98.5-N130I	Glass, 20 × 0.3	Static	18 % HCl, 150°C, $BaCO_3$		100.5	1700/m real		100
ITFPCBE	C135S163N-280I	Glass, 12.5 × 0.3	Static	18% HCl, $BaCO_3$, Carbowax		165	700/m real		101

[a] C = Crystal; S = smectic; N = nematic; I = isotropic liquid.

molecules with a rigid, rod-like core with semi-flexible side-groups. The effect of packing, connected with the molecular structure of the chromatographed substance, was considered to be an important element of the interactions.

The theory of separations on nematic liquid crystals is developed best, as it is on these that the best chromatographic separations are obtained. The theory of separation on smectics is much poorer, although there are more and more reports regarding the good chromatographic properties of some of them[28,29,31, 103]. In general it is assumed that smectics with a low degree of ordering of the mesophase (S_A. S_B) have better separation properties than those with a high degree of ordering. This problem requires more exhaustive studies, however.

The recent discovery of liquid crystals with re-entrant phases has created new possibilities for studying the relationship between the effectiveness of separation and other properties of liquid crystal stationary phases (*e.g.*, the column efficiency and the capacity ratio) depending on the kind of mesophase.

The re-entrant phases occur as a result of the effect of an anomalous sequence of phases shown by some liquid crystals. In such a case the nematic, cholesteric, smectic A and smectic C phases may appear twice in different temperature ranges. As a result, the same kind of phase (*e.g.*, a nematic) will appear at temperatures both higher and lower than another phase (*e.g.*, a smectic A). The phase appearing at the lower temperature is called the re-entrant phase. This phenomenon is related to the association of the liquid crystal molecules with the cyano terminal group and an alkyl or alkoxy group with at least eight carbon atoms. Most frequently the following sequence of phases with the re-entrant phase is observed: NS_AN_{re} or $NS_AN_{re}S_{Are}$ (where N = nematic; S_A = smectic A; N_{re} = nematic re-entrant; S_{Are} = smectic A re-entrant). However, instances are also known where only the low-temperature N_{re} phase occurs without the nematic phase at the higher temperature; in this event the following sequence of phases is observed: S_AN_{re}, $S_AS_CN_{re}$, $S_AS_CN_{re}S_{Cre}$ or $S_AN_{re}S_C$[104,105]. Re-entrant mesophases are observed not only in rod-like but also in disc-like liquid crystals[106].

The use of re-entrant phases in chromatography has so far been little studied[105,107–109]. Liquid crystals with a re-entrant phase can be used in a wide temperature range if they reveal a linear relationship between retention and transition temperature from one kind of phase to the other. An example of such a liquid crystal is 4-(4-nonyloxybenzoyloxy)-4′-cyanobenzene, which shows N, S_C and also N_{re} and S_{Cre} phases. This liquid crystal has a mesophase range of 67–220°C and when supercooled to 55°C preserves the S_{Cre} phase[108] (see Fig. 5). A similar, linear relationship between retention and temperature was observed[109] at the S_A–N_{re} phase transition. The observed[107] relationship log V_R = f(t) was, however, non-linear and connected with phase transition.

Some new interesting findings show that liquid crystal stationary phases can be used beyond the range of the true mesophase, *i.e.*, in the form of a solid, supercooled below the melting point, and in the form of an isotropic liquid, above the clearing point. Several examples are known of the use of liquid crystals in the solid state[71,110–112]. Karabanov *et al.*[110] described the determination of impurities in diethyl sulphide, which were separated much better on the solid than on the mesophase. The possibilities and limitations of using liquid crystal stationary phases in the solid state and in the mesophase have been described by Dmitreva and Gabitova[111].

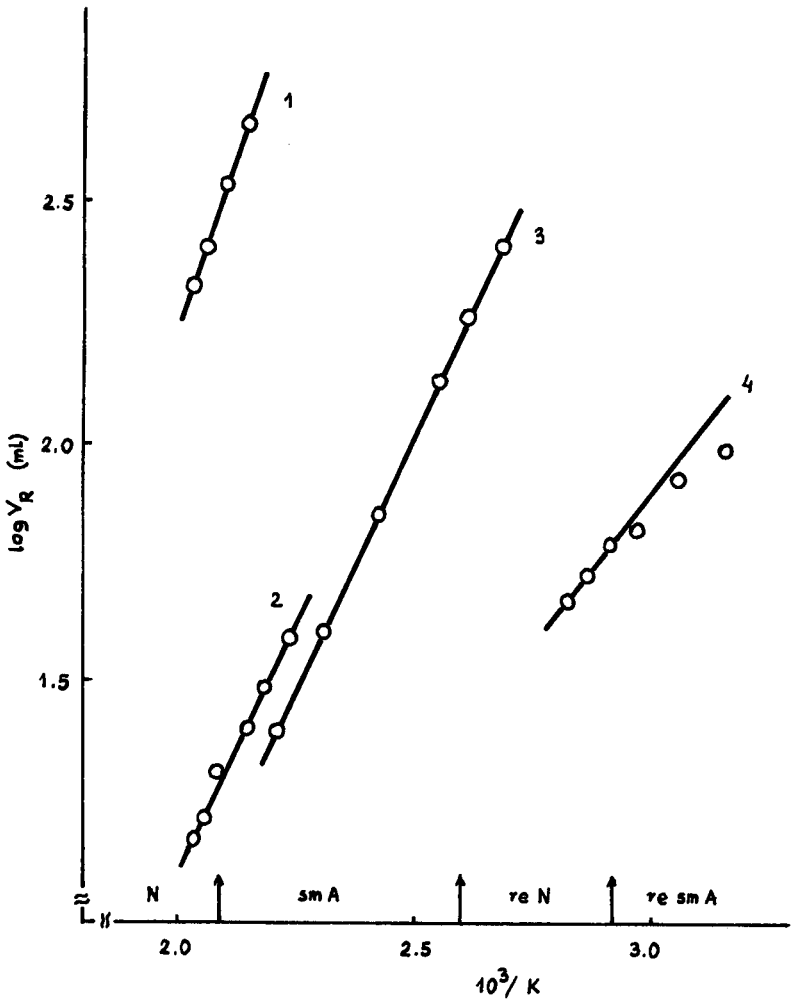

Fig. 5. Relationship between log (retention volume) and temperature for (1) phenanthrene, (2) *m*-chloroacetophenone, (3) *p*-dibromobenzene and (4) *o*-xylene[108].

In most instances the separations carried out on a solid stationary phase are much poorer than those conducted in the mesophase range and sometimes even poorer than those in which the isotropic liquid is used. For instance, the separation of isomers of trimethylbenzene on 4-butanoyloxy-4'-nitroazoxybenzene deposited in an amount of 20% on Chromaton N AW was possible with both the mesophase and the isotropic liquid but not on this stationary phase in the solid state[113]. The isotropic liquid is only seldom used, chiefly in those instances when the clearing point is not very high and the stationary phase reveals good thermal stability in the column. Industrial mixtures of methyl esters of C_8–C_{18} fatty acids were separated on methoxyethoxyazoxybenzene in short capillary columns at temperatures corresponding to the isotropic liquid[81].

In isothermal chromatography we usually utilize the initial temperature range

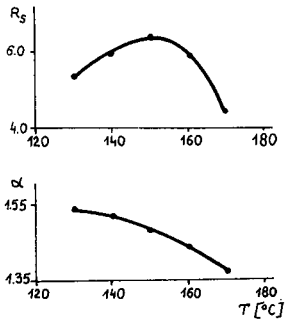

Fig. 6. Relationship between resolution (R_s) and relative separations (α) and temperature for anthracene and phenanthrene solutes on liquid crystalline polysiloxane stationary phase[30].

of the mesophase (above the melting point). The whole mesophase range or a large part of it is used when the temperature is programmed. Often also use is made of the mesophase supercooling range in which the ordering of the liquid crystal structure is greater than that in the true mesophase[76,79,87,93,96,114,115]. The supercooling may be very stable. For instance, after 12 months of mesophase supercooling at ambient temperature and 24 h at 0°C, the separations of mixtures were no worse than directly after supercooling[76].

The supercooling of the liquid crystal and the stability of the supercooled state are affected by, among other things, the molecular structure of the liquid crystal and also by the presence of lateral substituents[76,79,114,115]. The stability of the supercooled state is improved by the presence of halogen substituents.

Liquid crystal siloxane polymers are also liable to supercooling[30]. The relative retentions, α, of the chromatographed substances increase with supercooling and the resolution, R_S, achieves a certain optimum value (see Fig. 6). It is assumed that the corresponding temperature is the lowest point at which the stationary phase preserves its practical usefulness.

The possibility that a liquid crystal is supercooled depends on the thickness of its layer. The supercooled state is more stable when the liquid crystal film on the

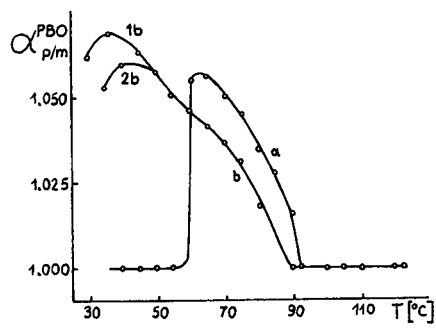

Fig. 7. Variation of the relative retention of *p*- and *m*-xylene ($\alpha_{p/m}$) on 4-*n*-pentylacetophenone (O-4-n-pentyloxybenzoyl oxime) (PBO) on heating the column (previously conditioned at ambient temperature) to the isotropic liquid temperature (a) and successive cooling to 30°C (b); 1b, cooling from the isotropic liquid (122.5°C); 2b, cooling from the mesophase (70°C).

capillary column wall is thicker[87]. If that film is thin (less than 40 nm), supercooling does not take place.

It has been found that the selectivity of a liquid crystal stationary phase in the supercooled state may depend on whether the cooling is started from the mesophase or from the isotropic liquid. It is more advantageous when the isotropic liquid is subjected to supercooling (see Fig. 7)[96].

Another method of chromatography on liquid crystals below their melting point is to use mixed phases. The separations on mixed phases are usually better than those on the pure components of the mixtures[70,79,116,117]. An important, if not the most important, reason is that mixtures, especially eutectic ones, have lower melting points and probably a greater ordering of the mesophase[118–121], which leads to a better selectivity of such mixtures at temperatures lower than those which can be used with the single phases. This has been shown, for instance, for binary and ternary liquid crystal stationary phases[70,122]. It is possible to predict the retention of the substances chromatographed on mixtures of liquid crystals if we know their retentions on the individual components. This relates both to liquid crystal–liquid crystal[123] and liquid crystal–isotropic stationary phase[124] mixtures.

In general it may be ascertained that the chromatographic separation proceeds according to different mechanisms at different temperatures corresponding to different states of the phases. Thus, by a skilful choice of the column temperature, one can obtain good separations of the components of different mixtures[74].

4.2. Molecular structure of the liquid crystal and of the chromatographed substance

A knowledge of intermolecular reactions between the liquid crystals and the chromatographed substances is important for the understanding of the phenomena taking place in the chromatographic column. The structure of the molecules and their polarity and polarizability affect, among other things, the solubility of the chromatographed substances in the liquid crystal[125]. In the course of dissolution complexes may be formed[126]. Many studies have dealt with the solubilities of non-mesogenic substances in liquid crystals[127–129] but they rarely refer directly to chromatography[122,130–134].

Quite recently an important paper was published in this field[135], concerning the thermodynamic properties of 22 solutes at infinite dilution in the smectic, nematic and isotropic mesophases of 4,4′-bis(heptyloxy)azoxybenzene. The thermodynamic properties were discussed in relation to the solute–solvent (liquid crystal) interactions as conditioned by the degree of order in the liquid crystal.

The process of dissolution dominates in the column during chromatography on a liquid crystal. However, as liquid crystals are mostly phases of medium polarity, the mechanism of the retention of the substances chromatographed on them is usually a combination being accompanied by adsorption. Nevertheless, the contribution of adsorption to the total retention is usually much smaller than that of dissolution.

Studies on the dissolution of chromatographed substances in N-(4-hexyloxybenzylidene)-4′-toluidine have led to the conclusion that the selectivity of the liquid crystal stationary phase is determined by the structure of the liquid crystal molecule influencing the creation of specific intermolecular reactions with the chromatographed substance and by the ordering of the mesophase[136]. The effect of the molecular structure of twelve liquid crystals based on azo and azoxy compounds on their

selectivity and polarity and the efficiency of conventional analytical columns in which these liquid crystals were deposited was studied by Szulc and Witkiewicz[79].

The relationship was studied between the molecular structure and the retention of the test substance for a set of nine other liquid crystals (4-alkoxycarbonyl-4'-nitroazoxybenzenes)[137]. In general, azoxybenzenes have better separation properties than azobenzenes[78,96], and among azoxybenzenes those which have different terminal substituents are more selective than those with the same substituents[94,96]. The inferior selectivity of the azo compounds probably occurs because they are planar and have a more compact structure into which the chromatographed substances penetrate only with difficulty.

The properties of the liquid crystal stationary phases depend both on the structure of the main chain of the molecule and on the terminal substituents which strongly affect the polarity of the molecules[138–140]. However, an equally important or even greater effect on the chromatographic properties of liquid crystals is exerted by the lateral substituents present in the molecule[76,79,114,115,139,140–144]. These substituents not only affect the intermolecular reactions between the liquid crystal and the chromatographed substance but also the liquid crystal – liquid crystal reactions. The lateral substituents also affect the selectivity of the liquid crystal owing to the changes they produce in the distance between its molecules[140]. This relates not only to monomers but also to polymers[145].

The direct quantitative correlation between the retention of the chromatographed substances and their molecular structure has been considered in several studies[78,146,147]. It is generally assumed that the ratio of the length to the smallest transverse dimension of the molecule, *l/b* (shape factor), is a decisive quantity for the retention of chromatographed substances on liquid crystal stationary phases. However, Suprynowicz *et al.*[146] chromatographed dimethylnaphthalenes on 4-ethyl-4'-(*p*-methylbenzoyloxy)azobenzene and on the basis of the results called into question the truth of this opinion. They suggested that the retention of dimethylnaphthalene isomers is directly related to the geometry of arrangement of the methyl groups in the molecule. This provoked polemics with Lamparczyk *et al.*[148,149].

Considering the shape-factor of twelve monomethylbenz[*a*]anthracenes and their separation on OV-17 stationary phase, Lamparczyk[147] predicted their retention and separation on BBBT and BABT liquid crystals.

The chromatography of olefinic hydrocarbons (pheromones) on liquid crystal derivatives of cholesteryl cinnamate has shown that the separation of geometric isomers of these compounds is affected either positively or negatively depending on the position of the multiple bond in the pheromone molecule[150].

The factors affecting the retention of polynuclear hydrocarbons and their weakly polar analogues containing sulphur in the molecule on a smectic siloxane polymer were studied[33]. In addition to the vapour pressure and volatility, the molecular structure of the chromatographed substances considerably affected the retention. The *l/b* ratio had a greater effect on the retention than other shape factors of the molecule, and this effect was more pronounced with a smectic than a nematic, the selectivity of the former being better. The shape of the molecule was, however, sufficiently important to affect the retention as determined from the *l/b* ratio.

The mutual effect of the molecular structures of the liquid crystal and the chromatographed substance was ascertained when determining the gas hold-up time

from the non-adjusted retention times of *n*-alkanes and methyl esters of fatty acids[151]. Methyl esters of mono- and bicyclic acids were chromatographed on the smectic and nematic phases of a high-temperature liquid crystal and the dependence of their retention on the molecular structure was studied. It was found that the selectivity is greatest at temperatures corresponding to the nematic range[75].

Little information is available on the mechanism of the separation ability of discotic liquid crystal stationary phases[56,57]. The existing information concerns only liquid crystal hexasubstituted triphenylene and benzene derivatives. A better knowledge and the generalization of the properties of this group of stationary phases require further studies of the already tested and other discotic liquid crystals.

The general principles underlying the interaction of the ordered structure of disc-like liquid crystals with molecules of chromatographed substances are the same as for rod-like liquid crystals. The molecular structure of discotic liquid crystals and their ordered structure are, however, the cause of the retention times of chromatographed substances whose molecules have an oblong shape on these liquid crystals being shorter than those of compounds with a compact molecular structure (cyclic–discotic). Such disc-like molecules are retained longer by the discotic liquid crystal stationary phase the flatter they are. The conclusions drawn for rod-like liquid crystals regarding good fitting, strong interaction with and easy penetration of such molecules into the ordered structure of the mesophase remain true for disc-like liquid crystals. The effect of planarity is probably even greater with discotic liquid crystal stationary phases. This has been shown for cyclohexane, cyclohexene and benzene and also for other compounds. Cyclooctatetraene (b.p. 142°C) is eluted after cyclooctane (b.p. 148.5°C), whose molecules are less flat. The importance of the planarity of the molecule is seen from the fact that the retention of cyclooctatetraene is almost the same as that of cyclooctanone (b.p. 200°C).

The separations of xylene isomers obtained on disc-like liquid crystal stationary phases are not as good as those obtained on rod-like phases. Even the order of elution is not changed with respect to conventional phases[56]. However, something is unclear here, as Goozner and Labes[152] reported that the solubilities of xylene isomers in mixtures of disc-like liquid crystals vary considerably. It is improbable that it will be possible to explain these differences only by the effect of the support.

Compounds with a linear structure of the molecule dissolve better than cyclic compounds in discotic liquid crystals. This has been shown for *n*-nonane and cyclooctane, which have similar boiling points (151 and 148°C, respectively) but very different retention times, cyclooctane being eluted second. If the chromatographed molecules do not have a disc shape (*e.g.*, *m*- and *p*-xylene isomers), the differences in their structure are immaterial and the boiling points are decisive for the order of their elution.

The ability of disc-like liquid crystal stationary phases to separate geometric isomers deserves attention. The separation of *cis*- and *trans*-decalin on a triphenylene derivative liquid crystal is illustrated in Fig. 8. The separation shown is very good and was obtained in a much shorter time than that on cyclodextrin as the stationary phase[56,153].

The relationship between the molecular structure of the chromatographed substances and their retention has been widely studied also with conventional stationary phases. Some results and conclusions from these studies may be applicable to liquid crystal stationary phases[154,155].

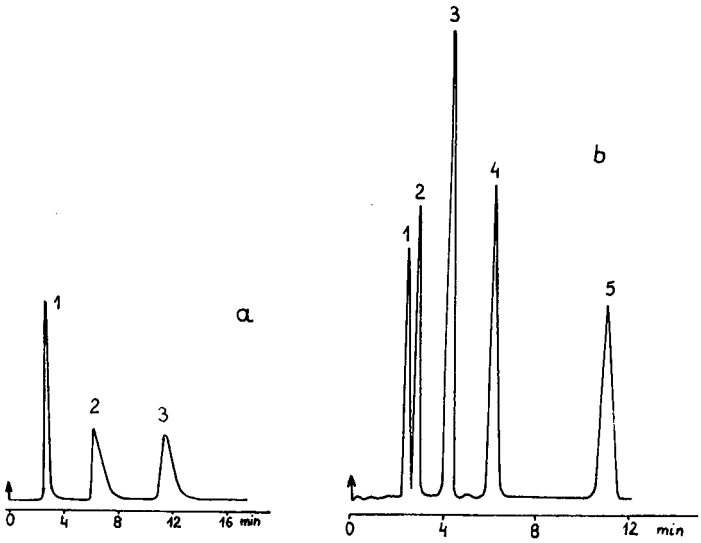

Fig. 8. Comparison of separation of a mixture of (1) *trans*-decalin, (2) *cis*-decalin, (3) tetralin, (4) naphthalene and (5) diphenyl. (a) Glass column (2 m × 4 mm I.D.) packed with 0.10 mol-% of β-cyclodextrin in formamide solution deposited on Celite; column temperature 70°C[153]; (b) glass column (2.1 m × 4 mm I.D.) packed with 10% of triphenylene hexa-4-octylbenzoate deposited on Chromosorb W AW; column temperature 198°C[56].

4.3. Effect of the support

Although great attention has been paid to the practical applications of liquid crystal stationary phases, relatively little concern has been devoted recently to fundamental studies of the interactions of the liquid crystals with the surface of the support. Such studies, however, will improve our knowledge of the properties of liquid crystal stationary phases and have not been totally abandoned, although sometimes they did not have a direct relation with gas chromatography[156].

The effect of the surface of the substrate on which the liquid crystal is deposited in the chromatographic column is rarely accounted for in analytical practice. This is also the case with other stationary phases. However, this effect may be important and should be taken into account. Usually the substrate has an adverse effect on the separations, although sometimes this effect may be positive. These problems are considered in detail by Berezkin[157].

The surface of the support or the column wall may not only contribute substantially to the retention of the chromatographed substances but may also influence the orientation of the liquid crystal molecules in various ways. Some information on this subject may be gathered from studies connected with the design of liquid crystal displays[158]. In view, however, of the special treatment of the surface of glass used in these displays, the possible correlations are very limited. A better knowledge of the effect of the condition of the surface and of the arrangement on this surface of the liquid crystal molecules should allow us to obtain systems with an optimum arrangement of the liquid crystal molecules in the column and hence with optimum properties as regards chromatographic separations.

The activity or neutrality of the surface of the support on which the liquid crystal is deposited has an effect on the mechanism of retention of polymethylbenzenes and *n*-alkylbenzenes[96]. Practice has shown that the use of an active support with a developed specific surface area (Silochrom, 80 $m^2 g^{-1}$) may give better separations of dimethylmercury and its contaminants and of diethyl sulphide and its contaminants on the nematic *p-n*-butyloxybenzoic acid deposited on that support compared with the same liquid crystal deposited on Chromaton N AW[71,110,159].

The distribution of the liquid crystal on the support and hence the properties of the whole system are affected not only by the chemical character (silanized or non-silanized surface) and porous structure of the support, but also by the amount of the liquid crystal deposited on its surface[160,161]. The effect of the support surface also manifests itself by the changes in the phase transition temperatures of the deposited liquid crystal. This effect is related to the conditions under which the column filling is heat treated. During heating, a redistribution of the liquid crystal on the support takes place and as a result the properties of the system are changed[96,162,163]. In some instances conditioning at high temperatures leads to a more advantageous ordering of the liquid crystals in the column. Therefore, if this treatment is not long enough or is conducted at an insufficiently high temperature, sometimes the selectivity of the column may change in the course of its use[164]. It should be borne in mind that the occurrence of this phenomenon is related to the kind of liquid crystal used and the properties of the surface on which it has been deposited.

The selectivity and also other properties of the system depend strongly on the kind of support used and on the amount of the liquid crystal deposited on it[165,166], as shown in Fig. 9. The selectivity also depends on the thickness of the liquid crystal layer on the capillary column wall and on the character of the wall surface[87,90,91,96]. The reproducibility and reliability of the retention data are the better the more inactive is the surface of the capillary wall and the greater is the thickness of the liquid crystal layer (>140 nm)[87].

The liquid crystal molecules may occur on the support in two states[162,163,166]: as a film on the surface or in bulk form in the capillaries. The proportion of the two states influences the properties of the system and depends on the kind of the support and the kind and amount of the deposited liquid crystal.

Studies of the dependence of retention on the coverage of the support make it possible to determine the thickness of the monolayer of the liquid crystal on the support surface and hence to establish how the molecules of the liquid crystal are arranged on the surface[160,161]. It has been calculated that the molecule of the liquid crystal shown schematically in Fig. 10 occupies an area 0.806 nm^2 on the surface of a silanized support. If the length of the bonds in the molecules is taken into account, it can be calculated that the liquid crystal molecule may occupy maximally about 1.2 nm^2 in the planar position and minimally 0.21 nm^2 in the homeotropic position. The surface area of the flat, rigid part of the molecule (hatched in Fig. 10) is about 0.75 nm^2. By comparing these values it can be seen that the molecule may lie on the surface of the support planarly on its rigid, flat part with the hydrocarbon chain raised upwards. In this way a sort of modified surface of the support is formed on which successive layers of the liquid crystal are deposited. The formation of a monolayer is not, however, a sufficient condition for a further uniform distribution of the liquid crystal on the support surface. As the amount of the liquid crystal on that surface increases it accumulates in the pores with diameters greater than 1 μm[160,161].

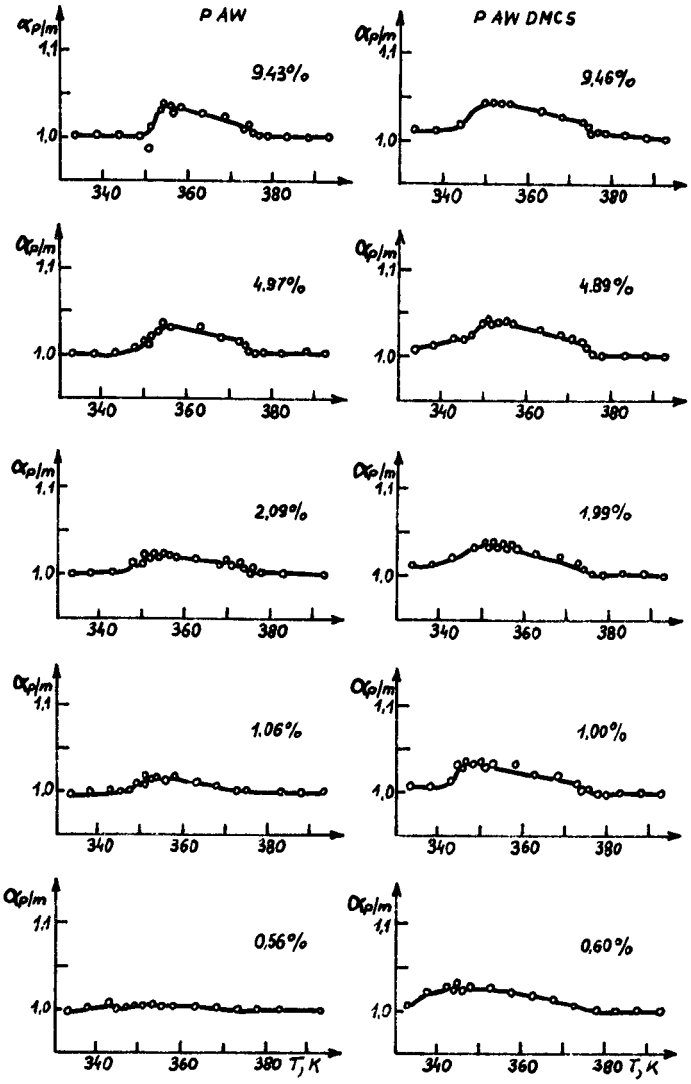

Fig. 9. Relationship between relative retention of *p*-xylene and *m*-xylene and column temperature with different amounts of liquid crystalline stationary phase deposited on Chromosorb P AW and Chromosorb P AW DMCS[165].

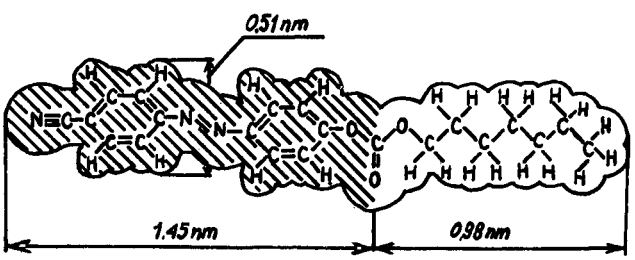

Fig. 10. Liquid crystalline 4-cyano-4′-*n*-heptyloxyformyloxyazobenzene molecule[160].

The interaction of the support surface with the liquid crystal stationary phase may give specific effects. One is a lowering of the melting point (by 7°C) of part of the liquid crystal owing to its contact with the silanized surface of various diatomite supports. The occurrence of this effect was detected for two liquid crystals prepared from the 4-cyano-4′-*n*-alkoxycarboxyazobenzenes with seven and nine carbon atoms in the alkyl chain[162,163,167]. The lowering of the melting point is due to the formation, under the influence of the support, of a layer of the phase with a crystalline structure different from that of the bulk liquid crystal beyond the support. This effect is not related to the kind of substance chromatographed but depends on the kind and amount of the liquid crystal deposited on the support and is a feature of the liquid crystal–silanized support system. It appears when the amount of the liquid crystal on the support exceeds *ca.* 3% and manifests itself by a new phase transition not observed thermo-optically. Hence this effect differs from the normal interactions of the liquid crystal with the support, which at small coverages of the support manifest themselves by a shift of the phase transition connected with the liquid crystal melting point and not by a new phase transition.

The variation of the specific retention volume accompanying the considered effect with the amount of the liquid crystal on the support is illustrated in Fig. 11. Below the melting point of the crystal (83°C), an additional phase transition appears at 76°C. The increases in retention related to the latter transition are approximately equal for all three supports. The increase in retention related to the second transition (at 83°C) depends on the amount of the stationary phase on the support. This effect is not observed for non-silanized supports. It follows that the interaction of the silanized support with the two considered liquid crystals is different from that of the non-silanized supports.

Fig. 12 and 13 show the relationships between the retention volume of *o*-xylene,

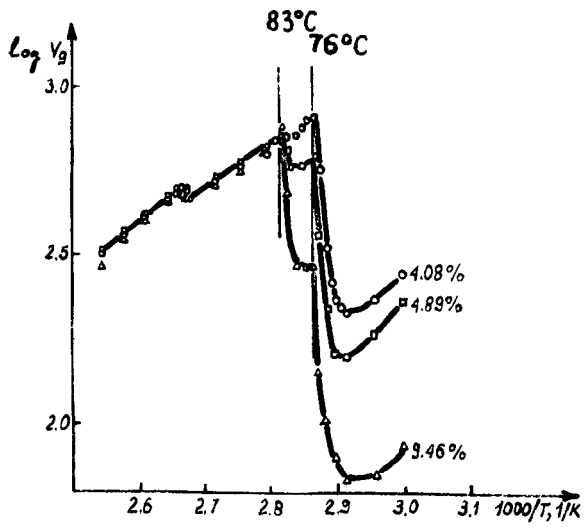

Fig. 11. Variation in retention volume of *o*-xylene with temperature on liquid crystalline 4-cyano-4′-*n*-heptyloxyformyloxyazobenzene deposited in different amounts on Chromosorb P AW DMCS. The additional phase transition at 76°C is visible[167].

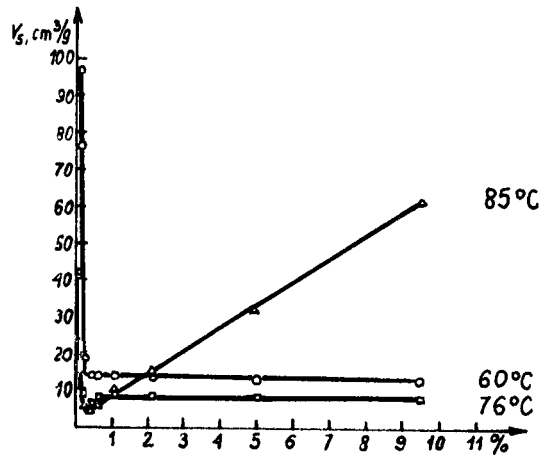

Fig. 12. Variation of the retention volume of *o*-xylene with the amount of liquid crystalline 4-cyano-4′-*n*-heptyloxyformyloxyazobenzene deposited on Chromosorb P AW[160].

calculated in terms of the support mass (V_S), and the amount of 4-cyano-4′-*n*-heptyloxycarboxyazobenzene for fillings in which Chromosorb P AW and Chromosorb P AW DMCS were used as supports. The dependence of the retention volume on the amount of the liquid crystal is given at the temperatures corresponding to the solid (60°C), mesophase (85°C) and the additional phase transition observed on silanized surfaces. The differences between the columns with silanized and non-silanized supports are visible at each of these temperatures. The results indicate that in some chromatographic systems the inactive silanized support may show a specific activity which may considerably affect the properties of such systems.

The effect of the support on the properties of liquid crystal stationary phases is also observed when these phases are supercooled. A given liquid crystal may undergo stable supercooling on one support and resist supercooling on another. On some supports liquid crystals undergo stronger supercooling than in their absence, but

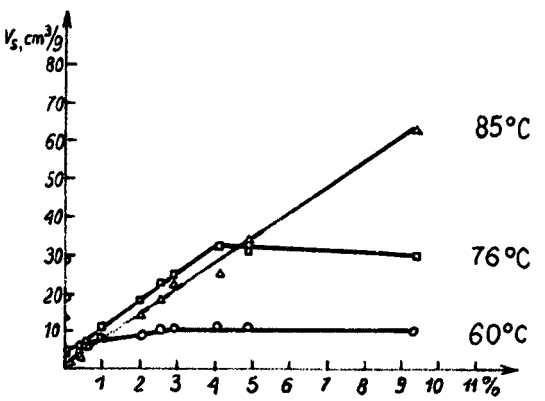

Fig. 13. Variation of the retention volume of *o*-xylene with the amount of liquid crystalline 4-cyano-4′-*n*-heptyloxyformyloxyazobenzene deposited on Chromosorb P AW DMCS[160].

opposite examples are also known. This depends on the properties of the whole liquid crystal – support system, which may manifest themselves in that below a certain amount of the liquid crystal on a silanized support supercooling does not take place at all or is very unstable, whereas above a certain coverage of the support with the same liquid crystal, characteristic of a given system, supercooling by several tens of degrees below the melting point may be stable. This characteristic coverage for 4-cyano-4′-*n*-heptyloxycarboxyazobenzene and silanized Chromosorb G, P and W was about 2.5%, 3% and 8%, respectively.[165]

The character of the surface of the support considerably affects the relative retention of the chromatographed substances[167]. On silanized and non-silanized Chromosorb P the relative retentions are greater at the same, small coverages of the supports with the liquid crystal stationary phase on the silanized support. The observed difference decreases with increasing coverage of the support (see Fig. 9).

5. PRACTICAL APPLICATIONS OF LIQUID CRYSTAL STATIONARY PHASES

Progress in the improvement of the physico-chemical properties and separation abilities of liquid crystal stationary phases and improvements in the efficiencies of columns filled with these phases has led to a continuing increase in the use of these phases in chromatographic practice.

Most examples of separations relate to various kinds of isomers, mainly positional isomers of benzene and naphthalene derivatives, *cis* and *trans* isomers of unsaturated hydrocarbons and isomers of polynuclear hydrocarbons and their derivatives. All these compounds often occur in mixtures of industrial importance. The separations of these substances on liquid crystal stationary phases are usually better and often faster than those on common isotropic stationary phases. This also relates to such highly selective isotropic stationary phases as cyclodextrins[56,153] and polysiloxanes.

Liquid crystals allow the separation of complex mixtures not only in the conventional way. For instance, Watabe *et al.*[168] developed a method for identifying and determinating components in the overlapping peaks of two or even three substances. They used a capillary column, provided with an internal and an external electrode, filled with 4,4′-di-*n*-amyloxyazoxybenzene to which they applied a constant electric field. Under the action of this field polar compounds are adsorbed in the column, the amount of the substance adsorbed increasing with the electric field. The adsorption is also affected by the dielectric constant and structure of the chromatographed substance and the kind of liquid crystal used.

5.1. Separation of isomers of benzene and naphthalene derivatives

For the separation of isomers of benzene derivatives, the use is recommended of methoxyazoxybenzene (MEAB), a nematic with the mesophase ranging from about 90 to 150°C and classified in the group of medium-temperature liquid crystal stationary phases. MEAB is the most frequently tested and practically used liquid crystal[74,81,82,90–98,169–174] and can also be applied when supercooled to 70°C[93]. Vigdergauz and co-workers are of the opinion that this liquid crystal should be included in the set of stationary phases recommended for the universal system of chemical analysis[74,175–177]. They suggested that 4,4′-ethoxypropoxyazoxybenzene should also be included in that set[132,178].

Alkylbenzenes are well separated on MEAB[90,92,93,169,170]. For instance, a mixture of C_{10}–C_{13} was succesfully separated on MEAB in a 23-m capillary in the mesophase temperature range[92], and at 100°C a good separation of 22 C_{14}–C_{17} alkylbenzenes was achieved in 22 min[170]. Xylene and ethylbenzene isomers can be separated in a 20-m column filled with MEAB in 70 s[93]. For the separation of a similar mixture, in addition to MEAB its eutectic mixture with 4,4′-azoxyphenetole was also used[172].

On MEAB a high relative retention of *p*- and *m*-xylene ($r_{p/m}$) is obtained, being 1.12–1.13 in the mesophase range. On supercooling, the value of $r_{p/m}$ at 80°C is 1.14 and at about 40°C it is 1.25[96,173]. The supercooled state at the latter temperature is, however, probably unstable.

The *p*- and *m*-xylene isomers are often used for measuring the selectivity of columns with liquid crystal stationary phases in view of the difficult separation of these isomers on common stationary phases. Some workers, however, are of the opinion that these isomers are not ideal test substances, as in the chromatographic system the interactions between the liquid crystal stationary phase and the chromatographed substance connected with their polarity are much greater than solute–solvent steric hindrance[83]. Some observations and conclusions regarding the differences in the retentions of *meta* and *para* disubstituted benzene derivatives on common stationary phases may be helpful when the mechanism of separations of these isomers on liquid crystal stationary phases is considered[179].

Apart form MEAB, other liquid crystal stationary phases also give very good separations of alkylbenzene isomers. For instance, the separations of alkylbenzenes obtained on 2-methyl-4′-*n*-butyl-4″-ethoxybenzoyloxyazobenzene in an analytical aluminium column were better than those obtained on SE-30 or OV-17[180]. A mixture of benzene, toluene, ethylbenzene, xylenes, isopropylbenzene, styrene and *n*-propylbenzene is better separated (with a different order of elution) and in a shorter time on 4-*n*-pentylacetophenone (O-4-*n*-ethyloxybenzoyl oxime)[95] than on a heavy alkylated benzene stationary phase modified with Bentone 34[181]. Various alkylbenzenes have also been successfully separated in a 1-m conventional analytical column on the liquid crystal hydroquinone *p*-heptoxybenzoate[182].

Other liquid crystals have also been used for separations of mixtures containing isomers of alkylbenzenes[183–185].

Very good separations of alkylbenzenes are obtained on recently obtained liquid crystal isothiocyanates[100]. The mesophase ranges of some of these liquid crystals compare well with those of the MEAB mesophase. Others reveal much wider ranges of the mesophase and may be used in capillary columns with temperature programming for the separation of mixtures containing components differing significantly in boiling temperature. Thus, on isothiocyanate stationary phases, mixtures containing alkylnaphthalenes in addition to alkylbenzenes may be separated[100]. For instance, dimethylnaphthalene isomers, and also pairs, which are very difficult to separate on other stationary phases, were separated.

The separation of methylnaphthalenes and of nine dimethylnaphthalenes on 4-ethyl-4′-(*p*-methylbenzoyloxy)azobenzene was studied by Suprynowicz *et al.*[146]. A liquid crystal containing a naphthalene ring in its molecule was applied to the separation of α- and β-naphthols and certain dihydroxynaphthalenes, and the effect was better than when OV-17 or OV-225 was used[186]. The results were better when the hydroxynaphthalenes were converted into acyl derivatives.

Very good separations of various positional and geometric isomers, including methylnaphthalenes, dichlorobenzenes and pheromones, were obtained when the liquid crystal stationary phase was used in the supercooled state[187].

5.2. Separation of alkene and alkane isomers

The isomers of alkenes and alkanes can be separated on conventional stationary phases especially when long capillary columns are used. The above described MEAB liquid crystal is useful for separating alkenes. This asymmetric azoxybenzene was compared with other symmetric and asymmetric azoxybenzenes and with methoxyethoxyazobenzene for the separation of C_{15}–C_{17}. MEAB showed the best separating properties[94].

On the MEAB mesophase and supercooled phase in a capillary column good separations of C_{15}–C_{18} alkanes and of mixtures of alkene isomers and cyclic hydrocarbons[91] and also C_{10}–C_{14} alkenes[93] were obtained. A change in retention occurs in which *trans*-5-decene is eluted before *trans*-4-decene. In general, it can be concluded that the *trans* isomers are retained longer than the *cis* isomers on liquid crystal stationary phases[96]. In Fig. 14 it is shown that the analysis of *n*-pentadecene isomers and *n*-pentadecane is much faster when chromatography is carried out on MEAB instead of conventional stationary phases[96,188].

A procedure for the analysis of hydrocarbon mixtures has been patented. One of the claims is the use of MEAB[189,190] and of a eutectic mixture of two other liquid crystal stationary phases[191].

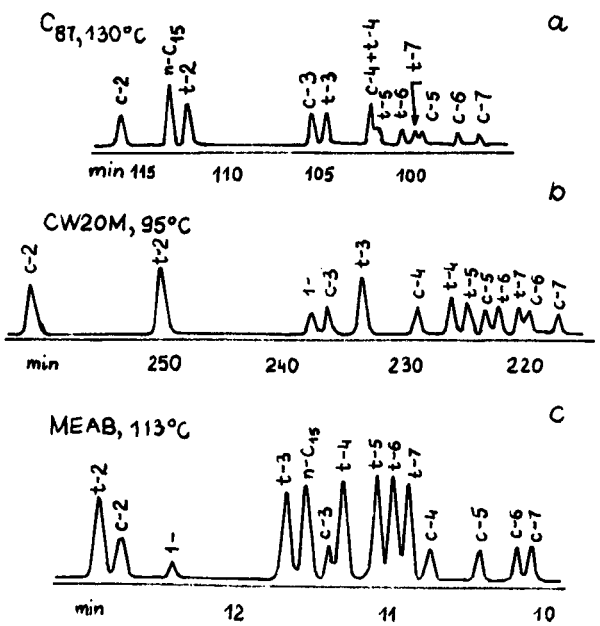

Fig. 14. Chromatograms of the separation of *n*-pentadecene isomers on columns coated with (a) Apolan-87 (200 m × 0.25 mm I.D., N = 670 000 plates), (b) Carbowax 20M (300 m × 0.25 mm I.D., N = 400 000 plates) and (c) MEAB (90 m × 0.25 mm I.D., N = 200 000 plates). c- = *cis*-isomer; t- = *trans*-isomer; n-C_{15} = *n*-pentadecane; 1- = *n*-pentadecene[173].

In general, 4-*n*-pentylacetophenone (O-4-*n*-pentyloxybenzoyl oxime; PBO) is less selective than MEAB, but the isomers of C_9–C_{11} *n*-alkenes and *n*-alkanes are separated better on it than on MEAB[97]. The diastereoisomers of C_8–C_{10} alkanes are separated better on PBO than on conventional stationary phases[98]. It has been shown that PBO has advantages as a stationary phase in the separation of alkyne isomers[192]. As in other systems, alternation of retention of the particular isomers was observed depending on their molecular structure (even or odd number of atoms in the molecule and position of the multiple bond in the chain). The separation of alkynes on a liquid crystal is easy as they are eluted in the order of corresponding to the shift of the triple bond from the centre towards the end of the chain, the selectivity increasing in the same order. Particularly good results were obtained when alkynes with ten or more carbon atoms in the molecule were separated[192].

C_{18}–C_{20} isoprenoid diastereomeric alkanes are separated worse on PBO than on isotropic phases[98]. In contrast, diastereomeric arylalkenes are separated better on a cholesteric liquid crystal than on a non-polar isotropic stationary phase[193]. By comparing the results obtained it can be concluded that if an isomer with a "more elongated" racemic molecule is eluted from a conventional stationary phase in the same or longer time than an isomer with a "less elongated" *meso* molecule, then these isomers are separated better on a liquid crystal stationary phase. This situation prevails with C_8–C_{10} hydrocarbons. In contrast, if the "more elongated" isomers is eluted earlier, then its shift beyond the "less elongated" isomer, typical of liquid crystal stationary phases, becomes diffcult and therefore the separation on liquid crystals is worse[98].

The separation of a multi-component mixture of paraffinic hydrocarbons in a common 1-m analytical column filled with the hydroquinone ester of *p*-heptoxybenzoic acid, although requiring a long time, is an illustration of the separation possibilities with liquid crystal stationary phases[182].

5.3. Separation of mixtures of benzene and aliphatic hydrocarbon derivatives containing heteroatoms

Apart from hydrocarbons, many other organic compounds of different polarity and volatility, including those with oxygen in the molecule, may be analysed on liquid crystal stationary phases.

As regards non-alkyl benzene derivatives, the separation was studied of dichlorophenol isomers on 4,4′-dimethoxyazoxybenzene (DMAB) and on isotropic stationary phases. Although the separation on DMAB was generally good, the separation of 2,6- and 2,5-dichlorophenols proved impossible. No succes was achieved in this respect when the above liquid crystal was mixed with dioctyl phthalate or silicone KF-54[194]. The separation of dichlorophenols also presented problems when 4,4′-diethoxyazoxybenzene was used as the stationary phase[195]. In contrast, chlorophenol isomers and the esters of *p*-hydroxybenzoic acid were well separated on liquid crystal polyacrylates[145].

Among other compounds chromatographed on liquid crystal stationary phases are lower aliphatic amines[196], pesticides[197], mono- and bifunctional impurities in terephthalic acid and dimethyl terephthalate[198], organic compounds in aqueous medium[199], alcohols, esters, glycols[200], pentanol isomers, esters[201], diene aliphatic alcohols and their acetates[202], gemfibrosil [2,2-dimethyl-5-(2,5-xylyloxy)valeric acid] con-

taining an isomeric contaminant[203], isomers of C_3–C_5[132], C_1–C_5 and C_3–C_8 aliphatic alcohols[111], pheromones[204] and oxygen-containing 4,4-dimethyl-1,3-dioxane synthesis products[112]. On polymeric liquid crystal siloxanes, *cis* and *trans* isomers of saturated fatty acid esters were separated[99], the *trans* isomers being retained longer than the *cis* isomers.

5.4. Separation of polynuclear hydrocarbons

Gas chromatography with liquid crystal stationary phases allows the analysis of various mixtures of polynuclear hydrocarbons. Such analyses have become fairly common, on the one hand in view of the high toxicity of these compounds, many of them being carcinogenic[205], and on the other because liquid crystal stationary phases are particularly well suited for this analysis as they allow the separation of isomers.

Recently, polymeric siloxane liquid crystal stationary phases have become increasingly used for separating polynuclear hydrocarbons[28–33,35,67,99,103,206–209]. These phases are suitable for use in capillary columns[80], primarily glass and fused-silica columns. They are usually used individually, although sometimes they are mixed with isotropic siloxane polymers, *e.g.*, SE-30 (polydimethylsiloxane)[99]. It is interesting that most of these polymers are smectics and that they reveal very good separation properties[28,29,31–33,103,206,207,209,210]. In addition to siloxane polymers, polymers without silicon atoms in the molecule are also used for this purpose[35,145]. All polymers can be used when the temperature is programmed over a wide range. Some of them may be used for a longer time at 280°C and a shorter time at 300°C.

The nematic siloxane polymers reveal mesophase ranges of 70–300°C[67] or 91–319°C[99], and their separation properties are superior to those of SE-52. This has been shown by way of example for methyldibenzothiophenes and tetranuclear aromatic compounds with sulphur in the ring, and also for other polyaromatic hydrocarbons and their methyl isomers. For the last separation columns filled with SE-52 and a liquid crystal were combined in different configurations, various separations being obtained[67].

On smectic liquid crystal siloxane polymers the following separations were, for instance, carried out: polychlorodibenzodioxine and polychlorodibenzofuran isomers[103,208], hydroxy thiophene derivatives (both primary and modified by treatment with trimethylsilylimidazole)[206] and isomers of methylchrysene and methylbenz[*a*]anthracene[32]. On the polysiloxane smectic phase, dimethyldibenzothiophene isomers[29], coal tar polynuclear hydrocarbons[28], isomers of methylphenanthrene, methylchrysene, hydroxydibenzothiophene, aminophenanthrene and of other compounds[31] are separated better than on SE-54. Naikwadi *et al.*[210] separated, in addition to polynuclear hydrocarbons and polychlorinated compounds, also halogen-disubstituted benzene derivatives. The separation of mixtures on liquid crystal siloxane polymers, as on other stationary phases, depends on the molecular structure of these polymers. It has been found that an increase in the number of mesomorphic groups in the smectic polymers leads to a higher smectic–isotropic liquid phase transition temperature and to a higher selectivity towards isomers. However, this is accompanied by a slow decrease in the efficiency of the system. We can illustrate this effect by using a mixture of methylphenanthrene and methylcarbazole isomers by way of example[28].

It has been found that an analogy exists between the dependence of retention on

the shape of the molecule of the substance being chromatographed on the liquid crystal smectic siloxane polymer in gas chromatography and on the polymeric octadecylsilane (C_{18}) in liquid chromatography[209]. The similar order of elution of isomeric polynuclear hydrocarbons in both instances indicates that the surface of the polymeric C_{18} phases is more ordered than that of the monomeric type[211]. The similarity of the selectivities on reversed phases in liquid chromatography and on liquid crystal stationary phases in gas chromatography may be extended beyond polyaromatic compounds by suggesting that the excellent separations of other isomeric compounds on liquid crystals may also be achieved in liquid chromatography on polymeric C_{18} phases.

It should be noted that the separations of polynuclear hydrocarbons on polymeric smectics are so good that they have been used to establish the quantitative composition (with the use of an internal standard) of standard solutions of these hydrocarbons[207].

On liquid crystal polyacrylates, in addition to mixtures of aromatic compounds also the isomers of chlorophenols and esters of *p*-hydroxybenzoic acid and of methoxynaphthalenes and naphthols have been separated[35,145,212]. Polyacrylates have also been used as stationary phases in supercritical fluid chromatography in glass and fused-silica capillary columns with carbon dioxide as the mobile phase. These stationary phases resist pressures of 200 MPa at 160°C. They have also been used to separate the isomers of naphthols and phenylphenols[213]. It can be expected that liquid crystal stationary phases will be increasingly used in supercritical fluid chromatography as this method develops further, its current development being very rapid and promising[214–218].

In the analysis of polynuclear hydrocarbons, monomeric liquid crystals from the group of Schiff bases, mainly BMBT, BBBT, BPhBT and BHxBT, find continued application[68,69,72,73,219–224]. In view of their features, these stationary phases have been chiefly used in conventional analytical columns[69,72,73,219–223] and only seldom in capillary (fused-silica) columns[68,84,224]. On these phases separations were effected of, *e.g.*, biphenyl polychloro derivatives[222], hydrocarbons containing 3–6 rings in the molecule and their derivatives and also isomers[72,73,219–221,223]. Some hydrocarbons were isolated from graphitized carbon black[72] and airborne particulates[221,223]. On BBBT phase mixed with SE-52 (1:1), 4-5-ring hydrocarbons containing sulphur in the molecule were separated[68]. The mixed phases BMBT – OV-17 and BBBT – SE-30 were used in combined columns or in one column to determine the content of anthracene, phenanthrene and carbazole in carbochemical products[69]. It has been shown in this connection that gas chromatography gives superior determination to polarography and UV spectrometry.

Anthracene, phenanthrene and other polynuclear hydrocarbons, including benzopyrenes, have also been separated on other high-temperature liquid crystal stationary phases with very good results[77,143,144,225–231].

The earlier considered liquid crystal polysiloxane stationary phases reveal very good separating properties and high thermal stability. Some of them, however, have the disadvantage that it is almost impossible to use them in the whole range of the mesophase and supercooled state as their viscosity is high at low temperatures[30]. This disadvantage does not apply to liquid crystal isothiocyanates, which are characterized by a wide range of the mesophase, comparable to that of the siloxane polymers, and

which have a low viscosity. Owing to these properties the liquid crystal isothiocyanates are potentially superior to liquid crystal Schiff bases as stationary phases. The easier synthesis, good solubility and good coating of capillary column walls are also features that place isothiocyanates ahead of siloxane polymers. Studies of the liquid crystal isothiocyanates as stationary phases were started only recently, so not all their properties are yet known. However, the first results regarding the separation of polynuclear hydrocarbons are very promising[101,232].

In discussing the analyses conducted on liquid crystal stationary phases, it should also be noted that various chromatographic methods have been used for testing the purity of and separating liquid crystal mixtures, *e.g.*, thin layer chromatography[233,234], high-performance column chromatography combined with mass spectrometry[235], gas chromatography[236] and supercritical fluid chromatography[237].

To assess chromatographic separations on liquid crystal stationary phases one can use the universal retention indices[174,178,238].

In Table 2 examples are given of separations on liquid crystal stationary phases and the parameters of the chromatographic processes. In Figs. 15-17 examples of chromatograms of separated compounds are shown.

6. APPLICATIONS OF LIQUID CRYSTALS IN LIQUID CHROMATOGRAPHY

6.1. Column chromatography

It has already been mentioned that liquid crystal siloxane polymers find application in supercritical fluid chromatography[213]. A recent paper dealt with the use of liquid crystals in column liquid chromatography[241]. It described the behaviour and properties of two liquid crystals, *viz.*, 4-ethoxybenzylidene-4′-*n*-butylaniline (EBBA) and cholesteryl oleate (ChO). The mesophase range of EBBA lies in the range 35.5–77.5°C and that of ChO in the range 20–33°C. These liquid crystals were deposited on Silasorb-600 (silica gel) in amounts of 20% and 40%, respectively. The phase transition points of these liquid crystals measured chromatographically were lower than those measured thermo-optically. With EBBA the clearing point was about 30°C lower. This lowering of the phase transition points was related to the adsorption of the liquid crystals on the surface of silica gel, although this has not been confirmed. Possibly the effect of hexane used as the mobile phase, which could influence the properties of the mesophase, was wrongly neglected. This seems the more likely as the properties of the mesophase are influenced even by the presence or absence of a gas[242].

With an increase in temperature the capacity factor of nitrotoluene isomers increased on both liquid crystals (EBBA and ChO). However, no distinct differences in the α values of *o*- and *m*-dinitrobenzene (DNB) at the melting point of EBBA were observed on uncoated Silasorb and Silasorb coated with the EBBA liquid crystal, although there were certain differences in the course of the plot of $\alpha_{o/m\text{-DNB}}$ *versus* temperature.

Examples have been given of the separation of nitrotoluene isomers on ChO (see Fig. 18). The separation was better the higher the column temperature. However, this separation should not be related directly to the liquid crystal properties of ChO as at least two separations (better) were obtained beyond the mesophase range. It is difficult to explain the results obtained and the mechanism of the observed phenom-

TABLE 2

EXAMPLES OF LIQUID CRYSTAL STATIONARY PHASES AND MIXTURES SEPARATED ON THEM[a]

Stationary phase	*Transition temperature to phase (°C)*			*Column (m × mm I.D.)*	*Temperature of column (°C)*	*Separated substances and separation time (min)*	*Ref.*
	Smectic	*Nematic*	*Isotropic*				
4-Methoxy-4′-ethoxyazoxybenzene (MEAB)		91	150	Glass capillary		22 C_{14}–C_{17} alkylbenzenes and *o*-dialkylbenzenes obtained from dehydrogenation of *n*-alkanes (32)	19
					140	20 C_{10}–C_{13} phenylalkanes (17.5)	
4-*n*-Pentylacetophenone (O-4-*n*-ethyloxybenzoyl oxime) (EBO)		97	112	Glass, 48 × 0.25	40	9 C_6–C_9 aromatic hydrocarbons (2.5)	
Polysiloxanes	98		226	Fused silica, 12 × 0.2	120–200	5 methylphenanthrene isomers	28
	103		288				
	92	246	286	Fused silica, 12 × 0.2	120–230	4 methylcarbazole isomers	
	120		230–260	Fused silica, 13 × 0.2	40–280	16 polycyclic aromatic hydrocarbons in a coal tar	
	60		226				
Polysiloxane (PMPS-1,2)	130	219	235	Fused silica, 18 × 0.3	80–200	7 isomeric dimethyldibenzothiophenes	29
Polysiloxane	118		300	Fused-silica capillary	70–170	5 isomeric methylphenanthrenes (19)	31
				Fused-silica capillary	70–250	6 isomeric methylchrysenes (38)	
				Fused-silica capillary	70–210	4 isomeric hydroxydibenzothiophenes (29)	
				Fused-silica capillary	70–210	5 isomeric aminophenanthrenes (30)	
				Fused-silica capillary	120–220	4 nitrogen heterocyclics and 5 *n*-alkanes (20)	
				Fused-silica capillary		5 isomeric four-ring polycyclic aromatics	
Polysiloxane	104		308	Fused silica, 10 × 0.2	40–250	16 methylbenz[*a*]anthracene and methylchrysene isomers (30)	32
	95		306				

Polysiloxanes	130 118	219	235 300	Fused silica, 10-30 × 0.2 or 0.3		Polycyclic aromatic compounds and slightly polar sulphur heterocyclics	33
Polyacrylates		75	246	Glass, 32 × 0.25	130	Diphenylmethane, biphenyl, 1,2-diphenylethane, 1-methoxynaphthalene, 2-methoxynaphthalene, fluorene, *o*-terphenyl, triphenylmethane, phenanthrene, anthracene (37)	35
		69	230	Glass, 30 × 0.25	150	*o*-, *m*- and *p*-toluic acids (15)	
		70	270	Glass, 32 × 0.25	180	1- and 2-naphthol (11)	
		85	291	Glass, 31 × 0.25	135	Methyl, ethyl, propyl, butyl, pentyl and hexyl benzoates (14)	
Mixture of two disc-like liquid crystals (1:1)				Glass, 2.1 × 4	78	Pentane, hexane, cyclohexane, methylcyclohexane, octane (4.5) Cyclohexane, cyclohexene, benzene, cyclooctene, cyclootatetraene, cyclooctadiene (15) Heptane, octane, nonane, *p*-xylene, *o*-xylene, decane (16)	57
N,N′-Bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT) + GE SE-30 N,N′-Bis(*p*-butoxybenzylidene)-α,α′-bi-*p*-toluidine (BBBT) + GE SE-30				Steel, 2 × 2	230	Technically pure anthracene, (naphthalene, 1- and 2-methylnaphthalenes, diphenyl, dimethylnaphthalene, diphenyl oxide, fluorene, triphenylmethane, phenanthrene, anthracene, carbazole, fluoranthene, pyrene (19)	66
Polysiloxane (PMMS)		70	300	Fused silica, 19 × 0.32	120–260	16 methyldibenzothiophenes and four-ring aromatic sulphur heterocyclics (31)	67
					120–260	17 compounds of the sulphur heterocyclics fraction of a coal liquid (40)	
					120–270	32 polycyclic aromatic hydrocarbons (57)	
					230	6 methylchrysene isomers (16)	
PMMS + SE-52					230	6 methylchrysene isomers (21)	
BBBT + SE-52 (1:1)				Fused silica, 19.6 × 0.32	220	12 four-ring aromatic sulphur heterocyclics (10)	68
					230	10 five-ring aromatic sulphur heterocyclics (25)	

(Continued on p. 68)

TABLE 2 (*continued*)

Stationary phase	*Transition temperature to phase (°C)*			*Column (m × mm I.D.)*	*Temperature of column (°C)*	*Separated substances and separation time (min)*	*Ref.*
	Smectic	*Nematic*	*Isotropic*				
Mixture of three liquid crystals				Glass, 3.1 × 3	104	Mono- and dialkylbenzenes (20)	70
BBBT	159	188	303	Glass, 0.7 × 3	210	Polycyclic aromatic hydrocarbon fraction of a carbon black (300)	72
BMBT		181	320	Glass, 1.9 × 2	190	17 tricyclic and tetracyclic aromatic hydrocarbons (35)	73
				Glass, 3 × 2	240	Mixture of isomers of pentacyclic aromatic hydrocarbons	
				Glass, 1.9 × 2	260	Mixture of isomers of hexacyclic aromatic hydrocarbons	
4,4′-Biphenylene dibenzoate		250	350	Steel, 1 × 2	240	Isomeric four-ring compounds (44)	77
					280	Isomeric five-ring compounds (7)	
				Steel, 3 × 2		Isomeric disubstituted benzenes and naphthalene derivatives	
MEAB		91	150	Brass, 10 × 0.35	178	10 methyl esters of fatty acids (40)	81
				Brass, 6 × 0.35	160	10 alcohols (14)	
				Brass, 8 × 0.35	180	9 cyanides of fatty acids (28)	
MEAB		97	147	Steel, 50 × 0.25	120	Isomers of chlorotoluene (8)	82
4-*n*-Pentylacetophenone (O-4-*n*-heptylbenzyl oxime)		41	69.5	Glass capillary		Isomers of C_8 cyclic and aromatic hydrocarbons	83
BBBT		188	303	Glass, 18 × 0.25	200–260	Methylchrysene isomers (44)	84
					200	3 four-ring polycyclic aromatic hydrocarbons (18)	
					280	Benzopyrenes, perylene and benzofluoranthenes (41)	
N,N′-Bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine (BPhBT)		257	403	Glass, 20 × 0.25	280	10 polycyclic aromatic hydrocarbons (25)	
4-*n*-Pentylacetophenone (O-4-*n*-pentyloxybenzoyl oxime) (PBO)		63	94	Glass capillary		*cis* and *trans* isomers of *n*-tridecenes and *n* tetradecenes	86
BBBT		188	303	Glass, 10 × 0.22	280	Phenanthrene, anthracene, pyrene, benz[*a*]anthracene, chrysene (5)	89

				Glass, 10 × 0.22	280	Mixture of 4–6 polycyclic aromatic hydrocarbons (55)	
MEAB		97	147	Steel, capillary		C_5–C_{18} *n*-alkanes, isoalkanes and alkylcyclic hydrocarbons	91
MEAB		91.5	150	Glass, 23 × 0.25	90	20 C_{10}–C_{13} phenylalkanes (44)	92
					140	20 C_{10}–C_{13} phenylalkanes (15)	
MEAB		95.8	148	Glass, 20 × 0.25		Isomers C_8 alkylbenzenes and *cis*, *trans* isomers of C_{10}–C_{14} *n*-alkenes	93
MEAB		95.8	148	Glass capillary		*cis* and *trans* isomers of C_{15}–C_{17} *n*-alkenes	94
4-Propoxy-4′-ethoxyazoxybenzene		101.5	145				
4-Propoxy-4′-propoxyazoxybenzene		115.5	123.6				
4-Butoxy-4′-ethoxyazoxybenzene		100	146				
4-Methoxy-4′-ethoxyazobenzene		132.2	134.5				
EBO		56	93	Glass capillary	40	Benzene, toluene, ethylbenzene, *m*- and *p*-xylene, isopropylbenzene, *o*-xylene, *n*-propylbenzene, styrene (2.5)	95
MEAB		95.8	148	Glass, 20 × 0.25	95	Isomers of C_8 alkylbenzenes (1.2)	96
				Glass, 86 × 0.25	80	Isomers of C_{10}–C_{13} *n*-alkenes (44)	
EBO		56	93	Glass, 48 × 0.25	40	Benzene, toluene, ethylbenzene, *m*- and *p*-xylene, isopropypbenzene, *o*-xylene (2)	
PBO		63	94	Glass, 100 × 0.25	56	25 isomeric C_9–C_{11} *n*-alkenes and the corresponding *n*-alkanes (72)	97
PBO		63	94	Glass, 100 × 0.25	40	Diastereomeric C_8–C_{10} alkanes (30)	98
Polysiloxanes (MEPSIL)				Glass, 15 × 0.25	140–200	12 fatty acid methyl esters (16)	99
MEPSIL + SE-30 (1:1)				Glass, 15 × 0.25	100–290	20 polycyclic aromatic hydrocarbons (56)	
					240	Isomeric methyl-substituted benz[*a*]-anthracenes (22)	
					275	Isomeric methyl-substituted benzo[*a*]pyrenes (32)	
Isothiocyanates		58	132	Glass, 15.5 × 0.3	50	Alkylbenzenes and dialkylbenzenes (16)	100
					127	Dialkylnaphthalenes (24)	
	55.8	98.5	130	Glass, 20 × 0.3	100.5	Alkyl- and dialkylbenzenes (10)	
					129.5	Isomers of dimethylnaphthalenes (25)	
Isothiocyanate	135	163	280	Glass, 12.5 × 0.3	246–279	13 polycyclic aromatic hydrocarbons (33)	101

(Continued on p. 70)

TABLE 2 (*continued*)

Stationary phase	*Transition temperature to phase (°C)*			*Column (m × mm I.D.)*	*Temperature of column (°C)*	*Separated substances and separation time (min)*	*Ref.*
	Smectic	*Nematic*	*Isotropic*				
SB-smectic				Glass, 25 × 0.32	180–280	2,3,7,8-Class congeners of polychlorodibenzodioxins and polychlorodibenzofurans	103 164
4-(4-Nonyloxybenzoyloxy)-4′-cyanoazobenzene		70	250	Glass, 1.5 × 3	220	Fluorene, phenanthrene, anthracene (11)	108
					140	*m*- and *p*-dibromobenzene, *m*- and *p*-chloroacetophenone (6)	
					75	3 xylene isomers (2.5)	
					55	3 xylene isomers (3.5)	
4-*n*-Butyloxybenzoic acid		147	160	Glass, 2 × 3	100	Methane, methyl bromide, ethyl bromide, ethyl iodide, ethyl acetate, ethanol diethyl sulphide (15)	110
2-Cyano-5-phenylpyrimidine		121	128	Glass, 2.2 × 3	80	Benzene, toluene, ethylbenzene, *m*- and *p*-xylene, isopropylbenzene, *o*-xylene	111
					124	4 isomers of butanol	
					80	8 C_1–C_5 alcohols	
					80	6 C_3–C_5 alcohols	
					124	methyl ethyl ketone, methyl propyl ketone, methyl butyl ketone	
4,4′-Azoxyphenetole		138	168	Glass, 6 × 3	92	6 heterocyclic compounds of the dioxane and pyrane group (16)	112
4,4′-Ethoxypropoxyazoxybenzene		102.5	148			Isopropanol, *n*-propanol, isobutanol, *n*-butanol, isopentanol, *n*-pentanol	132
Bis(4-methylene-4′-*n*-butoxy-azobenzene		201	310	Steel, 1 × 4	243	13 polycyclic aromatic hydrocarbons (60)	144
Bis(4-methylene-4′-*n*-butoxy-3′-cyanoazobenzene)	197	212	283	Steel, 1 × 4	220	11 polycyclic aromatic hydrocarbons (50)	
					180	1- and 2-naphthylamine (8)	
Bis(4-methylene-4′-*n*-butoxy-3′-methylazobenzene		115	204	Steel, 1 × 4	120	1- and 2-ethylnaphthalene (12)	

Polyacrylates	95	250	Glass, 35 × 0.25	160	10 aromatic hydrocarbons (29)	145
	70	270	Glass, 32 × 0.25	140	3 chlorophenol isomers (8)	
				190	4 alkyl *p*-hydroxybenzoates (10)	
4-*n*-Butyloxybenzoic acid	147	160	1 × 3	150	3 isomers of trimethylbenzenes (7)	159
			2 × 3	149	Impurities in dimethylmercury (4.5)	
				149	Impurities in diethyl sulphide (57)	
4,4′-Azoxyphenetole	138	168	Copper, 50 × 0.36	138	Octane, benzene, toluene, *m*-, *p*- and *o*-xylene (30)	169
MEAB	96	148	Copper, 50 × 0.36	100	Octane, benzene, toluene, ethylbenzene, *m*-xylene, isopropylbenzene, *p*- and *o*-xylene (70)	
MEAB	91.5	150	Glass, 23 × 0.25	100	22 C_{14}–C_{17} alkylbenzenes (32)	170
MEAB	91	150	Copper, 40 × 0.35	108	Hexane, benzene, toluene, ethylbenzene, xylene isomers (28)	172
MEAB	91	150	Glass, 90 × 0.25	80	Isomeric C_{10}–C_{13} *n*-alkenes (44)	173
				113	*n*-Pentadecene isomers (13)	
2-Methyl-4′-*n*-butyl-4-(4″-ethoxybenzoyloxy) azobenzene	107	199	Aluminium, 1.84 × 4	107	13 mono-, di- and trialkylbenzenes (21)	180
					3 vinylbenzenes (28)	
Hydroquinone bis(*p*-heptyloxybenzoate)	121	195	1 × 3	150	20 aromatic hydrocarbons (24)	182
					Isomeric C_6–C_{19} *n*-alkanes (125)	
2,6-Naphthalene bis(*p*-*n*-heptyloxycinnamate)	158	291	Glass, 1 × 3	160	1- and 2-naphthol	186
				170	1- and 2-naphthyl acetate	
					1- and 2-naphthyl propionate	
				225	1- and 2-naphthyl benzoate	
2-Methyl-4′-methoxy-4-(*p*-methoxycinnamoyloxy) azobenzene	149	298	Glass, 2.6 × 2.6	135 (solid)	1- and 2-methoxynaphthalene (6)	187
				135 (nematic)	1- and 2-methoxynaphthalene (30)	
2-Methyl-4′-ethoxy-4-(*p*-methoxycinnamoyloxy)azobenzene	154	300	Glass, 2.6 × 2.6	104–170	9,11-Tetradecadienyl acetate isomers (insect sex pheromones) (55)	
2-Methyl-4′-*n*-butyl-4-(*p*-methoxycinnamoyloxy)azobenzene	109	253	Glass, 2.6 × 2.6	60	*m*- and *p*-xylene (9)	
				110	*m*- and *p*-dichlorobenzene (5)	
MEAB	93	150	Glass, 80 × 0.25	113	13 *cis* and *trans*	188

(Continued on p. 72)

TABLE 2 (*continued*)

Stationary phase	*Transition temperature to phase (°C)*			*Column (m × mm I.D.)*	*Temperature of column (°C)*	*Separated substances and separation time (min)*	*Ref.*
	Smectic	*Nematic*	*Isotropic*				
						n-pentadecene isomers (13)	
MEAB		96	148	Glass, 90 × 0.25	87	47 isomers of C_9–C_{13} *n*-alkenes and C_9–C_{13} *n*-alkanes (45)	189
MEAB		93	150	Steel, 50 × 0.25	97	15 isomers of C_9–C_{11} *n*-alkenes and C_9–C_{11} *n*-alkanes (23)	190
						26 isomers of C_9–C_{11} *n*-alkenes, C_9–C_{11} isoalkenes and C_9–C_{11} *n*-alkanes	
4-*n*-Pentylacetophenone-(O-4-*n*-pentyloxybenzoyl oxime) (PBO)		63	94	Glass, 90 × 0.25	70	*n*-Decyne isomers (12)	192
						n-Undecyne isomers (17)	
				Glass, 62 × 0.25	70	*n*-Tridecyne isomers (27)	
					80	*n*-Tetradecyne isomers (35)	
Cholesterol *p*-chlorocinnamate				Glass, 26 × 0.2	175	Diastereomeric arylalkenes (17)	193
				Glass, 13.6 × 0.2	190	Diastereomeric arylalkenes (54)	
4,4′-Azoxyphenetole		136	167	Glass, 2 × 2	138	4 isomers of pentanol	201
N,N′-Bis (*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT)		181	320	Glass, 1.8 × 2	135	Gemibrozil containing 2,4-xylyloxy isomer contamination (13)	203
					160	2,5-Xylenol containing 2,4-xylenol contamination (7)	
SB-smectic				30 × 0.25	30–280	104 polycyclic aromatic hydrocarbons (52)	207
Polysiloxane				Fused silica, 20 × 0.25	100–270	Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (39)	208
SB-smectic				Fused silica, 20 × 0.25	100–270	9 disubstituted benzene isomers (11)	210
						8 chlorinated compounds (95)	
						17 isomeric polycyclic aromatics (69)	
						25 isomeric polycyclic aromatics (46)	
						80 isomeric polycyclic aromatics (72)	

2-Methyl-4′-methoxy-4-(4″-methoxycinnamyloxy)-azobenzene		126	262	2.6 × 2.6	150 90°C for 20 min then programmed at a rate of 2°C/min	Methoxynaphthalenes (18) 6 isomeric insect sex pheromone (67)	212
Polymer		95	280	Glass, 38 × 0.25	215 225	Naphthols (13) Dihydropyrene, fluoranthene, pyrene (14)	
Polymer		88	245	Glass, 45 × 0.15	180 149	10 aromatic compounds (43) 12 aromatic compounds by supercritical fluid chromatography (SFC) (42)	
Polyacrylates		75	246	Glass, 45 × 0.15	149	Diphenylmethane, diphenyl, diphenylethane, 1-methoxynaphthalene, 2-methoxynaphthalene, fluorene, *o*-terphenyl, triphenylmethane. phenanthrene, anthracene by SFC (27)	213
		95	250	Fused silica, 10 × 0.1	100 150	*o*-Phenylphenol, 1- and 2-napthol, *m*- and *p*-phenylphenol by SFC (12) 2,6-, 2,5-, 3,5- and 3,4-xylenol (15)	
N,N′-Bis(*p*-methoxyphenyl-benzylidene)-α,α′-bi-*p*-toluidine (BABT)		253	370	Glass, 1.8 × 2	235	N-Trimethylsilylimidazole ethers of monohydroxybenzo-[*a*]pyrene isomers (21) Methylbenzo[*a*]pyrene isomers (33)	219
N,N′-Bis(*p*-phenylbenzyli-dene)-α,α′-bi-*p*-toluidine (BPhBT)		257	403	Glass, 1.8 × 2	270	N-Trimethylsilylimidazole ethers of monohydroxybenzo-[*a*]pyrene isomers (21) Methylbenzo[*a*]pyrene isomers (34)	
BPhBT + N,N′-bis(*p*-hexy-loxybenzylidene)-α,α′-bi-*p*-toluidine (1:1)				Glass, 1.5 × 2	120–255	14 polycyclic aromatic hydrocarbons (59) 10 polycyclic aromatic hydrocarbons (59)	221
N,N′-Bis(*p*-butoxybenzylide-ne)-α,α′-bi-*p*-toluidine (BBBT)	159	188	303	1.2 × 3	190–265	28 polycyclic aromatic hydro-carbons in benzene extract of coal fly ash (65)	
Bis(4′-ethyldiphenylidene)-1,2-di(4-aminophenyl)ethane	234	282	400	Steel, 2 × 2.2	285	10 polycyclic aromatic hydro-carbons (25)	226

(Continued on p. 74)

TABLE 2 (*continued*)

Stationary phase	*Transition temperature to phase (°C)*			*Column (m × mm I.D.)*	*Temperature of column (°C)*	*Separated substances and separation time (min)*	*Ref.*
	Smectic	*Nematic*	*Isotropic*				
						Anthracene oil (phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*e*]pyrene, perylene, benzo[*a*]pyrene) (25)	
4′-(*trans*-4-Propylcyclohexyl)-4-biphenylcarboxylic acid 4′-(*trans*-4-pentylcyclohexyl)-4-biphenyl ester	200	224	462	2 × 2.2	290	*p*-5-(*cis*/*trans*-4-Pentylcyclohexyl)-2-pyrimidinylbenzonitrile (16)	228
4′-(*trans*-4-Pentylcyclohexyl)-4-biphenylcarboxylic acid *trans*-4-(*p*-cyanophenyl) cyclohexyl ester		148.6	347	2 × 2.2	150	Methyl oleate (*Z*)-isomer and methyl elaidate (*E*)-isomer (21)	
4′-{*trans*-4-[2-(4′-(*trans*-4-pentylcyclohexyl)-4-biphenylyl)ethyl]cyclohexyl} 4-biphenylcarbonitrile		194.5	440	2 × 2.2	230–300	Phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene (28)	
Polymer	195		265	Glass, 1.7 × 4	230	Benzene, naphthalene, diphenyl, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene (22)	
Bis(4′-ethyldiphenylidene)-1,2-di(4-aminophenyl)ethane	234	282	400	Glass, 1.1 × 4	238	11 polycyclic aromatic hydrocarbons (12)	231
					282	11 polycyclic aromatic hydrocarbons (10)	

Bis(4′-penthyldiphenylidene)-1,2-di(4-aminophenyl)ethane	130		387	Glass, 1.1 × 4	316	11 polycyclic aromatic hydrocarbons (7)	
Polysiloxanes	43		177			Xylene isomers	239
	75		176			Xylene isomers	
	67		156			Xylene isomers	
BPhBT		257	403			Chrysene, benzo[*a*]pyrene, benzo[*e*]pyrene, benz[*a*]anthracene	240
Polysiloxane		139	319	Glass, 15 × 0.25	180	Fluorene, phenanthrene, anthracene (3.5)	30
					235	Fluoranthene, pyrene, 1,2- and 2,3-benzofluorene, triphenylene, benz[*a*]anthracene, chrysene (9.5)	
					275	Benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, perylene, benzo[*a*]pyrene (11)	
					140–280	17 polycyclic aromatic hydrocarbons (54)	
Hexapentyloxytriphenylene (disc-like from 65°C)			118	Glass, 2.1 × 4	70	Heptane, octane, nonane, *p*- and *o*-xylene, undecane (29)	56
					78	Cyclooctene, cyclooctane, cyclooctatetraene, cyclooctadiene (17)	
Triphenylene hexa-4-octylbenzoate (disc-like from 137°C)			206	Glass, 2.1 × 4	198	*trans*- and *cis*-decalin, tetralin, naphthalene, diphenyl (12)	

[a] A few examples of very good separations of polynuclear aromatic hydrocarbons mixtures by gas chromatography on BPhBT (SP-301) liquid crystal stationary phase are given in Supelco Bulletin 773D (1983). Four excellent chromatograms of androstanol and androstanediol and -triol isomers and also estrogens and sulphur-containing polycyclic aromatic hydrocarbons are shown in refs. 270 and 271. The chromatograms were obtained by supercritical fluid chromatography.

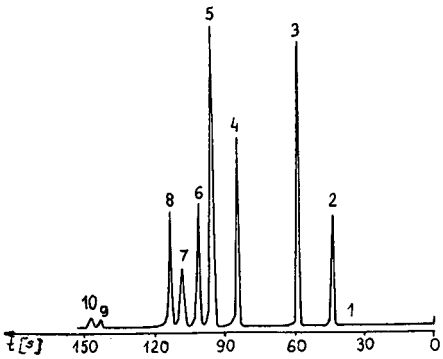

Fig. 15. Separation of alkylbenzenes on 4-*n*-pentylacetophenone (O-4-*n*-ethyloxybenzoyl oxime) in a glass capillary column (48 m × 0.25 mm I.D.) at 40°C. 1 = Methane; 2 = benzene; 3 = toluene; 4 = ethylbenzene; 5 = *m*-xylene; 6 = *p*-xylene; 7 = isopropylbenzene; 8 = *o*-xylene; 9 = *n*-propylbenzene; 10 = styrene[95].

ena. It is intriguing, for instance, why an increase in temperature improves the separation of the isomers, as this conflicts with the properties of liquid crystal stationary phases observed in gas chromatography, where the best separations are obtained at a temperature several degrees above the liquid crystal melting point.

The results obtained of applying liquid crystals in column liquid chromatography are promising although not necessarily related to their liquid crystal properties. The observations made require conformation and complementing, however. Further studies are also required. The use of siloxane polymers as column fillings seems interesting. Such polymers should be obtained by bonding the monomeric liquid crystal with the surface of silica. Suitable reagents that would allow such polymers to be obtained are under investigation[30].

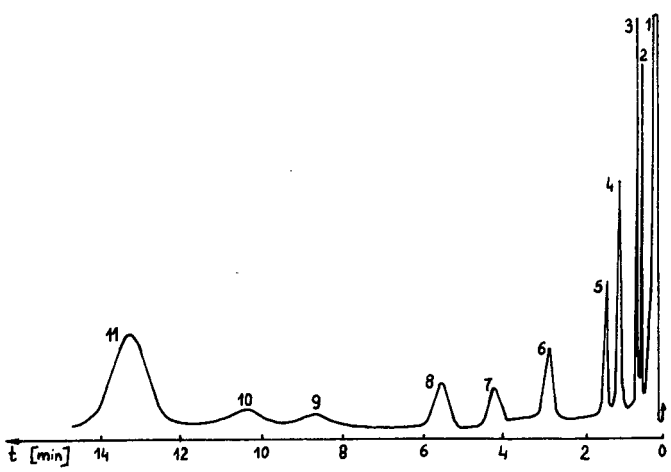

Fig. 16. Separation of hydrocarbon mixture on bis(4′-ethyldiphenylidene)-1,2-di(4-aminophenyl)ethane deposited in an amount of 5% on Chromaton N AW DMCS. Glass column (1.1 m × 4 mm I.D.); column temperature, 282°C. 1 = Benzene; 2 = phenanthrene; 3 = anthracene; 4 = fluoranthene; 5 = pyrene; 6 = triphenylene; 7 = 1,2-benzanthracene; 8 = chrysene; 9 = naphthacene; 10 = perylene; 11 = 3,4-benzopyrene[231].

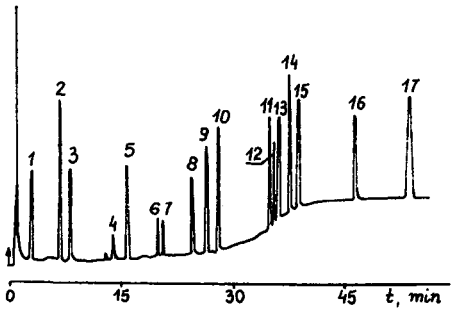

Fig. 17. Separation of polycyclic aromatic hydrocarbons on polysiloxane liquid crystalline stationary phase in a Pyrex glass column (15 m × 0.25 mm I.D.); column temperature, 140°C for 3 min, 140–280°C at 4°C/min. 1 = Fluorene; 2 = phenanthrene; 3 = anthracene; 4 = fluoranthene; 5 = pyrene; 6 = 1,2-benzofluorene; 7 = 2,3-benzofluorene; 8 = triphenylene; 9 = benz[*a*]anthracene; 10 = chrysene; 11 = benzo[*b*]fluoranthene; 12 = benzo[*k*]fluoranthene; 13 = benzo[*e*]pyrene; 14 = perylene; 15 = benzo[*a*]pyrene; 16 = 1,2,3,4-dibenzanthracene; 17 = benzo[*ghi*]perylene[30].

6.2. *Thin-layer chromatography*

Liquid crystals may be used in liquid crystal detectors for visualizing thin-layer chromatograms[243–246]. Until recently, liquid crystal detectors were used for detecting air pollutants[247]. Their practical value is small, however, as they are not selective. The good detectability and short response time are advantages, and the lack of selectivity presents no obstacle in using liquid crystal detectors for detecting the components on a chromatographic plate after their separation.

Liquid crystals may be used to detect various substances as their properties are changed by those substances. A small amount of the admixture disturbs the order of the liquid crystal structure, and a large amount of a foreign substance destroys that structure completely and the liquid crystal is converted to the isotropic liquid. The amount of the admixture that destroys the ordered structure of the mesophase is the

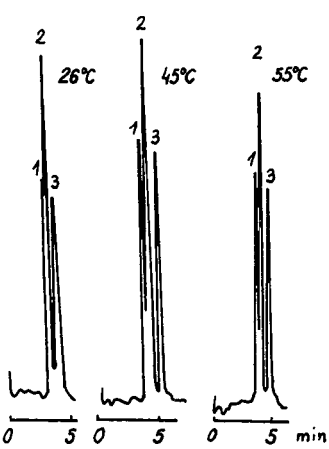

Fig. 18. Chromatograms of nitrotoluene isomers (1 = *ortho*; 2 = *meta*; 3 = *para*) at different temperatures. Column, 40% cholesteryl oleate on Silasorb-600, 5 μm (10 cm × 0.4 mm I.D.); eluent, hexane at a flow-rate of 1.5 cm^3/min[241].

smaller the closer the temperature at which this occurs is to the liquid crystal clearing point. The changes in the ordering of the liquid crystal structure affect the physicochemical properties, *e.g.*, optical, of the liquid crystal. These changes are greatest on transition from the mesophase to the isotropic liquid. The detector is therefore so designed (the liquid crystal is so selected) that the transition to the isotropic liquid is produced by the least possible amounts of the substances to be detected.

A liquid crystal detector may be produced by impregnating the pores of porous foil with a nematic liquid crystal[243,244]. The foil may have a thickness of 0.12 μm and a pore diameter of 0.12 – 0.15 μm, the surface pore density being, *e.g.*, 4.5%. The detector, when observed in polarized light, has a characteristic colour. Its intensity and homogeneity depend on the thickness of the liquid crystal layer (*i.e.*, on the foil thickness) and on the diameter and surface density of the pores.

In order to detect a substance on the chromatographic plate a detector is used with dimensions the same as those of the plate. After developing the chromatogram in any chromatographic chamber, the plate is sprinkled with water. Next the liquid crystal detector is placed on the chromatographic plate and pressed to the plate at a pressure of 0.3 – 0.4 MPa for 1–2 min. Such conditions are optimal for obtaining good mapping of the chromatogram on the detector[245]. The detector is pressed to the plate in a chromatographic pressure chamber[248] or in a special device of similar design[245]. It is recommended, however, that devices be used in which the pressure is exerted on the detector and plate by means of compressed gas. In such devices the required pressure is achieved much quicker than in devices in which water is used as the pressure medium. The best contact between the detector and chromatographic plate is ensured if an elastic foil is used as the pressure transfer medium.

The sprinkling of the adsorbate with water prevents the transfer of the liquid crystal from the foil to the adsorbent and facilitates the migration of the substances to be detected to the liquid crystal in the foil.

After the detector has been removed from the chromatographic plate it is placed between crossed polarizers, when spots of different colours than that of the detector become distinctly visible. These spots correspond to the spots on the chromatographic plate as regards shape and position.

The detectability of the chromatographed substances depends primarily on their solubility in the liquid crystal, the detectability being better the greater is the solubility. Some pesticides may be detected in a chromatographic plate in amounts of 10^{-7} – 10^{-8}g in the spot. This detectability is better than or comparable to that achieved by other methods. Liquid crystal detectors allow compounds to be visualized that cannot be detected at the required low level by a colour reaction or UV radiation. The sensitivity of the liquid crystal detector depends on the kind of liquid crystal used (the best are nematics), its clearing point, temperature and the kind of substance to be detected.

Thin-layer chromatograms should be visualized at ambient temperature. From the characteristics of liquid crystal detectors it follows that these detectors are most sensitive at temperatures close to the clearing point of the liquid crystals used. The clearing points of the liquid crystals used in the detectors should therefore lie in the range 295–305 K. In addition to single liquid crystals, their mixtures may also be used. In some instances it is advisable to heat the liquid crystal detector to increase its sensitivity. At the maximum sensitivity of the detector the spot of the detected sub-

stance has a diameter 1–2 mm greater than that detected by usual chemical methods.

The liquid crystal detector makes it possible to determine the amount of a substance in a chromatographic spot. Advantage is taken here of the linear variation of the clearing point of the liquid crystal with the amount of the isotropic substance (to be determined) dissolved in the mesophase. This relationship may serve as a means for determining the distribution of the concentration of the substance in the spot or the apparent better separation of the spots on the chromatogram. This is possible because at a low sensitivity of the detector the sites of greatest concentration of the chromatographed substance, *i.e.*, the centres of the spots, appear first. The connection and subsequent spreading of the spots proceeds as the sensitivity of the detector increases and sites of low concentration of the substance to be detected are revealed.

In the course of detection only part of the substance to be detected passes from the adsorbent (chromatographic plate) to the foil saturated with the liquid crystal. Hence it is possible to repeat the visualization of one chromatogram several times. When the chromatogram is visualized by the liquid crystal method, the chromatographic plate does not become contaminated and the substances being determined do not undergo chemical conversion. The method is therefore suitable for visualizing chromatograms in preparative thin-layer chromatography.

The chromatographic plate visualized by means of a liquid crystal detector may be used several times after regeneration with a solvent of high eluting power, preferably in a pressure or continuous flow chamber.

The use of liquid crystal detectors for visualizing chromatograms allows the wide laboratory application of plates with adsorbents of various colours and not only the white type that were used almost exclusively hitherto. One can mention here, for instance, various carbon adsorbents differing considerably in their properties from the white type and also between each other. At least some of them may be suitable for thin-layer chromatography.

The liquid crystal visualization method has several advantages over the method of visualizing chromatograms on carbon plates advanced by Prochazka and Starka[249], who used aluminium plates with a silica gel layer on which the spots were mapped and detected by a conventional method. The latter method is less sensitive and slower than the liquid crystal method.

7. FINAL REMARKS

The publications discussed in this review show that the application of liquid crystals in chromatography is an important development. Despite the opinions of some sceptics the liquid crystals now have a static position in chromatographic practice, I am convinced that developments in this field will continue rapidly. We now have at our disposal a wide range of liquid crystals which have not yet been tested as stationary phases. Some of them seem very promising in this respect. New liquid crystals are continuously becoming available and I believe that the best of them are yet to come.

So far only thermotropic liquid crystals have been used as stationary phases in chromatography, and no use has been made of lyotropic liquid crystals. The application of lyotropic liquid crystals as stationary phases seems promising as it has been

shown that gas chromatography may be used for determining the phase diagrams of these liquid crystals[250].

It has been suggested that, analogously to conventional isotropic stationary phases, colloidal stationary phases including a liquid crystal as a component could be used. These colloidal phases make it possible to control the sorptive capacity and the general selectivity of chromatographic columns over a wide range. This is due to the change in the contribution of dissolution to the adsorption ratio at the phase boundaries in the general retention of chromatographed substances. The colloidal stationary phases include mineral filling agents which adversely affect the ordering of the mesophase structure and hence the selectivity of the liquid crystal stationary phase. This decrease in selectivity varies, however, depending on the kind of liquid crystal (probably depending chiefly on its molecular structure). The efficiencies of columns with colloidal stationary phases including liquid crystals are higher than those of columns with normal liquid crystal stationary phases. As the above information was taken from the only paper so far published on this subject[251], further research is necessary to improve our knowledge of colloidal liquid crystal stationary phases and especially to allow some generalizations regarding their properties and practical usefulness.

From this review of studies published in recent years it can be seen that work dealing directly or indirectly with the use of liquid crystals in chromatographic practice for separating multi-component mixtures has dominated. Much less attention has been devoted to the testing of liquid crystals by gas chromatography. Only one paper reported studies of the thermotropic properties of polymeric liquid crystals by reversed-phase gas chromatography[252].

Investigations on the use of liquid crystals for visualizing thin-layer chromatograms have hardly been started and there is a lot to be done in this respect. It remains to be established whether the liquid crystal method of detection in thin-layer chromatography is of real practical use and what its range of applicability is. There are probably other possibilities of utilizing liquid crystals in chromatography.

Some work, mainly by Chinese authors, has not been considered in this survey[253–269].

8. SUMMARY

On the basis of the literature published since 1982, the applications of liquid crystals in chromatography are reviewed. The properties are described of new liquid crystals that may be used as stationary phases in gas chromatography; the most important in this group are liquid crystal siloxane polymers, followed by isothiocyanate liquid crystals and disc-like liquid crystals. The properties are discussed of conventional and capillary columns in which liquid crystal stationary phases are applied and the effects are considered of various factors that influence the separation of components of mixtures on these stationary phases. Among these factors, the effects of the kind of mesophase of the liquid crystal used, the structure of the liquid crystal molecule and that of the chromatographed substance and the constitution of the surface of the substrate (support, wall of the capillary column) on which the liquid crystal has been deposited are given special consideration. A large part of the review is devoted to practical applications of liquid crystal stationary phases, examples being given of separations of isomers of benzene and naphthalene derivatives, of polycyclic

hydrocrabons and of other compounds. Attention is drawn to the possibility of using liquid crystals, apart from in gas chromatography, also in liquid chromatography and especially in thin-layer chromatography for visualizing the chromatograms.

NOTE ADDED IN PROOF

Recently, various articles on liquid crystals in chromatography appeared[272–274].

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REVIEW

BIOGENIC AMINES AND THEIR METABOLITES IN BODY FLUIDS OF NORMAL, PSYCHIATRIC AND NEUROLOGICAL SUBJECTS[a]

BRUCE A. DAVIS

Neuropsychiatric Research Unit, Medical Research Building, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)

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CONTENTS

[a] *Editor's note:* This review is a unique discussion of the determination of traces of amines in biological fluids. As such it is of interest not only in the medical field but also to toxicologists, food scientists, nutritionists, etc., and was hence accepted for publication in the general section of this journal.

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1. INTRODUCTION AND SCOPE OF THE REVIEW

The biogenic monoamines have been implicated in the neurochemistry and physiology of mental illness and neurological disorders. Because the direct assessment of the monoamines and their metabolites in the brain of living human subjects is not possible and *post-mortem* samples suffer from a number of limitations, peripheral measures of these compounds in urine, blood plasma and cerebrospinal fluid have been carried out for the purpose of assessing the presence of a dysfunction in a given condition. The results of hundreds of such investigations have been published over the last 40–50 years.

These studies have been interesting not only for purely scientific reasons, but also because they have potentially important practical applications for clinicians in the diagnosis and treatment of these disorders. Originally it was hoped that analysis of accessible body fluids of patients and normal controls would reveal statistically reliable differences in metabolite concentrations which could serve as biological "state" or "trait" markers. Despite a wealth of published papers and increasing

sophistication of biochemical and analytical methodology, however, few marker candidates have been unambiguously established. More hopeful has been some psychopharmacological work in which metabolite concentrations have been used as predictors of drug response or have been observed to respond to drug treatment in a manner which parallels clinical response.

Part of the difficulty in obtaining consistently significant differences in the metabolite concentrations of patients and controls undoubtedly reflects the complexity and heterogeneity of psychiatric disorders; it is also caused, however, as a consequence of biochemical individuality (sex, age, weight, height, ethnic origin, circadian rhythms), environmental factors (diet, drug or alcohol ingestion, cigarette smoking, physical and mental stress, posture) and for analytical reasons (sample storage conditions and the specificity and sensitivity of the analytical methods). The importance of using specific and sensitive methods for quantification becomes evident when comparing some of the earliest work with more recent results. Improvements in and the introduction of new types of chromatography and derivatives have produced ever better component resolution (and therefore greater specificity of the measurements), and new and more sensitive means of detection have permitted ever smaller amounts to be quantified.

This review is a summary of the results obtained over the last 25–30 years of investigations of the concentrations of the biogenic monoamines and their metabolites in the biological fluids of normal, psychiatric and neurological subjects. In the first section, concentration values for normal controls and the analytical methods employed are presented; in the second section, the concentrations for psychiatric and neurological patients are tabulated; in the final section the individual and environmental factors which may influence metabolite concentrations are described.

2. CONCENTRATIONS OF BIOGENIC MONOAMINES AND THEIR METABOLITES IN BODY FLUIDS OF NORMAL SUBJECTS

2.1. Explanation of the tables

The calculation of the weighted mean of the mean concentrations for each metabolite and fluid (and weighted mean of the standard errors of the mean) was carried out as follows. For each study selected for inclusion, the reported concentration (converted to common units) and standard error (converted from standard deviation where required) was multiplied by the number of subjects in the study. All the products were added to give a grand total concentration and standard error, which was then divided by the total number of subjects to give the weighted mean value ± standard error of the mean (S.E.M.) reported in the tables. For a paper to be cited in a table, it was necessary that the method of analysis, number of subjects and concentration in a biological fluid be reported. Papers in which the results clearly conflicted with those in the majority of similar papers were excluded. For those metabolites for which few analyses have been carried out, markedly different results from different studies are presented separately. In the column headed "References and methods", the abbreviations refer to the method of isolation/separation and detection/quantification. A reference number followed by an asterisk (*) indicates that the paper cited

includes full details of the procedure for the isolation and quantification of the metabolite. The abbreviations are those commonly used in the literature, as follows:

IE = ion exchange;
PC = paper chromatography;
TLC = thin-layer chromatography;
Fl = spectrophotometric detection and quantification (mostly fluorimetric);
GC = gas chromatography;
ECD = electron-capture detection;
FID = flame ionization detection;
ND = nitrogen detection;
NCI = negative chemical ionization;
MS = mass spectrometry;
HPLC = high-performance liquid chromatography;
ED = electrochemical, amperometric or coulometric detection;
REA = radioenzymatic assay;
RIA = radioimmunoassay;
n.d. = not detected.

2.2. *Creatinine as a unit of urinary concentrations*

Expression of excretion rates in units of metabolite per milligram of creatinine has been suggested as a means of correcting for incomplete urine collections. However, several investigators have concluded that this may obscure important metabolic variations in some mental patients[1–3]. For example, a high correlation between excretion rates of homovanillic acid (HVA), indoleacetic acid (IAA), 5-hydroxyindoleacetic acid (5-HIAA) and tryptamine (TRA) and the excretion rate of creatinine has been noted[2–4]. Further, daily excretion of creatinine in normals subjects[5] and schizophrenics[1–3,6] has been found to be highly variable, with unmedicated schizophrenics excreting significantly lower amounts than normal subjects[4]. Treatment of schizophrenics with sulpiride or chlorpromazine, however, increases creatinine excretion[4]. Some of the daily variation may be due to dietary factors[6], but this does not appear to be the sole cause of the variability[1,3,7]. A diurnal variation in creatinine excretion has also been reported[8]. McDonald and Weise[3] explained the difference in excretion rates of creatinine between normal subjects and schizophrenics to be due to the fact that the schizophrenics were older and heavier, a finding corroborated by Jenner *et al.*[9], who found that creatinine excretion was related to body weight. That women, who generally weigh less than men, also excrete significantly less creatinine than men lends further confirmation to these results[8,10,11]. It has also been shown that there is a high correlation between urine volume and creatinine excretion[6,12]. As the daily urine volume is extremely variable, it was concluded that the use of creatinine excretion as a correction factor for incomplete 24-h sample collection is not justified. Soliman *et al.*[13] found that urinary creatinine is stable for only 24 h when frozen, and not even for 24 h if the samples had been acidified before freezing. However, in an HPLC analysis of creatinine in CSF (at physiological pH), Swahn and Sedvall[4] observed no correlation between creatinine level and storage time. In view of the problems associated with creatinine excretion measurements, urinary excretion rates are expressed by most authors in units per 24 h, and this convention is followed in this review.

2.3. Correlations between concentrations in body fluids and the brain

In contrast to an assessment of the activity of a given neuronal system in the brain, the quantification of neurotransmitters and their metabolites in the CSF or peripheral body fluids permits the control of such factors as diet, age and sex of the subjects, medication and general physical health through experimental design. Such studies also permit the accumulation of large sample sizes, and repeated measurements in the same individual. It is necessary, however, to assess how well the concentrations of the metabolites in the biological fluids correlate with brain concentrations before it is valid to relate the results of an experiment to brain neuronal activity.

It has been suggested that lumbar CSF levels of the biogenic amine metabolites partially reflect metabolism in the spinal cord[492,884] and may have some relevance to neurotransmission in the brain[14,15]. Stanley *et al.*[16] measured HVA and 5-HIAA in 48 individuals at autopsy and determined that their concentrations in the cerebral cortex were positively correlated with their levels in the CSF for the same individual. They concluded, as did other authors[17,18], that the CSF concentrations of HVA and 5-HIAA do, in large part, reflect brain metabolism and may offer a valid means of determining the central activity of the serotonin and dopamine systems. Davis *et al.*[19] ascertained that about 50% of plasma HVA originates in the brain, and by measuring venous samples from both the internal jugular vein and the radial artery Maas *et al.*[20] were able to calculate the average production of HVA and 5-HIAA in brain. The data were related to the urinary excretion of these metabolites and indicated that approximately 33% of urinary HVA and 34% of 5-HIAA are derived from the brain.

Elevated levels of noradrenaline (NA) in the brains of schizophrenics have been shown to be correlated with elevated NA levels in the CSF[21]. Other investigations have led to the conclusion that in the CSF the major metabolite of noradrenaline, 3-methoxy-4-hydroxyphenyl glycol (MHPG), arises largely from central metabolism[22,23]. Estimates of the percentage of urinary MHPG originating in the brain range from a low of 20%[24] to as much as 60%[25]. There are considerable data showing a large peripheral contribution to MHPG levels in CSF, plasma and urine, so it may be that such measurements will not prove useful for the assessment of central noradrenergic activity[26]. Further, although as much as 60% of plasma MHPG has been claimed to be produced in the brain[27], it has been shown that plasma MHPG is oxidized to vanilmandelic acid (VMA), raising more questions about the validity of its use as an index of central NA activity[28].

A significant positive correlation between the concentrations of the catecholamines and their metabolites in plasma and in CSF[29–31] and between the concentrations of MHPG in urine and CSF[32–34,686] has been reported by several authors, although not by all[35]. As the CSF concentrations of these metabolites may reflect central nervous system metabolism, it may be permissible in some instances to conclude that plasma, and even urine, concentrations also reflect central activity. A positive correlation between plasma total MHPG and 24-h urinary MHPG in psychiatric patients has been reported[36]; an earlier study showed no correlation[37]. Low-dose administration of debrisoquin has been reported to eliminate about 80% of the peripheral contribution to plasma HVA and MHPG, resulting in a situation in which at least 75% of these metabolites in plasma originated in the brain[38]. Thus, debrisoquin potentially provides a method for studying brain catecholamines through measurement of their metabolites in plasma.

Sandler and co-workers[39,40], observing a high correlation between both free and conjugated phenylacetic acid (PAA) in plasma and CSF taken at the same time, suggested that plasma PAA measurements might justifiably be employed clinically to provide an estimate of central phenylethylamine (PE) changes.

Probenecid is a drug used to block transport of monoamine acid metabolites out of the cerebrospinal fluid. It has generally been assumed that the resultant rise in the CSF concentrations of the metabolites will reflect presynaptic turnover of the parent amine in the central nervous system. However, it has been remarked that the CSF levels of probenecid correlate with the levels of metabolites, suggesting that the blockade may be incomplete at the probenecid concentrations obtainable in human studies[41]. A review of the probenecid test, including its theoretical basis, the assumptions on which it is based and its limitations has been published[42]. It shows that the test can be extended from the measurement of dopamine and serotonin turnover to include comparisons of turnover of other monoamines, such as octopamine, *p*-tyramine and tryptamine. The differential effects of probenecid on the CSF concentrations of the monoamine metabolites in controls and the psychiatric and neurological disorders are presented in the relevant sections of this review.

2.4. Comparison of analytical methods

In order to assess the merits of the various analytical procedures, studies comparing GC, LC, mass fragmentography, radioenzymatic assay and fluorimetry have been carried out. The subject has also been reviewed for the catecholamines[43] and for the biogenic amines and their metabolites[44].

Comparisons of the results of analyses on the same samples by radioenzymatic and LC assays have shown that the former tend to produce higher values with greater scatter, but are more reliable for concentrations below 100 pg/ml[45–48]. In an interlaboratory comparison of plasma catecholamine concentrations, 34 laboratories employing radioenzymatic, HPLC and fluorimetric assay produced, in some instances, considerable differences in results when analyzing the same sample by the same method[46]. The fluorimetric assays gave unacceptable results. Tracy *et al.*[49] found that measurement of urinary 5-HIAA by a colorimetric assay gave excellent agreement with an LC assay only for high concentrations. On the other hand, a high correlation in the results of measurements of the urinary catecholamines by fluorimetry and by LC has been obtained[50]. Although some researchers have been able to obtain accurate and precise quantifications by fluorimetry, many have not. The key factor seems to be adequate purification, without which the results may be too high owing to contributions to the fluorescence from other substances[51], or too low owing to quenching of the fluorescence by other substances[52,53].

GC–MS and fluorimetry have been compared for the quantification of 5-HIAA[54,55], HVA[52,54–56] and serotonin[57]. For HVA in CSF, the values were found to be lower when measured by fluorimetry[52,54–56], but for 5-HIAA[54] good agreement between the two methods was obtained. Good agreement in the results of measurements of 5-HIAA and HVA in CSF by HPLC and GC–MS have also been reported[55]. Plasma serotonin (5-HT) levels were determined to be lower when measured by fluorimetry than by GC–MS[57]. GC using a nitrogen detector gave results comparable to those obtained by GC–MS for the determination of the isothiocyanate derivatives of biogenic amines[58].

Various aspects of the use of GC–MS with selected ion monitoring[59–62], GC–MS with negative ion chemical ionization[63] and HPLC[64,65] for the determination of biogenic amines and their metabolites have been reviewed.

2.5 Intra-individual variation

Most reported concentrations of metabolites are the result of measurements on a single sample from each subject. Large intra-individual day-to-day variations in concentrations may obscure real differences between groups of subjects, particularly if the size of the groups is small. This problem has been addressed by longitudinal studies for some metabolites in some fluids.

Urinary excretion of the biogenic amines and their metabolites is characterized, in general, by large day-to-day variations. The trace amines *m*- and *p*-tyramine (mTA and pTA), PE and TRA were quantified in urine from one subject daily for 28 days; the concentration of the unconjugated mTA and pTA remained fairly constant, but the unconjugated PE and TRA and all the conjugated amine levels were extremely variable[67]. Using the same urine samples, the excretion of the acidic metabolites of the trace amines also exhibited very large daily variations[68]. In a study on the reliability of urinary monoamine and metabolite output measurements, Linnoila *et al.*[69] showed that the urinary concentrations of normetanephrine, 5-HT, dopamine (DA), NA, PE, HVA and 5-HIAA were highly variable with time and that at the very least two measurements on different days were required for the data to be reliable. Only MHPG and VMA proved to be relatively stable from one 24-h sample to the next. Hollister *et al.*[70], however, observed that the urinary excretion of MHPG, although stable for three consecutive 24-h collections, was not stable over a period of several weeks, suggesting that excretory pattents are not traits. Large daily variations in the urinary excretion of pTA, PE, normetanephrine (NMN) and metanephrine (MN)[71], PAA[72] and 3,4-dimethoxyphenylethylamine[73] have also been noted in other studies.

In plasma, over periods of up to 3 days, MHPG concentration shows minimal variation within subjects[37,74–76], but across a 5-week study period considerable variation was reported[75]. Plasma noradrenaline concentration appears to be stable for at least a few days[76].

2.6 Determination of conjugates

Conjugates of the amines and their metabolites, mostly sulfates and glucuronides, may be determined after either acid[145,298] or enzymatic hydrolysis[239,241,242] or directly by a radioenzymatic assay[236] or by HPLC[162,191]. Techniques for the determination of phenolic amine neurotransmitter conjugates have also recently been reviewed[66].

2.7 Tables of values calculated as weighted means

In Tables 1–18 amines in urine are usually expressed as μg per 24 h, whereas in plasma and CSF the units are pg/ml. The acidic metabolites, on the other hand, are usually given in mg per 24 h in urine and ng/ml in plasma and CSF. Any other units are listed directly in the tables, as is the nature of the conjugates when this is known. If

TABLE I

UNCONJUGATED BIOGENIC AMINES IN URINE

Amine	*References and methods*[a]	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*
Histamine	Bioassay: 77*, 78, 79*, 80*; IE–Fl: 81*, 82*; PC-GC-Fl: 83* REA: 84*, 85*, 86, 87*; HPLC–Fl: 88*	248	19.7 ± 2.3
N^{τ}-Methylhistamine	PC–GC–Fl: 83*; HPLC–Fl: 88*	28	276 ± 26
Phenylethylamine	Fl: 89; GC–FID: 90*; GC–ECD: 91*, 92*, 93, 94*; TLC–MS: 67*, 95*, 96*, 97*; GC–MS: 98*, 99, 100, 101, 102, 103*, 104*, 105*	266	8.1 ± 1.8
m-Tyramine	GC–ECD: 94*; TLC–MS: 67*, 95*, 96*, 97*; GC–MS: 99, 102*, 103*	93	83 ± 10
p-Tyramine	IE–Fl: 106, 107, 108, 109, 110*; PC–Fl: 111; Fl: 112, 113; GC–ECD: 94*; TLC–MS: 67*, 95*, 96*, 97*; GC–MS: 99, 102, 103*, 114	189	579 ± 75
Tryptamine	IE–Fl: 115*, 116*; Fl: 111, 112, 117*, 118; GC–ECD: 94*; TLC–MS: 67*, 95*, 96*, 97*; GC–MS: 119, 120	147	103 ± 19
p-Octopamine	REA: 121, 122, TLC–MS: 123*	38	0.80 ± 0.15
p-Synephrine	TLC–MS: 123*	4	6.9 ± 0.7
Noradrenaline	Fl: 43, 112, 124; alumina–Fl: 125*, 126*, 127*, 128*, 129, 130*, 131, 132, 133, 134; IE–Fl: 50*, 135*, 136*, 137*, 138*, 139, 155; bioassay: 132; GC–ECD: 140*; GC–MS: 103*, 141; REA–TLC: 142, 143*; HPLC–ED: 144*, 145*, 146*, 147*, 148, 149, 150, 151, 160*, 161*; HPLC–Fl: 144*, 152*	981	40.1 ± 3.9

Adrenaline	Fl: 43, 112, 124; alumina–Fl: 125*, 126*, 127*, 128*, 129, 130*, 131, 132, 133, 134, 153*; IE–Fl: 50*, 126*, 135*, 136*, 137*, 138*, 139, 155*; bioassay: 132, 134; GC–ECD: 140*; GC–MS: 141; REA–TLC: 142, 143; HPLC–ED: 144*, 145*, 146*, 147*, 148, 151, 161*; HPLC–Fl: 152*	777	10.1 ± 1.2
Dopamine	Fl: 43, 112; alumina–Fl: 111, 130*, 133, 156*; IE–Fl: 50*, 138*, 139, 155*, 157; GC–ECD: 140*; GC–MS: 103*; REA: 158; REA–TLC: 142, 143*; alumina–REA–IE–TLC: 159*; HPLC–EC: 145*, 146*, 147*, 148*, 151, 160*, 161*, 162*; HPLC–Fl: 152*	719	263 ± 34
5-Hydroxytryptamine	IE–Fl: 115*, 116*, 163, 164; GC–ECD: 94*	55	120 ± 23
Normetanephrine	Alumina–Fl: 130*; PC–Fl: 165*; IE–Fl: 118, 139, 166*, 167*, 168*, 169, 170; GC–ECD: 94*, 171*; REA: 174*; GC–MS: 76, 103*, 172, 173*; HPLC–ED: 175*	326	194 ± 17
Metanephrine	Alumina–Fl: 130*; PC–Fl: 165*; IE–Fl: 118, 139, 166*, 167*, 168*, 169, 170; GC–ECD: 171*; RIA: 176*; GC–MS: 76, 172, 173*; HPLC–ED: 175*	305	95 ± 9
3-Methoxytyramine	IE–Fl: 177*, 178*; GC–ECD: 171*, 94*; GC–MS: 103*	86	83 ± 11
3,4-Dimethoxy-phenylethylamine	IE–Fl: 179*, 180*, PC–Fl: 181*; RIA: 182*; TLC–radioactivity: 183	96	0.35 range: n.d.–1.71
N,N-Dimethyltryptamine	GC–ND: 184; GC–MS: 185*	19	0.38 ± 0.12
N-Methyltryptamine	GC–ND: 184	19	0.86 ± 0.22
Bufotenin	GC–MS: 186	10	1.71
Melatonin	Bioassay: 187	5	9.6 ± 1.2
6-Hydroxymelatonin	GC–MS: 188, 189*; RIA: 190*	39	14.7 ± 1.7

[a] In all tables, reference numbers followed by an asterisk (*) indicates that the paper cited includes full details of the procedure for the isolation and quantification of the metabolite.

TABLE 2

CONJUGATED BIOGENIC AMINES IN URINE

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*
Histamine	Bioassay: 79, 80	54	27 ± 5
Phenylethylamine	GC–FID: 90*; TLC–MS: 67*, 96*, 97*	31	19.9 ± 7.4
m-Tyramine	TLC–MS: 67*, 96*, 97*	19	90 ± 14
p-Tyramine	IE–PC–Fl: 107; TLC–MS: 67*, 96*, 97*	21	455 ± 135
Noradrenaline	REA–TLC: 142; HPLC–ED: 145*, 191*	23	139 ± 37
	HPLC–Fl: 191*	11	3-Sulfate: 124 ± 41
	HPLC–Fl: 191*	11	4-Sulfate: 21.6 ± 6.1
Adrenaline	IE–Fl: 155*; REA–TLC: 142; HPLC–ED: 145*	24	20.0 ± 3.0
	HPLC–Fl: 191*	11	3-Sulfate: 18 ± 2.1
	HPLC–Fl: 191*	11	4-Sulfate: 3 ± 0.3
Dopamine	REA–TLC: 142; GC–MS: 193; HPLC–ED: 145*, 162*, 192*; HPLC–Fl: 191*, 194*	68	785 ± 167
	HPLC–ED: 162*; HPLC–Fl: 191*, 194*	20	3-Sulfate: 392 ± 72
	HPLC–ED: 162*; HPLC–Fl: 191*, 194*	20	4-Sulfate: 69 ± 10
5-Hydroxytryptamine	IE–Fl: 164	5	34 ± 9 (glucuronide)
		5	52 ± 13 (sulfate)
Normetanephrine	IE–Fl: 135*, 166*, 195*; GC–MS: 193	71	Total: 183 ± 23
Metanephrine	RIA: 176*	15	13.4 ± 2.7
6-Hydroxymelatonin	RIA: 190*	18	Sulfate: 9.7 ± 1,7

no standard error of the mean is shown, this is because none was reported in the reference cited.

3. PSYCHIATRIC DISORDERS

3.1. Depression

3.1.1. Introduction

Biogenic amine hypotheses of affective disorders have mainly been derived from pharmacological and biochemical findings in experimental animals and from animal models of depression. These hypotheses state that some depressive disorders

TABLE 3

UNCONJUGATED BIOGENIC AMINES IN PLASMA

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*
Histamine	IE–Fl: 197* REA: 85*, 87, 198*, 199*, 200, 201*; REA–TLC: 202*, 203, 204*; HPLC–Fl: 205*, 206*; HPLC–ED: 207*; GC–MS: 208*, 209*	428	537 ± 49
N^t-Methylhistamine	GC–MS: 87*, 210;	15	1492 ± 167
Phenylethylamine	GC–MS: 211, 212;	19	124 ± 27
m-Tyramine	GC–MS: 102	10	540 ± 150
p-Tyramine	GC–MS: 99	15	680 ± 90
Tryptamine	GC–ND: 58*	4	n. d.
p-Octopamine	REA: 213	14	400 ± 60
Noradrenaline	Alumina–Fl: 133, 214*, 215, 216*, 217*, 218*, 219, 220, 221, IE–Fl: 136*, 222*, 223*, 292*; REA–TLC: 31, 142*, 143*, 172, 224*, 225*, 226, 227, 228, 229*, 230*, 231, 232*, 233, 234, 235*, 236, 237, 238, 239, 240, 241*, 242, 243, 244*, 245, 246, 294; alumina–REA: 76, 247*, 248*, 249*, 250, 251, 252, 253, 254, 255; IE–REA: 256*; IE–REA–TLC: 257, 258, 259*; REA–HPLC: 260*, 261*, 262, 272*; PC–REA–TLC: 263; REA: 264, 265, 266, 267, 268, 269*, 270, 271, 293; GC–ECD: 273*, 274*; GC–MS: 275*; HPLC–ED: 45, 47, 276*, 277*, 278, 279, 280, 281, 282*, 283*, 284, 285, 286*, 287, 288, 289, 290, 299*; HPLC–Fl: 291*	1651	275 ± 32
Adrenaline	Alumina–Fl: 43*, 214*, 215, 216*, 217*, 218*, 219, 220; IE–Fl: 223*, 292*; REA–TLC: 31, 142, 143*, 172, 224*, 225*, 226, 228, 229*, 230*, 231, 232*, 233, 234, 235*, 236, 238, 239, 240, 241*, 242, 244*, 246, 269*, 293, 294*; IE–REA: 256*; alumina–REA: 249*; IE–REA–TLC: 257, 258, 259*;	1067	63 ± 11

(Continued on p. 100)

TABLE 3 (*continued*)

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*
	REA–HPLC: 260*, 262, 272*; REA: 265, 267, 271; GC–ECD: 274*; HPLC–ED: 45, 47, 277*, 278, 280, 281, 283*, 286*, 287, 290, 299*; HPLC–Fl: 291		
Dopamine	IE–Fl: 295*; alumina–REA–IE–TLC: 159, 296*; REA–TLC: 142, 143*, 228, 229*, 230*, 232*, 233, 234, 236, 239, 240, 241*, 242, 244*, 269*, 294*, 297; REA–HPLC: 260*, 262; IE–REA–TLC: 259; REA: 158; 270, 271, 298*; GC–ECD: 274*; HPLC–ED: 47, 280, 281, 283, 286*, 287, 290, 299*	535	86 ± 15
5-Hydroxytryptamine	REA: 300*, 301*; RIA: 301*; HPLC–ED: 290, 303*, 304*, 305; HPLC–Fl: 305, 306, 307*, 308*; IE–Fl: 309*;	226	1440 ± 142 (platelet-poor)
	HPLC–Fl: 307*	7	387 ± 84 (ultrafiltrate)
Normetanephrine	IE–REA–TLC: 310*; GC–MS: 311*	12	116 ± 17
Metanephrine	GC–MS: 311*	3	118 ± 92
3-Methoxytyramine	GC–MS: 311*	3	418 ± 84
N,N-Dimethyl-tryptamine	GC–MS: 312*, 313	44	~ 500
Melatonin	GC–MS: 314*; RIA: 315, 316, 317*, 318	26	Day: 11 ± 2 Night: 47 ± 9
	GC–MS: 319*; RIA: 320*	18	24 h: 44 ± 8

TABLE 4

CONJUGATED BIOGENIC AMINES IN PLASMA

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*
Noradrenaline	REA–TLC: 142, 236, 238, 239, 241*, 242, 244*, 269*, 321; REA–HPLC: 262; HPLC–ED, Fl: 283*	111	782 ± 259
	REA–TLC: 239	6	Glucuronide: 139 ± 49
	REA–TLC: 236, 238, 239, 241*, 321	46	Sulfate: 636 ± 57
Adrenaline	REA–TLC: 142, 236, 238, 239, 241*, 242, 244, 269*, 321; REA–HPLC: 262; HPLC–ED, Fl: 283*; GC–MS: 273*	116	221 ± 78
	REA–TLC: 239	6	Glucuronide: 22 ± 6
	REA–TLC: 236, 238, 239, 241*, 321	46	Sulfate: 216 ± 43
Dopamine	REA–TLC: 142, 236, 239, 241*, 242, 244*, 269*, 321, 322; REA–HPLC: 262; REA: 158, 298; HPLC–ED: 283*, 323*; HPLC–Fl: 194*, 283*; GC–ECD: 324*	183	2926 ± 696
	REA–TLC: 239	6	Glucuronide: 924 ± 121
	REA–TLC: 236, 239, 241*, 242, 321, 322	84	Sulfate: 3178 ± 343
Normetanephrine	GC–MS: 311*	3	494 ± 37
Metanephrine	GC–MS: 311*	3	335 ± 63
3-Methoxytyramine	GC–MS: 311*	3	668 ± 217

TABLE 5

UNCONJUGATED BIOGENIC AMINES IN CEREBROSPINAL FLUID

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*
Histamine	REA: 87*	11	43.1 ± 5.7 ng/ml
N^{τ}-Methylhistamine	GC–MS: 325*	5	2.0 ± 0.1 ng/ml
Phenylethylamine	GC–MS: 99	15	600 ± 100
p-Tyramine	GC–MS: 99	15	790 ± 250
Tryptamine	GC–ND: 58*	4	1–6 ng/ml
Noradrenaline	IE–Fl: 326; alumina–Fl: 220; REA–TLC: 31, 143*, 229*, 243; alumina–REA: 327; PC–REA–TLC: 263; REA–HPLC: 262; REA: 21, 270, 328; HPLC–ED: 329, 330, 331*, 332, 333*, 334, 336; HPLC–Fl: 335	455	119 ± 16
Adrenaline	REA–TLC: 31, 143*, 229*; REA–HPLC: 262; REA: 270; HPLC–ED: 336	118	47 ± 23
Dopamine	REA–TLC: 143*, 263; REA: 270; REA–HPLC: 262; HPLC–ED: 329, 331*, 336	165	48 ± 14
5-Hydroxytryptamine	HPLC–ED: 303*, 337*, 338*, 339*	42	678 ± 94
Normetanephrine	GC–MS: 340*, 341*	19	1800 ± 420
Metanephrine	GC–MS: 341*.	6	80 ± 20
3-Methoxytyramine	GC–MS: 341*	6	635 ± 184
Melatonin	RIA: 342; GC–MS: 319*	17	59 ± 28
N,N-Dimethyltryptamine	GC–MS: 343*, 344*	10	n.d. – 1500

TABLE 6

CONJUGATED BIOGENIC AMINES IN CEREBROSPINAL FLUID

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*
Noradrenaline	REA–HPLC: 262	40	199 ± 137
Adrenaline	REA–HPLC: 262	40	30 ± 34
Dopamine	REA–HPLC: 262; HPLC–ED: 332, 345*	117	594 ± 108

TABLE 7

UNCONJUGATED ACID METABOLITES IN URINE

Acid metabolite	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (mg per 24 h)*
Imidazoleacetic	IE–REA: 346*; HPLC–Fl: 347*	20	1.6 ± 0.2
N^{τ}-Methylimidazoleacetic	GC–FID: 348*, 349, 350; HPLC–Fl: 347*	76	2.6 ± 0.2
N^{π}-Methylimidazoleacetic	GC–FID: 349, 350 HPLC–Fl: 347*	42	2.2 ± 0.3
Phenylacetic	GC–MS: 96*, 97*, 351*, 352	73	8.5 ± 1.7
o-Hydroxyphenylacetic	GC–FID: 93	27	1.0 ± 0.1
m-Hydroxyphenylacetic	GC–FID: 93; GC–MS: 96*, 97*; GC–ECD: 972*	74	6.6 ± 0.8
p-Hydroxyphenyl acetic	IE–PC–Fl: 107; GC–ECD: 102; 972*; GC–FID: 93, 108, 109; GC–MS: 96*, 97*, 103*	113	19.0 ± 2.4
Indoleacetic	IE–Fl: 116*; GC–MS: 96*, 97*, 353*, 354*	67	10.2 ± 1.3
o-Hydroxymandelic	GC–MS: 355*	10	7.3 ± 1.1 μg/g creatinine
m-Hydroxymandelic	GC–MS: 355*, 356*	7	59 ± 14 μg per 24 h
p-Hydroxymandelic	GC–FID: 93 GC–MS: 96*, 97*, 103*	50	2.8 ± 0.5
Mandelic	GC–MS: 97*	8	0.23 ± 0.01
5-Hydroxyindoleacetic	Colorimetry 131, 357, 358, 359, 360, 361, 362, 363; Sephadex–FL: 120; Bioassay: 364; IE–FL: 116*, 118, 164; Fl: 365, 366, 367; GC–ECD: 972*; GC–MS: 141, 353*, 354*; HPLC–ED: 49*, 146*, 368*, 369, 370*, 371*, 372*; HPLC–Fl: 373*	741	4.56 ± 0.38

(*Continued on p. 104*)

TABLE 7 (*continued*)

Amine	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (mg per 24 h)*
Homovanillic	IE–Fl: 139, 374*, 375*, 376, 377, 378*; silica–Fl: 379*, 380*; TLC–Fl: 381*; alumina–Fl: 130*, 156*; PC–Fl: 382*, 383*, 384*; GC–FID: 93, 385, 386*, 387*; GC–ECD: 388*, 389*, 972*; GC–MS: 97*, 103*, 141, 354*; HPLC–ED: 146*, 151*, 390, 391*, 392*	729	4.85 ± 0.36
Isohomovanillic	PC–colorimetry: 393*; GC–ECD: 388*	15	0.19 ± 0.04
Vanilmandelic	Colorimetry: 394*, 395*, 396*, 397*, 398*, 399*; PC–colorimetry: 367, 384*, 400; alumina–Fl: 130*; IE–Fl: 8*, 135*, 139, 157, 376, 378*, 401*, 402*, 403*, 404*; GC–FID: 93, 385, 386*, 405*; GC–ECD: 118, 169, 170, 388*, 406*, 407; GC–MS: 76, 97*, 103*; alumina–REA: 174*, 408*; HPLC–ED: 151*, 390, 391*, 392*, 409*; HPLC–Fl: 410	1408	4.07 ± 0.25
3,4-Dihydroxy-phenylacetic	Alumina–Fl: 156; electrophoresis–Fl: 411; IE–Fl: 138*, 377, 378*; GC–ECD: 412*; GC–MS: 103*, 193; HPLC–ED: 151*	134	1.87 ± 0.19
3,4-Dihydroxy-mandelic	IE–Fl: 135*, 378*, 413*; electrophoresis–Fl: 411*; alumina–REA: 408*; HPLC–ED: 151*	79	0.29 ± 0.03
3,4-Dimethoxy-phenylacetic	GC–tritium: 180*	8	Trace
5-Methoxyindole-acetic	GC–MS: 189*, 414*	33	5–38

TABLE 8

CONJUGATED ACID METABOLITES IN URINE

Acid metabolite	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (mg per 24 h)*
Imidazoleacetic	IE–REA: 346*	10	3.30 ± 0.22
Phenylacetic	GC–ECD: 939*; GC–FID: 72*, 93, 415*, 416, 629; GC–MS: 97*, 103*, 105*, 351*, 352, 417; HPLC–Fl: 418*	338 (includes 276 totals)	145 ± 15
m-Hydroxyphenylacetic	GC–MS: 97*	8	1.2 ± 0.7
p-Hydroxyphenylacetic	IE–Fl: 107; GC–FID: 109; GC–MS: 97*	18	6.1 ± 2.8
Mandelic	GC–MS: 97*	8	0.12 ± 0.06
5-Hydroxyindoleacetic	GC–MS: 419*	5	0.38 mg/l
Homovanillic	IE–Fl: 377; GC–MS: 419*	13	−0.6 to +5.8 mg/l
3,4-Dihydroxyphenylacetic	IE–Fl: 377; GC–ECD: 412*	20	1.05 ±0.08
3,4-Dihydroxymandelic	IE–Fl: 135*	9	0.17 ± 0.04

are associated with or may be the result of deficiencies in central catecholamine or indoleamine transmission. In man amine metabolite and pharmacological studies have tentatively identified two biochemical sub-types of depressive illness: one subtype claims an abnormality of central noradrenaline systems, and the other an abnormality in central serotonin systems. Several in-depth reviews of these theories have been published in the last few years[616–623,748]. More recently, the putative neuromodulator phenylethylamine has been implicated in the aetiology of affective disorder and this amine has been recently reviewed[624,625]. Other suggested mechanisms have involved abnormal conjugation of *p*-tyramine as a trait marker in major depression[717–722], although a recent study comparing conjugation of ingested deuterium-labelled tyramine in healthy volunteers and depressed patients showed no difference in conjugation between the two groups[970].

The weighted means (calculated as for the normal subjects) of the results of studies on depressed subjects are presented in Tables 19–28. The depressed subjects here have not been differentiated according to the various clinical sub-types of depression. These studies in aggregate indicate that depressed patients as a group show reduced 5-HIAA concentrations in plasma and CSF (but not in urine), reduced MHPG and 3,4-dihydroxyphenylglycol (DHPG) concentrations in urine, plasma and CSF and reduced homovanillic acid and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in urine and CSF (but not in plasma) of depressed subjects. Although many of these reductions are not very substantial, the large total number of subjects suggests that these trends may be significant. The parent amines do not appear to be reduced in any of the biological fluids of depressed subjects.

An examination of each of the references cited shows that in over half of them

TABLE 9

UNCONJUGATED ACID METABOLITES IN PLASMA

Acid metabolite	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
Imidazoleacetic	IE–REA: 346*	10	277 ± 13
N^{τ}-Methylimidazoleacetic	GC–MS: 420*, 421*	6	11.7 ± 1.6
N^{π}-Methylimidazoleacetic	GC–MS: 420*	5	10.3 ± 2.0
Phenylacetic	GC–MS: 40, 97*, 422*, 423, 424*, 425, 426, 427, 428	327	124 ± 14
m-Hydroxyphenylacetic	GC–MS: 59*, 97*, 424*, 425, 426, 427, 428	293	13.4 ± 2.2
p-Hydroxyphenylacetic	GC–MS: 59*, 97*, 424*, 425, 426, 427, 428	293	69.0 ± 8.8
Indoleacetic	GC–MS: 97*, 428; HPLC–ED: 429*; HPLC–Fl: 430*	144	293 ± 38
Mandelic	GC–MS: 431*, 432*	16	13.6; 41.0
m-Hydroxymandelic	GC–MS: 432*	10	n.d.
p-Hydroxymandelic	GC–MS: 59*, 97*, 428, 432*	106	9.7 ± 0.8
5-Hydroxyindoleacetic	GC–MS: 20, 433; HPLC–ED: 434*, 435*; HPLC–Fl: 430*	45	10.5 ± 1.1
Homovanillic	GC–MS: 20, 59*, 97*, 428, 433, 436*, 437, 438, 439*, 440, 441*, 442, 443; HPLC–ED: 444*, 445, 446, 447*, 448	311	10.0 ± 1.0
Vanilmandelic	GC–MS: 59*, 279, 428, 432*, 436*, 439*, 449*; HPLC–ED: 447*, 451*	163	7.0 ± 0.8
3,4-Dihydroxyphenylacetic	IE–REA–TLC: 259*; REA–TLC: 452*; GC–MS: 59*, 441*; HPLC–ED: 286, 453*	73	3.4 ± 0.5
3,4-Dihydroxymandelic	REA–TLC: 31, 235*, 244*, 245, 452*; IE–REA–TLC: 259*	99	1.94 ± 0.35

TABLE 10

CONJUGATED ACID METABOLITES IN PLASMA

Acid metabolite	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
Imidazoleacetic	IE–REA: 346*	10	25 ± 3
Phenylacetic	GC–MS: 40, 97*, 422*, 423, 424*, 425, 426, 428, 454*	273	345 ± 35
m-Hydroxyphenyl-acetic	GC–MS: 97*, 424*, 425, 426, 428	219	2.1 ± 1.1
p-Hydroxyphenyl-acetic	GC–MS: 97*, 424*, 425, 426, 428	219	13.6 ± 4.3
3,4-Dihydroxy-mandelic	REA–TLC: 244*	5	7.9 ± 1.0

no comparison with normal controls was made, but in the remainder of the studies the equivocal evidence for the proposed hypotheses of depression suggests that the serotonergic and noradrenergic systems may indeed be deficient in depression, which appears also to be the case for PAA. Hence cerebrospinal fluid 5-HIAA concentrations in depressed subjects have been reported to be significantly reduced[470,471,473,474,482,496,510,626–628], to exhibit a trend to be reduced[466,478,487,498,517] and to be no different[170,330,332,463,480,488,493,494,503,505,518,531,536,630–633] than normal controls. Plasma 5-HIAA tends to be reduced[305], whereas urinary 5-HIAA mostly shows no difference between depressed and normal subjects[118,120,361,366]. Most investigators have found no significant differences between the urinary[93,103,193], plasma[440] or CSF[330,332,480,485,494,498,499,503,505,509,518] concentrations of HVA in normal and depressed subjects. In four studies a significant reduction was claimed[487,496,510,528], and a trend to decreased HVA in depression has been reported in a few others[170,493,517,536,631,633]. Urinary[103,193] and CSF[330,332,503] concentrations of DOPAC are also not significantly different between depressed and normal populations. A slight decrease in urinary dopamine (DA)[103] and non-significant increases in plasma[287,332,345] and CSF[634] dopamine levels in depression have been reported.

In the noradrenergic system, the major central metabolite, MHPG, has been found to be significantly reduced[120,169,193,557,562,564,565,568], to show a trend to be reduced[76,118,477,551,563] or to be unchanged[32,103,170,566,571,572] in the urine of depressives. In plasma[27,76,440,448,587,588,593,607] and CSF[35,170,330,332,494,498,503,505,517,518,610,612,633], however, MHPG concentrations in depressed and normal subjects have not been found to be significantly different, with one exception in which MHPG in the CSF of depressed subjects was significantly reduced[500]. The concentrations of two other noradrenergic metabolites, DHPG in plasma[603] and VMA in CSF[541], have been reported to be significantly lower in depression. Dajas and co-workers[149,150] have claimed a significant elevation of urinary noradrenaline in depression, and a trend to elevated plasma[76,284,287,288,326,723] and CSF[255] levels in depression has been reported by other investigators. Urinary VMA[76,93,103,118,169,170,193,562,637] and normetanephrine, the methylated metabolite of noradrenaline[76,103,118,169,170,193], were not significantly different in depressed and control subjects.

In a major study on a large number of severely depressed patients and healthy

TABLE 11

UNCONJUGATED ACID METABOLITES IN CEREBROSPINAL FLUID

Acid metabolite	*References and methods*[a]	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
N^{τ}-Methylimidazoleacetic	GC–MS: 420*, 421*	15	0.14; 3.19
N^{π}-Methylimidazoleacetic	GC–MS: 420*	10	11.3 ± 2.6
Phenylacetic	GC–MS: 39, 40, 422*, 785, 454*, 455*, 456; HPLC–ED: 336	125	24.0 ± 3.1
m-Hydroxyphenylacetic	GC–MS: 432*	10	~0.5
p-Hydroxyphenylacetic	GC–MS: 457; HPLC–ED: 458*	32	8.8 ± 1.4
Indoleacetic	HPLC–Fl: 270, 459, 460, 461	184	4.35 ± 0.55
p-Hydroxymandelic	GC–MS: 457	5	1.3 ± 0.4
5-Hydroxyindoleacetic	Sephadex–Fl: 18, 462*, 463, 464, 465, 466; TLC–Fl: 328, 467, 468, 469, 470, 471, 472, 473, 478, 479, 480, 481, 482; column chromatography–Fl: 15, 154, 474, 475, 476; IE–Fl: 483; Fl: 22, 131, 450, 484, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498; GC–ECD: 500*; GC–MS: 54, 419*, 433, 450, 501, 502, 503, 504*, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514;	1553	26.2 ± 2.2

	HPLC–Fl: 270; HPLC–ED: 303*, 330, 332, 333*, 334, 336, 337*, 339*, 515*, 516*, 517*, 518, 519*, 520*, 521*, 522*, 523, 524, 525, 526		
Homovanillic	Sephadex–Fl: 263, 464, 480, 481, 499, 527, 528*; IE–Fl: 328, 529, 530; column chromatography: 468, 531; Fl: 15, 18, 22, 131, 270, 467, 469, 472, 475, 476, 479, 484, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 532, 533, 534, 535, 536; GC–ECD: 498, 500* GC–MS: 54, 170, 419*, 433, 441*, 450, 457, 501, 502*, 504*, 505, 506, 507, 508, 510, 511, 512, 513, 514, 537*, 538, 539*; HPLC–ED: 330, 332, 333*, 334, 339*, 515*, 516*, 517*, 518, 519*, 520*, 521*, 522*, 524, 525, 526	1606	40.7 ± 4.3
Vanilmandelic	GC–MS: 450, 457, 501, 538, 541	44	0.98 ± 0.16
3,4-Dihydroxy-phenylacetic	GC–MS: 441*, 450, 502*, 503, 513, 540; HPLC–ED: 330, 331*, 332, 339*, 524	252	0.49 ± 0.04
3,4-Dihydroxy-mandelic	REA–TLC: 31	36	2.35 ± 0.26

TABLE 12

CONJUGATED ACID METABOLITES IN CEREBROSPINAL FLUID

Acid metabolite	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
Phenylacetic	GC–MS: 40, 454*, 455*; HPLC–ED: 336	54	24.5 ± 4.5
Homovanillic	GC–MS: 502*	23	0.25 ± 0.58
3,4-Dihydroxy-phenylacetic	GC–MS: 502*, 503, 540	40	0.22 ± 0.01

controls, two sub-groups of depressed patients were identified, one excreting normal levels of adrenaline, metanephrine, noradrenaline and normetanephrine and the other excreting very high levels[971]. Interestingly, the HVA concentration in the CSF of subjects in the high excretion group was significantly low.

In addition to the major neurotransmitters and their metabolites, the comparisons indicate that there may also be deficiencies in PE and pTA metabolism in depression. Although the parent amine concentrations in the urine of depressives appear to be normal, PAA and *p*-hydroxyphenylacetic acid concentrations in urine, plasma and CSF are all lower in depressed than in normal subjects. A statistically significant reduction in urinary PAA has been reported and proposed as a biological "state" marker for unipolar depression[416,417,629,663], but other research groups reported no reduction at all in the urinary excretion of PAA in depression[72,352]. In a study of PE excretion in depression, DeLisi *et al.*[417] reported that those patients forming a sub-

TABLE 13

UNCONJUGATED ALCOHOL AND GLYCOL METABOLITES IN URINE

Alcohol or glycol	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*
Phenyl glycol	GC–MS: 542*	1	22
p-Hydroxyphenyl-ethanol	GC–MS: 542*	1	n.d.
p-Hydroxyphenyl-glycol	GC–MS: 542*	1	13
3-Methoxy-4-hydroxyphenyl glycol	Fl: 543*; IE–Fl: 544; GC–ECD: 11*, 545; GC–MS: 97*, 419*, 546*, 547*; HPLC–ED: 548*	109	113 ± 17
3-Methoxy-4-hydroxyphenyl-ethanol	GC–MS: 546*	9	n.d.
3,4-Dihydroxy-phenyl glycol	REA–TLC: 549*	7	155 ± 42 μg/l

TABLE 14

CONJUGATED ALCOHOL AND GLYCOL METABOLITES IN URINE

Alcohol or glycol	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*
p-Hydroxyphenyl-ethanol	GC–MS: 542*	1	11
p-Hydroxyphenyl-glycol	GC–MS: 542*	1	78
3-Methoxy-4-hydroxyphenyl glycol	Fl: 543*; Sephadex–Fl: 551; IE–Fl: 108, 376, 544, 552*, 553*, 554*, 555; alumina–REA: 174*; GC–FID: 385, 387*, 477; GC–ECD: 11*, 70, 118, 120, 131, 170, 407, 545, 546*, 556*, 557, 558, 559*, 560, 561*, 562, 563*, 564, 565, 566, 567*, 568, 569, 570, 571, 572, 573, 574*; GC–MS: 10, 32, 76, 103*, 141, 193, 542*, 547*; HPLC–ED: 548*, 575*, 576*, 577*; HPLC–Fl: 578*	936	1854 ± 156
	IE–Fl: 544, 555; Sephadex–Fl: 551; GC–ECD: 11*, 407, 561*, 546*; GC–MS: 547*; HPLC–ED: 548*	141	Sulfate: 1070 ± 130
	IE–Fl: 544, 555; GC–ECD: 11*, 407, 546*; GC–MS: 547*; HPLC–ED: 548*	111	Glucuronide: 1010 ± 90
3-Methoxy-4-hydroxyphenyl-ethanol	GC–MS: 546*	9	239 ± 50
3,4-Dihydroxy-phenyl glycol	REA–TLC: 549*	7	584 ± 133 μg/l
	GC–MS: 579*	7	Total: 325 ± 60 μg/g creatinine
3,4-Dihydroxy-phenylethanol	GC–MS: 579*	4	Total: 16 ± 15 μ/g creatinine

TABLE 15

UNCONJUGATED ALCOHOL AND GLYCOL METABOLITES IN PLASMA

Alcohol or glycol	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
3-Methoxy-4-hydroxyphenyl glycol	GC–ECD: 74*, 580*; GC–MS: 26, 27, 29, 75, 76, 243, 440, 442, 449*, 581*, 583*, 584*, 585, 586, 587, 588, 589*, 590, 591, 592, 593; HPLC–ED: 447*, 448, 577*, 594*, 595*, 596*, 597, 598*, 599, 600*, 601*	402	3.55 ± 0.33
3,4-Dihydroxyphenylethanol	REA–TLC: 602*; IE–REA–TLC: 259*	22	0.17 ± 0.07
3,4-Dihydroxyphenyl glycol	REA–TLC: 31, 235*, 244*, 245, 452*, 549*, 602*, 603; IE–REA–TLC: 259*; GC–MS: 589*; HPLC–ED: 282*, 453*, 604	204	0.81 ± 0.07

TABLE 16

CONJUGATED GLYCOL METABOLITES IN PLASMA

Glycol	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
3-Methoxy-4-hydroxyphenyl glycol	GC–ECD: 74*, 573, 580*; GC–MS: 29, 279, 437, 449*, 581*, 584*, 585, 589*, 605*; HPLC–ED: 446, 606*, 607;	251	11.3 ± 0.8
3,4-Dihydroxy-phenyl glycol	REA–TLC: 244, 549*, 603; GC–MS: 589	70	1.18 ± 0.11

group with very high PE excretion (three times the highest value found in the controls) were clinically indistinguishable from depressed patients with low PE excretion. As PE excretion is not correlated with PAA excretion, these results suggest that depression is not associated with a generalized PE deficit and that reported PAA reductions may not reflect a PE abnormality.

3.1.2. Tables of values for depressed subjects (all clinical sub-types combined)

The studies discussed above and the comparisons of the weighted means in Tables 19–28 for depressed subjects with all clinical sub-types combined suggest that there may well be a monoamine dysfunction in depression. The current view, however, is that affective disease is a heterogeneous group of disorders. Therefore, the amine and metabolite concentrations in various diagnostic sub-types of depression are compared with each other and with controls in Tables 29–36.

3.1.3. Comparison of unipolar and bipolar depressed subjects

Comparisons of unipolar and bipolar depressed subjects are given in Tables 29–31.

To summarize, bipolar patients exhibited lower CSF concentrations than did unipolar patients in five of seven studies on 5-HIAA, three of four studies on MHPG and three of seven studies on HVA. Plasma MHPG concentrations were lower in bipolar patients in three of four studies and urinary MHPG concentrations in eleven of thirteen studies. Plasma noradrenaline levels were lower in the bipolar subjects in all five studies. There would appear, therefore, to be a noradrenergic dysfunction in bipolar subjects.

Some investigators have suggested that the unipolar depressive disorders can be subdivided into two or three sub-types based on differences in pretreatment urinary MHPG levels[169,562,568,571,637,740,741] and on CSF monoamine metabolite levels[745]. A bimodal distribution of 5-HIAA in the CSF of patients with endogenous depression has been found by several investigators[531,667,678,680,745], but could not be confirmed by others[518,683,686]. For unipolar depressive patients, multiple regression analysis has revealed strong correlations which suggest that high and low HVA, MHPG and 5-HIAA syndromes should be isolated[681]. Evidence has also been reported that in both unipolar and bipolar patients high and low monoamine syndromes are characterized by different symptomatology[681]. Schildkraut *et al.*[749] have

TABLE 17

UNCONJUGATED ALCOHOL AND GLYCOL METABOLITES IN CEREBROSPINAL FLUID

Alcohol or glycol	*References and methods*[a]	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
p-Hydroxyphenyl-ethanol	GC–MS: 457	8	12.3 ± 1.7
3-Methoxy-4-hydroxyphenyl glycol	Fl: 608; GC–ECD: 22, 35, 131, 494, 497, 498, 500*, 558, 609*, 610*, 611, 612; GC–MS: 170, 243, 419*, 433, 450, 501*, 502*, 504*, 505, 506, 507, 508, 510, 513, 538, 581*, 589*, 605*; HPLC–ED: 330, 332, 333*, 339*, 515*, 516*, 517*, 518, 520*, 522*, 526, 600*, 613*, 614*	884	10.5 ± 1.2 (mostly total MHPG)
3-Methoxy-4 hydroxyphenyl-ethanol	GC–ECD: 558	3	5.7 ± 0.9
3,4-Dihydroxy-phenylglycol	REA–TLC: 31, 549*; GC–MS: 589*	45	1.00 ± 0.07
3,4-Dihydroxy-phenylethanol	REA–TLC: 602*	8	1.62 ± 0.86
5-Hydroxytryptophol	GC–MS: 615*	24	0.78 ± 0.11

TABLE 18

CONJUGATED ALCOHOL AND GLYCOL METABOLITES IN CEREBROSPINAL FLUID

Alcohol or glycol	*References and methods*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*
3-Methoxy-4-hydroxyphenyl glycol	GC–MS: 419*, 502*, 581*, 589*; HPLC–ED: 613*, 614*	97	0.51 ± 0.10
3-Methoxy-4-hydroxyphenyl-ethanol	GC–ECD: 558	2	3.3 ± 1.0
3,4-Dihydroxy-phenyl glycol	REA–TLC: 549*; GC–MS: 589*	9	0.14 ± 0.04

developed an equation based on the concentrations of noradrenaline, MHPG, VMA, normetanephrine and metanephrine for calculating a depression score which has good predictive value. It has also been demonstrated that urinary MHPG output is highly reliable both during and between recurring depressive episodes[743], that is, low or high excreters are reliably low or high during different episodes. The concentrations of metabolites in biological fluids of bipolar patients on the depressed and manic phases are presented in Tables 32–34.

3.1.4. Comparison of bipolar-depressed and bipolar-manic subjects

To summarize the comparisons of bipolar-depressed and bipolar-manic subjects in Tables 32–34 urinary dopamine and noradrenaline are reported to be consistently higher in manic than in depressed subjects. The major metabolites of these amines, HVA in CSF and MHPG in urine and CSF, also tend to be higher in manic patients. Measurements of monoamine metabolites in CSF have shown abnormal, perhaps excessive, central noradrenergic activity in patients with mania[509]. Longitudinal studies of the switch process from depression to mania have shown highly significant correlations of high urinary[647] and plasma MHPG[585,739] and high urinary noradrenaline[642,643,645] and VMA[642,750] concentrations with the manic phase.

3.1.5. Comparison of psychotic and non-psychotic (neurotic) depressed subjects

This comparison is presented in Table 35.

3.1.6. Comparison of depressed subjects with and without melancholia

This comparison is presented in Table 36.

3.1.7. Panic disorder, agoraphobia and anxiety

Dysfunction of the central noradrenergic system has been postulated to play an important role in the neurobiology of both major depressive and panic disorders and there is evidence suggesting an overlap between these disorders[593]. In studying these disorders, plasma and urinary noradrenaline and MHPG have been used as an indirect measure of noradrenergic activity. The results, however, have been equivocal.

TABLE 19

UNCONJUGATED BIOGENIC AMINES IN URINE

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*	
			Depressives	*Normals*[a]
Phenylethylamine	69, 93, 102, 103, 635, 638, 639	81	7.7 ± 2.1	8.1 ± 1.8
m-Tyramine	102, 103	23	49 ± 8	83 ± 10
p-Tyramine	102, 103, 114, 638, 639	60	583 ± 64	579 ± 75
Tryptamine	7, 118, 119, 120, 640	67	96 ± 19	103 ± 19
Noradrenaline	69, 103, 131, 149, 150, 637, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652	441	52 ± 6	40 ± 4
Adrenaline	637, 641, 642, 643, 644, 645, 646, 649, 650, 651, 652, 653	299	19 ± 2	10 ± 1.2
Dopamine	103, 641, 642, 643, 645, 654	39	281 ± 52	263 ± 34
5-Hydroxytryptamine	69, 163	32	168 ± 34	120 ± 23
Normetanephrine	69, 76, 103, 118, 169, 170, 196, 562, 637, 638, 639, 644, 646, 648, 649, 650, 651, 655	463	277 ± 30	194 ± 17
Metanephrine	76, 118, 169, 170, 562, 637, 644, 646, 649, 650, 651, 655	405	133 ± 11	95 ± 9
3-Methoxytyramine	103, 639	10	12.9 ± 3.9	83 ± 11
N,N-Dimethyl-tryptamine	656	14	0.80	0.38 ± 0.12
N-Methyltryptamine	656	14	0.80	0.86 ± 0.22
Melatonin	187	5	11.6 ± 1.1	9.6 ± 1.2
6-Hydroxymelatonin	657	27	7.2 ± 1.2	14.7 ± 1.7

[a] Weighted normal means taken from Table 1.

TABLE 20

CONJUGATED BIOGENIC AMINES IN URINE

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h) (normals[a])*
Noradrenaline (total)	69, 149, 193, 196, 638, 639, 648	100	244 ± 36 (conjugated = 139 ± 37)
Dopamine (total)	69, 193, 639, 658	51	764 ± 148
Dopamine (conjugated)	157	13	304 (785 ± 167)
Normetanephrine (total)	193	28	195 ± 24 (total = 183 ± 23)

[a] Weighted normal means taken from Table 2.

TABLE 21

UNCONJUGATED BIOGENIC AMINES IN PLASMA

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*	
			Depressives	*Normals[a]*
Phenylethylamine	211	3	120 ± 60	124 ± 27
Noradrenaline	76, 246, 255, 284, 285, 287, 288, 659	209	263 ± 59	275 ± 32
Adrenaline	287	22	87 ± 9	63 ± 11
Dopamine	287	22	93 ± 20	86 ± 15
Melatonin	660	4	188 ± 38	44 ± 8

[a] Weighted normal means taken from Table 3.

TABLE 22

UNCONJUGATED AND CONJUGATED BIOGENIC AMINES IN CEREBROSPINAL FLUID

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*	
			Depressives	*Normals[a]*
Noradrenaline	28, 330, 332, 661, 662	78	128 ± 20	119 ± 16
Adrenaline	634	7	16.0 ± 3.4	47 ± 23
Dopamine	634	7	12	48 ± 14
Conjugated dopamine	332, 345, 662	196	756 ± 47	594 ± 108

[a] Weighted normal means taken from Tables 5 and 6.

TABLE 23

UNCONJUGATED (AND TOTAL) ACID METABOLITES IN URINE

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (mg per 24 h)*	
			Depressives	*Normals*[a]
Phenylacetic (total)	72, 93, 103, 352, 416, 417, 629, 663	243	119 ± 16	149 ± 15
o-Hydroxyphenyl-acetic	93	23	0.81 ± 0.10	1.0 ± 0.1
m-Hydroxyphenyl-acetic	93	23	4.45 ± 0.63	6.8 ± 0.9
p-Hydroxyphenyl-acetic	93, 103, 639	33	9.90 ± 1.00	18.6 ± 2.6
Indoleacetic	7, 640	29	3.60 ± 0.70	10.2 ± 1.3
p-Hydroxymandelic	93, 103	29	1.29 ± 0.20	2.8 ± 0.5
5-Hydroxyindole-acetic	7, 69, 118, 120, 131, 163, 361, 366, 639, 642, 645	147	5.10 ± 0.74	4.53 ± 0.37
Homovanillic	69, 93, 103, 193, 642, 639, 654, 658	96	4.32 ± 0.46	4.79 ± 0.32
Vanilmandelic	30, 69, 76, 93, 103, 118, 169, 170, 193, 196, 409, 562, 568, 637, 638, 639, 642, 644, 646, 648, 649, 650, 651, 664, 665	574	4.02 ± 0.31	4.07 ± 0.25
3,4-Dihydroxy-phenylacetic	103, 193, 658	41	1.08 ± 0.18	1.90 ± 0.19

[a] Weighted normal means taken from Tables 7 and 8.

TABLE 24

UNCONJUGATED ACID METABOLITES IN PLASMA

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Depressives	*Normals*[a]
Phenylacetic	427	46	98 ± 5	124 ± 14
m-Hydroxyphenyl-acetic	427	46	12.9 ± 2.4	13.4 ± 2.2
p-Hydroxyphenyl-acetic	427	46	59.4 ± 5.1	69.0 ± 8.8
Homovanillic	440, 448, 694, 708, 712	98	14.8 ± 2.0	9.7 ± 0.9
5-Hydroxyindole-acetic	305, 708	26	7.2 ± 2.6	11.4 ± 1.5

[a] Weighted normal means taken from Table 9.

TABLE 25

UNCONJUGATED AND CONJUGATED ACID METABOLITES IN CEREBROSPINAL FLUID

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Depressives	*Normals*[a]
Phenylacetic	39, 456	30	19.6 ± 2.0	24.0 ± 3.1
p-Hydroxyphenyl-acetic	458	4	4.0 ± 0.5	8.8 ± 1.4
Indoleacetic	461, 666, 667, 668	143	5.7 ± 0.7	4.35 ± 0.55
5-Hydroxyindole-acetic	131, 170, 330, 332, 463, 466, 470, 471, 473, 474, 478, 480, 487, 488, 493, 494, 496, 498, 503, 505, 510, 517, 518, 531, 536, 616, 626, 627, 628, 629, 630, 631, 632, 633, 636, 649, 653, 662, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694	1892	24.0 ± 2.1	26.2 ± 2.2
Homovanillic	131, 170, 330, 332, 480, 485, 487, 493, 494, 496, 498, 499, 503, 505, 509, 510, 517, 518, 528, 536, 616, 631, 633, 649, 653, 662, 668, 670, 671, 673, 674, 675, 676, 677, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 693, 694	1547	32.1 ± 3.4	40.7 ± 4.3
Conjugated Homovanillic	503	10	2.2 ± 1.7	0.25 ± 0.06
Vanilmandelic	541	19	0.70 ± 0.17	0.98 ± 0.16
3,4-Dihydroxy-phenylacetic	330, 332, 503, 662	59	0.34 ± 0.22	0.49 ± 0.04
Conjugated 3,4-dihydroxyphenylacetic	503, 662	37	0.46 ± 0.10	0.22 ± 0.10

[a] Weighted normal means taken from Tables 11 and 12.

TABLE 26

UNCONJUGATED AND CONJUGATED GLYCOL METABOLITES IN URINE

Glycol metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*	
			Depressives	*Normals*[a]
3-Methoxy-4-hydroxyphenyl glycol	555	36	64	113 ± 17
Conjugated 3-methoxy-4-hydroxyphenyl glycol	10, 25, 27, 28, 30, 32, 33, 35, 69, 76, 103, 118, 120, 169, 170, 193, 196, 477, 551, 555, 557, 562, 563, 564, 565, 566, 568, 571, 572, 616, 635, 637, 638, 639, 644, 645, 646, 647, 648, 650, 651, 683, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706	1373	1582 ± 138	1866 ± 165
3,4-Dihydroxyphenyl glycol (total)	706	20		352 ± 38 584 ± 133 μg/l

[a] Weighted normal means taken from Tables 13 and 14.

TABLE 27

UNCONJUGATED AND CONJUGATED GLYCOL METABOLITES IN PLASMA

Glycol metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Depressives	*Normals*[a]
3-Methoxy-4-hydroxyphenyl glycol	26, 28, 37, 76, 440, 448, 587, 588, 593, 707, 708, 709, 710, 711, 712	225	3.84 ± 0.34	3.55 ± 0.31
Conjugated 3-methoxy-4-hydroxyphenyl glycol	37, 585, 607 (total), 711	13	8.33 ± 0.66	11.3 ± 0.8
3,4-Dihydroxy-phenyl glycol	603, 711, 713	124	0.79 ± 0.07	0.82 ± 0.07
Conjugated 3,4-dihydroxyphenyl glycol	603, 713	101	0.81 ± 0.07	1.18 ± 0.11

[a] Weighted normal means taken from Tables 15 and 16.

TABLE 28

UNCONJUGATED AND CONJUGATED GLYCOL METABOLITES IN CEREBROSPINAL FLUID

Glycol metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Depressives	*Normals*[a]
3-Methoxy-4-hydroxyphenyl glycol	30, 35, 131, 170, 330, 332, 494, 498, 500, 503, 505, 509, 510, 517, 518, 610, 612, 616, 633, 649, 653, 662, 668, 679, 680, 681, 682, 683, 685, 686, 688, 690, 693, 694, 703, 714, 715, 716	897	9.68 ± 0.56	10.6 ± 1.2
Conjugated 3-methoxy-4-hydroxyphenyl glycol	494, 503, 714	41	2.2 ± 0.5	0.51 ± 0.10

[a] Weighted normal means taken from Tables 17 and 18.

TABLE 29

SEROTONIN SYSTEM

The significance tests reported in this and subsequent tables were performed by the authors of the papers cited, not by the author of this review.

Metabolite	*Reference*	*Biological fluid*	*Concentrations*		
			Unipolar	*Bipolar*	*Control*
5-Hydroxy-indoleacetic	120	Urine	–	4.13 ± 0.51 mg per 24 h	3.83 ± 0.25 mg per 24 h
	674	CSF	10 ± 2 ng/ml		18 ± 3
	496	CSF	16.4 ± 0.9[a]	14.6 ± 1.7[a] ng/ml	27.5 ± 1.2 ng/ml
	498	CSF	32.0 ± 3.2	23.0 ± 2.9	28 ± 6
	681	CSF	21.0 ± 1.8[b]	17.8 ± 10.5[b]	26.7 ± 13.4 (S.D.)
	687	CSF	29.4 ± 2.2	22.0 ± 3.4	
	170	CSF (male)	19.7 ± 0.9	20.4 ± 1.4	20.6 ± 0.8
	170	CSF (female)	25.4 ± 1.2	23.3 ± 1.7	21.8 ± 1.3
	330	CSF	–	17.6 ± 2.5	14.3 ± 0.9
		CSF weighted means ± S.E.M.	21.1 ± 1.4 (n = 175)	19.3 ± 2.1 (n = 97)	22.8 ± 1.9 (n = 132)

[a] $p < 0.001$ *vs.* control.
[b] $p < 0.01$ *vs.* control.

TABLE 30

NORADRENALINE SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Concentrations*		
			Unipolar	*Bipolar*	*Control*
Noradrenaline	637	Urine	45 ± 4 μg per 24 h	27 ± 4 μg per 24 h	
	650	Urine	46.2 ± 3.6	23.4 ± 3.1	
	723	Plasma	550* pg/ml	400* pg/ml	280 pg/ml
	284	Plasma	220 ± 41*	122 ± 15*	147 ± 10
	285	Plasma	233 ± 10†	161 ± 7*	264 ± 10
	76	Plasma	351 ± 81††	268 ± 30	232 ± 25
	288	Plasma	318 ± 49**	123 ± 17	142 ± 10
		Plasma weighted mean ± S.E.M.	346 ± 45 (n=65)	300 ± 15 (n=62)	191 ± 13 (n=135)
	330	CSF		57 ± 11 pg/ml	54 ± 8 pg/ml
	332	CSF		76 ± 13	67 ± 7
Adrenaline	637	Urine	9.8 ± 1.0 μg per 24 h	8.2 ± 1.2 μg per 24 h	
	650	Urine	25.8 ± 2.1	17.7 ± 1.8	
Normetanephrine	169	Urine	207 ± 25 μg per 24 h	187 ± 30 μg per 24 h	
	637	Urine	323 ± 43	225 ± 26	
	76	Urine	293 ± 23	275 ± 46	293 ± 24
	650	Urine	310 ± 22	227 ± 31	
		Urine weighted mean ± S.E.M.	296 ± 26 (n=102)	226 ± 31 (n=50)	
Metanephrine	637	Urine	144 ± 11	161 ± 19	
	169	Urine	92 ± 7	85 ± 16	75 ± 6
	76	Urine	138 ± 19	138 ± 49	130 ± 15
	650	Urine	141 ± 7	133 ± 10	
		Urine weighted mean ± S.E.M.	134 ± 9 (n=102)	135 ± 16 (n=50)	
Vanilmandelic acid	637	Urine	3.78 ± 0.25 mg per 24 h	4.04 ± 0.21 mg per 24 h	
	169	Urine	4.68 ± 0.51	3.93 ± 0.78	4.63 ± 0.44
	568	Urine	3.93 ± 0.17	4.04 ± 0.16	
	76	Urine	4.65 ± 0.28	4.30 ± 0.27	3.96 ± 0.22
	650	Urine	3.61 ± 0.49	3.44 ± 0.22	
		Urine weighted mean ± S.E.M.	3.91 ± 0.34 (n=145)	3.82 ± 0.25 (n=66)	
	330	CSF		386 ± 48 pg/ml	437 ± 34 pg/ml

(*Continued on p. 126*)

TABLE 30 (*continued*)

Amine or metabolite	*Reference*	*Biological fluid*	*Concentrations*		
			Unipolar	*Bipolar*	*Control*
3-Methoxy-4-hydroxyphenyl glycol[a]	25	Urine	1860 ± 170 μg per 24 h	1590 ± 250 μg per 24 h	
	25	Urine	1860 ± 170†††	830 ± 75[b]	
	169	Urine	1161 ± 142*	916 ± 153*	1348 ± 65
	120	Urine		1320 ± 190	1600 ± 80
	637	Urine	1590 ± 177	1209 ± 89	
	477	Urine	1580 ± 120		1680 ± 120
	701	Urine	904 ± 126	676 ± 115	
	566	Urine	1820 ± 120***	1090 ± 120 (group I)	1330 ± 120
	566	Urine	1820 ± 120	1440 ± 200 (group II)	1330 ± 120
	32	Urine (male)	3606 ± 241	3772 ± 631	3478 ± 280
	32	Urine (female)	3054 ± 222	2502 ± 205	2558 ± 280
	568	Urine (male)	2378 ± 133	1410 ± 186***	2021 ± 133
	568	Urine (female)	1853 ± 146	1336 ± 127***	1820 ± 157
	704	Urine	856 ± 136	1496 ± 266	
	571	Urine	2081 ± 143		1767 ± 117
	572	Urine	1790 ± 110	1440 ± 100	1850 ± 20
	572	Urine (male)	1890 ± 150	1600 ± 140	1890 ± 130
	572	Urine (female)	1670 ± 150	1370 ± 130	1830 ± 170
	76	Urine	2521 ± 245	2429 ± 359	2760 ± 190
	650	Urine	2155 ± 131	2123 ± 131	
	551	Urine	1480 ± 96	1037 ± 191	1451 ± 103
		Urine weighted mean ± S.E.M.	2038 ± 149 (n = 332)	1668 ± 180 (n = 151)	1856 ± 119 (n = 203)

3-Methoxy-4-hydroxyphenyl glycol[c]	27	Plasma	4.3 ± 0.6††† ng/ml	2.7 ± 0.4[d] ng/ml	
	27	Plasma	3.6 ± 0.3	3.0 ± 0.4	
	76	Plasma	3.2 ± 0.4	3.4 ± 0.3	3.0 ± 0.1
	448	Plasma	3.69 ± 0.62	2.89 ± 0.28	
		Plasma weighted mean ± S.E.M.	3.55 ± 0.36 (n=51)	3.16 ± 0.33 (n=18)	
	610	CSF	11.9 ± 0.6 (total)	6.1 ng/ml (total)	
	35	CSF	11.9 ± 0.6 (total)		10.8 ± 0.8 (total)
	498	CSF	14 ± 1.1***	15 ± 2.0	10 ± 0.9†
	681	CSF	10.7 ± 0.4	10.5 ± 0.5	11.0 ± 2.8 (S.D.)
	170	CSF	8.9 ± 0.3**	8.5 ± 0.3	8.0 ± 0.2
	330	CSF		8.1 ± 0.4	8.3 ± 1.8
		CSF weighted mean ± S.E.M.	10.4 ± 0.5 (n=117)	10.5 ± 0.5 (n=67)	8.7 ± 0.7 (n=115)

* $p < 0.05$ *vs.* control.
** $p < 0.001$ *vs.* control.
*** $p < 0.01$ *vs.* control.
† $p < 0.02$ *vs.* bipolar.
†† $p < 0.01$ *vs* bipolar.
††† $p < 0.001$ *vs.* bipolar.
[a] Conjugated or total.
[b] With history of mania.
[c] Unconjugated.
[d] With melancholia.

TABLE 31

DOPAMINE SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Concentrations*		
			Unipolar	*Bipolar*	*Control*
Dopamine	654	Urine	210 ± 30 μg per 24 h	170 ± 40 μg per 24 h	
Homovanillic acid	654	Urine	5.82 ± 0.71 mg per 24 h	4.97 ± 0.67 mg per 24 h	
	448	Plasma	10.7 ± 2.6 ng/ml	11.2 ± 3.8	
	674	CSF	20 ± 2	34 ± 4	
	498	CSF	88 ± 10***	78 ± 22*	45 ± 5 ng/ml
	681	CSF	37.0 ± 4.5**	33.3 ± 4.6***	54.6 ± 27.3 (S.D.)
	528	CSF	19.6 ± 13.2** (S.D.)	34.4 ± 14.2 (S.D.)	41.8 ± 4.2 (S.D.)
	687	CSF	27.7 ± 5.7	34.4 ± 8.3	
	170	CSF (female)	31.8 ± 2.1**	33.1 ± 1.9**	43.7 ± 26
	170	CSF (male)	38.4 ± 2.6**	36.9 ± 3.8**	40.0 ± 2.3
	330	CSF		32.4 ± 4.1	32.8 ± 2.2
	332	CSF		36.0 ± 5.5	32.0 ± 2.0
		CSF weighted mean ± S.E.M.	39.3 ± 4.1 (n=121)	37.1 ± 5.3 (n=89)	39.3 ± 2.9 (n=149)

* $p < 0.05$ *vs.* control.
** $p < 0.01$ *vs.* control.
*** $p < 0.001$ *vs.* control.

TABLE 32

SEROTONIN SYSTEM

Metabolite	*Reference*	*Biological fluid*	*Bipolar–depressed*	*Bipolar–manic*	*Control*
5-Hydroxy-indoleacetic acid	361	Urine	4.07 ± 1.56 mg per 24 h	2.91 ± 0.42 mg per 24 h	2.70 ± 0.48 mg per 24 h
	645	Urine	4.40 ± 0.80	7.60 ± 1.3	
	627	CSF	19.8 ± 1.5* ng/ml	19.7 ± 1.6* ng/ml	42.3 ± 3.2 ng/ml
	673	CSF	15.8 ± 2.7	8.8 ± 1.8	
	674	CSF	18 ± 3	15 ± 2	
	724	CSF	25	28	27
	496	CSF	14.6 ± 1.7*	13.9 ± 2.9*	27.5 ± 1.2
	170	CSF (male)[a]	20.1 ± 0.8	22.5 ± 2.8	20.6 ± 0.8
	170	CSF (female)[a]	24.8 ± 1.0	30.8 ± 3.5**	21.8 ± 1.3
	518	CSF	22.7 ± 1.5	22.8 ± 2.9	22.2 ± 1.8
		CSF weighted mean ± S.E.M.	22.1 ± 1.3 ($n=269$)	21.4 ± 2.4 ($n=90$)	25.8 ± 1.6 ($n=157$)

* $p < 0.001$ *vs.* control.

** $p < 0.01$ *vs.* control.

[a] These values are also reported in refs. 509 and 633.

TABLE 33

NORADRENALINE SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Bipolar–depressed*	*Bipolar–manic*	*Control*
Noradrenaline	641	Urine	27.9 ± 3.7†† μg per 24 h	93.9 ± 7.9 μg per 24 h	
	725	Urine	41.1	67.8	
	643	Urine	20.7 ± 2.1	56.2 ± 11.6	
	644	Urine	53 ± 12	102 ± 15	
	645	Urine	36.7 ± 7.1	61.1 ± 13.3	
	647	Urine	13.6 ± 1.8[a]††	36.0 ± 3.4[a]	
		Urine weighted mean ± S.E.M.	38.3 ± 2.1 ($n=58$)	72.9 ± 11.2 ($n=25$)	
	723	Plasma	315 ± 70 pg/ml	376 ± 65 pg/ml	280 pg/ml
	723	Plasma	400***	590***	280
	326	CSF	700	500	200 ± 80
	726	CSF	210††	460	225
Adrenaline	641	Urine	9.6 ± 1.1†† μg per 24 h	19.5 ± 1.8 μg per 24 h	
	725	Urine	26.9	35.9	
	643	Urine	5.6 ± 0.8	8.3 ± 1.4	
	645	Urine	10.6 ± 1.6	18.6 ± 6.5	
		Urine weighted mean ± S.E.M.	22.0 ± 1.2 (n=54)	23.1 ± 3.7 (n=21)	
Normetanephrine	727	Urine	99 ± 11[b]	206 ± 22[b]	
	170	Urine	279 ± 16	299 ± 35	192 ± 8 μg per 24 h
Metanephrine	170(M)	Urine	149 ± 8	157 ± 23	107 ± 7
	170(F)	Urine	122 ± 7	91 ± 11	88 ± 5
Vanilmandelic acid	728	Urine	2.34 ± 0.16†† mg/g creatinine	8.16 ± 0.51 mg/g creatinine	
	157	Urine	1.5 mg per 24 h	1.8 mg per 24 h	
	170	Urine	3.56 ± 0.20	3.55 ± 0.38	2.72 ± 0.16 mg per 24 h
	541	CSF	0.60 ± 0.20*** ng/ml	0.80 ± 0.15 ng/ml	1.05 ± 0.15 ng/ml

3-Methoxy-4-hydroxyphenyl glycol	696	Urine	1180 ± 90 μg per 24 h	2750 ± 150 μg per 24 h	
	645	Urine	2230 ± 220	2500 ± 340	
	727	Urine	805	1182	
	647	Urine[c]	1030††	1190	
	170(M)	Urine	2273 ± 144	2740 ± 512	2267 ± 143 μg per 24 h
	170(F)	Urine	1968 ± 136	1909 ± 751	1660 ± 121
	704	Urine	1496 ± 266	1647 ± 538	
	572	Urine	1440 ± 100	2110 ± 190	1850 ± 20
		Urine weighted mean ± S.E.M.	2001 ± 138 ($n=146$)	2266 ± 382 ($n=42$)	1933 ± 101 ($n=99$)
	585	Plasma[d]	13.7 ± 0.6 ng/ml	21.1 ± 1.3 ng/ml	
	611	CSF[d]	8.9 ± 1.1†*	15.5 ± 3.0	14 ± 1.5
	612	CSF	18.0	27.8*	16 ± 0.9
	729	CSF	15.8	23.0***	15.9
	724	CSF	10.5	15.5	16.0
	170	CSF	8.8 ± 0.2**	11.0 ± 1.0††**	8.0 ± 0.2
	518	CSF	8.0 ± 0.4	8.4 ± 0.4***	7.7 ± 0.4
		CSF weighted mean ± S.E.M.	9.3 ± 0.4 ($n=205$)	15.3 ± 1.3 ($n=69$)	11.4 ± 0.5 ($n=180$)

* $p < 0.01$ *vs.* control.
** $p < 0.001$ *vs.* control.
*** $p < 0.05$ *vs.* control.
† $p < 0.05$ *vs.* manic.
†† $p < 0.001$ *vs.* manic.
[a] Longitudinal study of 45 days.
[b] Longitudinal study of 20 days.
[c] Longitudinal study of 84 days.
[d] Unconjugated + conjugated MHPG.

TABLE 34

DOPAMINE SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Bipolar–depressed*	*Bipolar–manic*	*Control*
Dopamine	641	Urine	324 ± 88 μg per 24 h	423 ± 46 μg per 24 h	
	725	Urine	185	344	
	643	Urine	153 ± 31	276 ± 81	
	645	Urine	193 ± 20	218 ± 19	
	157	Urine	520	710	
	157	Urine[a]	180	410	
		Urine weighted mean ± S.E.M.	232 ± 41 ($n=60$)	415 ± 42 ($n=28$)	
Homovanillic acid	485	CSF	22.7 ± 5.0 ng/ml	22.2 ± 6.2 ng/ml	
	673	CSF	26.5 ± 12.1	44.2	
	496	CSF	15.9 ± 2.0*	44.4 ± 3.8*	33.4 ± 1.0 ng/ml
	170[b](M)	CSF	32.4 ± 1.6	43.7 ± 5.4	43.7 ± 2.6
	170[b](F)	CSF	38.0 ± 2.1	59.0 ± 10.4**	40.0 ± 2.3
	518	CSF	31.8 ± 2.9	38.0 ± 5.0	28.6 ± 2.5
		CSF weighted mean ± S.E.M.	31.4 ± 2.6 ($n=160$)	40.6 ± 5.5 ($n=45$)	36.1 ± 2.1 ($n=13$)

* $p < 0.01$ *vs.* control.

** $p < 0.02$ vs. control.

[a] Conjugated dopamine (values not included in weighted mean).

[b] These values are also reported in refs. 509 and 633.

TABLE 35

COMPARISON OF PSYCHOTIC AND NON-PSYCHOTIC (NEUROTIC) DEPRESSED SUBJECTS

Amine or metabolite	*Reference*	*Biological fluid*	*Psychotic depressed*	*Non-psychotic*
Noradrenaline	149	Urine	195 ± 58 μg per 24 h	133 ± 16 μg per 24 h
	287	Plasma	366 ± 119 pg/ml	438 ± 49 pg/ml
Adrenaline	730	Urine	15.7 μg per 24 h*	6.6 μg per 24 h
	287	Plasma	97 ± 12 pg/ml	85 ± 10 pg/ml
Dopamine	730	Urine	280 μg per 24 h	212 μg per 24 h
	287	Plasma	282 ± 12 pg/ml**	51 ± 4 pg/ml
Normetanephrine	730	Urine	49.6 μg per 24 h	40.5 μg per 24 h
5-Hydroxy-indoleacetic acid	7	Urine	6.0 ± 1.2 mg per 24 h	6.0 ± 0.9 mg per 24 h
	746	Urine	3.0 ± 0.2 mg/g creatinine[a]	3.3 ± 0.2 mg/g creatinine[b]
	673	CSF	12.8 ± 5.0 ng/ml	15.9 ± 2.0 ng/ml
Homovanillic acid	746	Urine	3.3 ± 0.3 mg/g creatinime[a]	3.0 ± 0.3 mg/g creatinime[b]
	440	Plasma(M)	10.8 ± 1.0 ng/ml	12.3 ± 1.6 ng/ml
	440	Plasma(F)	21.1 ± 3.2	16.2 ± 2.1
	448	Plasma	11.4 ± 2.5	10.8 ± 3.1
	673	CSF	28.6 ± 7.5	27.3 ± 9.1
Vanilmandelic acid	730	Urine	3.52 mg per 24 h*	2.18 mg per 24 h
3,4-Dihydroxy-phenylacetic acid	730	Urine	1.56	1.02
3,4-Dihydroxy-mandelic acid	730	Urine	0.74	0.39
3-Methoxy-4-hydroxyphenyl glycol	746	Urine	2006 ± 216 μg/g creatinine[a]	1472 ± 145 μg/g creatinine[b]
	448	Plasma	3.36 ± 0.74 ng/ml	3.53 ± 0.65 ng/ml

* $p < 0.01$ *vs.* non-psychotic depressed.
** $p < 0.001$ *vs.* non-psychotic depressed.
[a] Delusional depressed.
[b] Non-delusional depressed.

TABLE 36

COMPARISON OF DEPRESSED SUBJECTS WITH AND WITHOUT MELANCHOLIA

Amine or metabolite	*Reference*	*Biological fluid*	*With melancholia*	*Without melancholia*
Noradrenaline	193	Urine	331 ± 54 μg per 24 h	211 ± 29 μg per 24 h
	284	Plasma	370 ± 39 ng/ml	
	662	CSF	123 ± 15	
Dopamine	193	Urine (total)	662 ± 118 μg per 24 h	716 ± 136 μg per 24 h
	662	CSF (sulfate)	679 ± 98 ng/ml	
5-Hydroxytryptamine	305	Plasma	0.85	
Normetanephrine	193	Urine	227 ± 21 μg per 24 h	145 ± 24 μg per 24 h
5-Hydroxy-indoleacetic acid	510	CSF	17.8 ± 0.8 ng/ml	
	466	CSF	15.0 ± 1.7	
	662	CSF	16.6 ± 1.5	19.3 ± 2.6 ng/ml
	736	CSF	25.8	
Homovanillic acid	193	Urine	3.89 ± 0.37 mg per 24 h	5.41 ± 0.65 mg per 24 h
	712	Plasma	18.9 ± 2.4 ng/ml	
	510	CSF	36.3 ± 2.0	
	662	CSF	20.1 ± 2.7*	33.1 ± 6.4 ng/ml
Vanilmandelic acid	193	Urine	5.84 ± 0.85 mg per 24 h	5.27 ± 0.55 mg per 24 h
3,4-Dihydroxy-phenylacetic acid	193	Urine	1100 ± 250 μg per 24 h	1475 ± 220 μg per 24 h
	662	CSF (unconjugated)	230 ± 29 pg/ml*	319 ± 45 pg/ml
	662	CSF (conjugated)	390 ± 48*	538 ± 65
3-Methoxy-4-hydroxyphenyl glycol	193	Urine (total)	1240 ± 172 mg per 24 h	929 ± 176 mg per 24 h
	440	Plasma(M)	3.3 ± 0.2 ng/ml	
	440	Plasma(F)	4.1 ± 0.4	
	27	Plasma	3.9 ± 0.5	
	712	Plasma	5.1 ± 0.6	
	510	CSF	9.4 ± 0.3	
	662	CSF	9.0 ± 0.8	8.1 ± 0.4 ng/ml
Phenylacetic acid	629	Urine	103 ± 16 mg per 24 h	98.5 ± 10.2 mg per 24 h

* $p < 0.05$ *vs.* subjects without melancholia.

TABLE 37

NORADRENALINE AND METABOLITES IN PANIC DISORDER

Amine or metabolite	*Reference*	*Biological fluid*	*Without panic disorder*	*With panic disorder*
Noradrenaline	150	Urine	12.3 ± 1.4 ng/min	38.3 ± 6.9* ng/min
	246	Plasma	288 ± 22 ng/ml	161 ± 14 ng/ml
Adrenaline	246	Plasma	13 ± 2.5 ng/ml	16 ± 3.2 ng/ml
3-Methoxy-4-hydroxyphenyl glycol	569	Urine	1607 ± 181 μg per 24 h	727 ± 59* μg per 24 h
	705	Urine	1871 ± 171 μg per 24 h	2439 ± 235** μg per 24 h
	591	Plasma	3.6 ± 0.3 ng/ml	3.9 ± 0.2 ng/ml
	599	Plasma	4.4 ± 0.3	3.3 ± 0.2*
	592	Plasma	3.6 ± 0.5	3.3 ± 0.3
	593	Plasma	3.7 ± 0.3	3.6 ± 0.2

* $p < 0.01$; subjects with panic disorder *vs.* those without panic disorder.
** $p < 0.05$; subjects with panic disorder *vs.* those without panic disorder.

Some authors have claimed a reduction in noradrenergic activity in panic disorder and related phobias based on findings of lower plasma MHPG levels[599] and lower urinary excretion of MHPG[569] and noradrenaline[246]. Other workers have reported an elevation of urinary MHPG[705], plasma MHPG[894] and noradrenaline excretion[150] in panic disorder, suggesting that it may be associated with increased noradrenergic activity, and still other investigators found no significant differences in plasma MHPG between subjects with panic disorder and those without[591–593]. A survey of the results of these studies is presented in Table 37.

Yu *et al.*[427] measured the plasma concentrations of the acidic metabolites of the trace amines and found significantly lower *p*-hydroxyphenylacetic acid (PHPA) in agoraphobic patients if compared with healthy controls, but not if compared with depressed controls.

Anxiety has been reported to be correlated with plasma[26,709,894] and urinary[570,731] MHPG and with 5-HIAA[531] and HVA[732] in CSF.

3.1.8. Suicide

A positive correlation has often been found between strong suicidal thoughts, suicide attempts and self-aggressivity and low concentrations of 5-HIAA in the CSF[466,505,506,531,733–735]. Those attempting suicide by violent means show significantly lower 5-HIAA in CSF than non-violent suicide attempters[465], and violent offenders who have a history of suicide attempts have been reported to have a significantly lower level of 5-HIAA in their CSF than those who have no such history[636]. It has been claimed that the distribution of concentrations of 5-HIAA in CSF is bimodal[531,667,678,680,745]; Åsberg *et al.*[678] found that subjects with the lower concentrations are more likely to commit suicide. Furthermore, patients with low 5-HIAA concentrations in the CSF had significantly higher scores in easily evoked anxiety, general anxiety, hostility and depressive inhibition[738]. However, in contrast to the above, a number of studies have shown that subjects who had made a suicide attempt were no more likely to have low concentrations of 5-HIAA in their CSF than non-suicidal subjects[498,736,737]. A trend to low HVA concentrations in the CSF of suicidal subjects has been noted[465,505,506,733]. No statistically significant differences in MHPG concentrations in the CSF of non-suicidal and suicidal patients have been observed[505,506,733], but the item "Suicidal Tendencies Worst Week" score from the *Schedule for Affective Disorders and Schizophrenia* (SADS) was highly significantly and negatively correlated with the MHPG level in CSF and only to a slight extent with 5-HIAA levels[747]. The score on the item "Seriousness of Intent of Worse Suicide Attempt" earlier in life correlated significantly and negatively with both MHPG and 5-HIAA[747]. Studies of 5-HIAA in CSF in depression and suicidal behaviour have been reviewed[620].

3.1.9. Correlation of metabolite concentrations with severity of depression

On the basis of the amine hypotheses of affective disorders, one would predict that there would be a correlation between the severity of the disorder as measured by psychometric rating scales and the concentrations of the amine metabolites in biological fluids, particularly in CSF. In fact, the CSF concentrations of 5-HIAA and HVA (but not MHPG) have been reported by some groups to be significantly negatively correlated with scores on the Hamilton Depression Rating Scale (HDRS)[662,742],

whereas others found MHPG levels to be correlated with symptom ratings but HVA and 5-HIAA levels were not[509,518], and some other groups found no correlation for any of the CSF metabolites with the severity of the depression[33,633,671,689]. An explanation for these contradictory results may be found in another study in which the authors observed no correlation of CSF metabolite concentration with global depression severity, but did observe correlations with some symptoms of depression[531,633]. The urinary excretion of MHPG has not shown any correlation with ratings on the HDRS[120,700,702,743,744], except in one study[582]. Monoamine metabolite concentrations predicted from the SADS symptom items compared well with the true CSF values[681] and various symptoms and descriptive variables on the SADS were correlated univariately with urinary MHPG[32]. Using a computer program based on these relationships, it was possible to classify 20 out of 21 unipolar and all bipolar subjects correctly[681]. Using the Bech–Rafaelson Melancholia Scale, a modification of the HDRS, no significant correlation with any CSF metabolite was found[736].

Some anxiety symptoms are correlated with the plasma concentration of MHPG, but measures of global and state anxiety were not[593]. Within individuals, MHPG excretion and state anxiety have been observed to co-vary highly significantly[731]. However, in a population of depressed individuals, state anxiety and urinary MHPG do not co-vary significantly, so it is not possible to predict that an individual with a high baseline state anxiety will have a high baseline urinary MHPG excretion, or *vice versa*[731]. Easily evoked and general anxiety scores were found to be negatively correlated with the 5-HIAA concentration in CSF[738], whereas the HVA concentration has been reported to be positively correlated with anxiety[732]. Patients scoring high on anxiety and hostility in the Rorschach test had low CSF concentrations of 5-HIAA[510].

Phenylacetic acid excretion, which has been reported to be low in unipolar depression by some workers[416,417,629,663], but not by others[72,352], does not correlate with either the HDRS or the Carroll Rating Scale[72].

Probenecid, which inhibits the active transport of amine metabolites from the CSF to the blood, has been used to assess the central turnover of the monoamines in depression. Probenecid-induced accumulations of 5-HIAA[470,471,480,482,493] and HVA[480,493,499] in the CSF of depressed subjects have been shown to be significantly smaller than the accumulations in control subjects. The differences appeared to correlate with the depressive state[471]. A significant increase in MHPG concentrations can be attained only at very high doses of probenecid, and even at high probenecid doses MHPG sulfate concentration does not increase, suggesting that a probenecid-sensitive transport mechanism for MHPG sulfate does not occur[494]. At the usual doses of probenecid, MHPG accumulation is too meagre to be used for the assessment of noradrenaline turnover[493]. Mania and high levels of anxiety have been associated with a greater accumulation of noradrenaline after probenecid administration than either controls or depressed subjects, indicating that alterations in mood may be associated with changes in central noradrenaline metabolism[726]. In depressed patients suffering from severe motor retardation, central dopamine turnover is diminished, as evidenced by a sub-normal accumulation of HVA in the CSF after probenecid administration[499,759].

In the dexamethasone suppression test, induction by dexamethasone of plasma MHPG concentration increases have been directly correlated with the severity of

depressive symptoms[446,448,710,711,760]. Dexamethasone-resistant depressed patients showed elevated plasma noradrenaline and adrenaline levels[761] and urinary 3,4-dihydroxyphenyl glycol excretion[711]. No difference in MHPG concentrations in urine[744] or CSF[653] between dexamethasone suppressors and non-suppressors has been reported.

3.1.10. Effects of drugs and treatments

3.1.10.1. Effects of anti-depressant drugs on metabolite concentrations. These effects are shown in Table 38.

In addition to the studies on anti-depressant drugs, a study on anti-anxiolytic drugs demonstrated that urinary MHPG declined significantly ($p < 0.01$) after long-term use of chlordiazepoxide, clobazam, diazepam, nitrazepam, and oxazepam[570]. After withdrawal of treatment, urinary MHPG increases.

3.1.10.2. Metabolite values as predictor of response to drugs. For clinicians, a biochemical test predictive of a patient's therapeutic response to a drug would be a very useful treatment tool. Several investigators have demonstrated that pretreatment concentrations of MHPG or 5-HIAA in urine or CSF may be used as a basis for selection of anti-depressant drug therapy for depressed patients, and that this method of drug selection produces better clinical results than traditional selection methods. The differential responses of depressed patients to drug treatments permitted their classification as responders or non-responders. Statistically significant differences in the concentrations of some monoamines and their metabolites between the two groups were noted.

Low baseline urinary excretion of MHPG has been observed in patients who respond well to imipramine[686], L-deprenyl[635], D-amphetamine[560,698], nortriptyline[582,741] and desipramine[741], whereas subjects excreting large amounts of MHPG did not respond to treatment with these drugs. On the other hand, patients with high initial urinary MHPG levels responded well to alprazolam[751] and amitriptyline[700,741], whereas those with low concentrations did not. Other groups of workers, however, have reported that pretreatment urinary MHPG levels were not a predictor of response to amitriptyline[477,686,702]. The results of studies on the predictive value of pretreatment MHPG concentrations in CSF have been contradictory. High[716] and low[649] pretreatment values of MHPG in CSF have been claimed to predict which patients will respond to imipramine[649,716] and amitriptyline[649], and other groups have reported that MHPG in CSF is not a predictor of response to imipramine[688], amitriptyline[686,688], desimipramine[685] or femoxetine[685]. High urinary noradrenaline excretion may be a predictor of a positive response to alprazolam[651], whereas low excretion is a predictor of response to moclobemide[149].

A positive response to iproniazid[366,756], isocarboxazid[366] and *p*-chloro-N-methylamphetamine[163] has been predicted on the basis of low pretreatment concentrations of urinary 5-HIAA. Similarly, low pretreatment levels of 5-HIAA in CSF have been reported to be useful in predicting a positive response to treatment with imipramine[686] and zimelidine[682], and higher CSF concentrations of 5-HIAA predict a positive response to nortriptyline[667]. Some investigators have reported that 5-HIAA and HVA in CSF are not useful as predictors of response to desimipramine[682], amitriptyline[631,686,688] or imipramine[631,688] treatment. For depressed patients treated with a variety of anti-depressant drugs and grouped according to their responses, it

TABLE 38

EFFECTS OF ANTI-DEPRESSANT DRUGS ON METABOLITE CONCENTRATIONS

Drug	*Reference*	*Biological fluid*	*Category of depressive disorder*	*Effect on amines or metabolite*[a]
Alprazolam	751	Urine	Endogenous	MHPG (effect not stated)
	752	Plasma	Healthy normals	MHPG↓ ($p < 0.001$)
	651	Urine	Not classified	NA↓ ($p < 0.01$); A↓ ($p < 0.002$); NMN↓ ($p < 0.05$); VMA↓ ($p < 0.05$); MHPG↓ ($p < 0.05$)
	755	Plasma	Panic	MHPG↓
Amitriptyline	485	CSF	Not classified	5-HIAA↓ ($p < 0.02$); HVA↓ (N.S.)
	631	CSF	Unipolar + bipolar	5-HIAA↓ ($p < 0.05$); HVA↓ (N.S.)
	741	Urine	Unipolar + bipolar	MHPG↑ (low excreters); ↓ (high excreters)
	700	Urine	Bipolar	MHPG↑ ($p < 0.05$)
	659	Plasma	Severe depression	NA↓ ($p < 0.01$)
	707	Plasma	Endogenous	MHPG↓ ($p < 0.05$)
	688	CSF	Unipolar + bipolar	MHPG↓ ($p < 0.001$); 5-HIAA↓ ($p < 0.001$); HVA (N.S.)
	650	Urine	Unipolar	NA (N.S.); A (N.S.); VMA (N.S.); MHPG↓ ($p < 0.001$); NMN↓ ($p < 0.001$); MN ($p < 0.001$)
	650	Urine	Bipolar	NA (N.S.); A (N.S.); NMN (N.S.); MHPG↓ ($p < 0.001$); VMA↓ ($p < 0.001$); MN↓ ($p < 0.001$)
	736	CSF	Endogenous	MHPG (N.S.); 5-HIAA (N.S.); HVA (N.S)
D-Amphetamine	560	Urine	Endogenous	MHPG↑ (significant) (responders); ↓(N.S.) (non-responders)
	698	Urine	Endogenous	MHPG↓ ($p < 0.05$)
Bupropion	753	CSF	Not classified	HVA (N.S.); 5-HIAA (N.S.)
	694	CSF	Unipolar + bipolar	MHPG↓ (N.S.); HVA↓ (N.S.)
	694	Plasma	Unipolar + bipolar	HVA↑ (good responders); N.S. (poor responders)

(*Continued on p. 140*)

TABLE 38 (*continued*)

Drug	*Reference*	*Biological fluid*	*Category of depressive disorder*	*Effect on amines or metabolite*[a]
Chlorimipramine	679	CSF	Endogenous	5-HIAA↓ ($p < 0.001$); MHPG↓ ($p < 0.001$); HVA (N.S.)
	305	Plasma	Melancholia	5-HIAA↓ (N.S.); 5-HT↓ (N.S.); IAA (N.S.)
p-Chloro-N-methylamphetamine	163	Urine	Vital	5-HT↑ ($p < 0.01$); 5-HIAA↑ ($p < 0.05$)
Clonidine	587	Plasma	Endogenous	MHPG↓ ($p < 0.05$)
	894	Plasma	Panic, anxiety	MHPG↓ ($p < 0.002$)
	255	Plasma	Unipolar	NA↓ (N.S.); MHPG↓ ($p < 0.05$)
	255	Plasma	Bipolar	NA ↓ (N.S.); MHPG↓ (N.S.)
	604	Plasma	Not classified	DHPG↓ (N.S.)
Clorgyline	450	CSF	Not classified	HVA↓ ($p < 0.02$); MHPG↓ ($p < 0.001$); 5-HIAA↓ ($p < 0.02$); VMA (N.S.); DOPAC (N.S.)
	639	Urine	Bipolar	NA (N.S.); NMN↑ ($p < 0.01$); VMA↓ ($p<0.05$); MHPG↓ ($p < 0.05$); DA (N.S.); 3-MT↑ ($p < 0.05$); HVA↓ ($p < 0.05$); 5-HIAA↓ (N.S.); PE↓ (N.S.); pTA↑ ($p < 0.05$); pHPA↓ (N.S.)
	658	Urine	Unipolar + bipolar	DA↓ (N.S.); HVA↓ ($p < 0.01$); DOPAC↓ (N.S.);
	753	CSF	Not classified	5-HIAA↓ (significant); HVA↓ (significant)
	690	CSF	Not classified	MHPG↓ ($p < 0.001$); HVA↓ ($p < 0.01$); 5-HIAA↓ ($p < 0.01$)
L-Deprenyl	635	Urine	Atypical	PE↑ ($p < 0.05$); MHPG↓ ($p < 0.05$)
	753	CSF	Not classified	HVA↓ ($p < 0.005$); 5-HIAA↓
Desimipramine	741	Urine	Unipolar + bipolar	MHPG↑
	703	CSF	Unipolar + bipolar	MHPG↓ ($p < 0.05$)
	703	Urine	Unipolar + bipolar	MHPG↓ ($p < 0.01$)
	28	Plasma	Endogenous	MHPG↓ ($p < 0.002$)
	28	Urine	Endogenous	MHPG (N.S.)
	690	CSF	Not classified	MHPG↓ ($p < 0.001$); HVA↓ (N.S.); 5-HIAA↓ ($p < 0.01$)

	658	Urine	Unipolar + bipolar	DA↓ (N.S.); HVA↓ (N.S.); DOPAC↓ (N.S.)
	685	CSF	Not classified	5-HIAA↑ ($p < 0.02$); MHPG (N.S.); HVA (N.S.)
	753	CSF	Not classified	5-HIAA↓ (significant); HVA (N.S.)
Femoxetine	685	CSF	Not classified	5-HIAA↑ ($p < 0.05$); MHPG (N.S.); HVA (N.S.)
Fluvoxamine	318	Plasma	Healthy normals	Melatonin↑ ($p < 0.05$)
Imipramine	665	Urine	Endogenous	VMA↓ ($p < 0.01$); NMN↑ ($p < 0.05$)
	754	Urine	Endogenous	NMN↑
	664	Urine	Endogenous	VMA↓ ($p < 0.05$)
	118	Urine	Primary depression	MHPG↓ ($p < 0.05$); VMA (N.S.); NMN (N.S.); MN (N.S.); TRA↑ ($p < 0.025$); 5-HIAA (N.S.)
	631	CSF	Unipolar + bipolar	5-HIAA↓ ($p < 0.05$)
	676	CSF	Psychotic depression	HVA↑ (N.S.); 5-HIAA↑ (N.S.)
	741	Urine	Unipolar + bipolar	MHPG↑
	716	CSF	Endogenous	MHPG↓ (N.S.)
	688	CSF	Unipolar + bipolar	MHPG↓; 5-HIAA↓; HVA (N.S.)
	709	Plasma	Agoraphobia	MHPG↓ ($p < 0.001$)
	301	Plasma	Not classified	5-HT↑ ($p < 0.02$)
	650	Urine	Unipolar	NA↓ (N.S.); A↓ (N.S.); VMA↓ ($p < 0.01$); NMN↓ ($p < 0.001$); MN↓ ($p < 0.001$); MHPG↓ ($p < 0.001$)
	650	Urine	Bipolar	NA↑ (N.S.); A↑ (N.S.); VMA↓ ($p < 0.01$); NMN↑ (N.S.); MN↑ (N.S.); MHPG (N.S.)
Iproniazid	756	Urine	Not classified	5-HIAA↓ (N.S.)
	366	Urine	Melancholia, neurotic	5-HIAA↑ ($p < 0.001$)
Isocarboxazide	366	Urine	Melancholia, neurotic	5-HIAA↑ ($p < 0.001$)
	736	CSF	Endogenous	5-HIAA↓ (N.S.); MHPG↓ ($p < 0.01$); HVA↓ ($p < 0.001$)
Lithium carbonate	485	CSF	Manic	5-HIAA↓ (N.S.); HVA↓ (N.S.)
	157	Urine	Manic	DA↓ ($p < 0.01$); conjugated DA↓ ($p < 0.01$); VMA (N.S.)
	157	Urine	Depressed	DA↓ (N.S.); conjugated DA↑ (N.S.); VMA (N.S.)

(*Continued on p. 142*)

TABLE 38 (*continued*)

Drug	*Reference*	*Biological fluid*	*Category of depressive disorder*	*Effect on amines or metabolite*[a]
	25	Urine	Unipolar + bipolar	MHPG (acute, N.S.) MHPG↑ (chronic, N.S.)
	675	CSF	Manic	5-HIAA↑ ($p < 0.001$); HVA↑ ($p < 0.05$)
	698	Urine	Unipolar + bipolar	MHPG↑ (N.S.)
	659	Plasma	Bipolar, neurotic, melancholic	NA↑ ($p < 0.05$)
	658	Urine	Unipolar + bipolar	DA↓ (N.S.); DOPAC↓ (N.S.); HVA↓ ($p < 0.001$)
	332	CSF	Euthymic bipolar	5-HIAA↑ ($p < 0.05$); DOPAC↑ (N.S.); HVA↑ (N.S.); NA↑ (N.S.); DA·SO_4↓ (N.S.); MHPG↑ (N.S.)
Moclobemide	149	Urine	Unipolar	NA↓ (significant); A↓ (N.S.); DA↓ (N.S.)
Nortriptyline	667	CSF	Endogenous	5-HIAA↓ ($p < 0.005$); IAA↓ ($p < 0.05$)
	741	Urine	Unipolar + bipolar	MHPG↑
	27	Urine	Unipolar + bipolar	MHPG↓ (N.S.)
Pargyline	450	CSF	Not classified	HVA↓ ($p < 0.01$); MHPG↓ ($p < 0.01$); VMA↓ (N.S.); DOPAC↓ (N.S.); 5-HIAA↓ ($p < 0.05$)
Phenelzine	664	Urine	Endogenous	VMA↓ ($p < 0.01$)
	672	CSF	Not classified	HVA↓ ($p < 0.001$); 5-HIAA↓ (N.S.)
	646	Urine	Not classified	TRA↑ ($p < 0.01$); NA↑ (N.S.); A↑ (N.S.); NMN↑ ($p < 0.05$); MHPG↓ ($p < 0.05$); VMA↓ ($p < 0.05$)
	757	Plasma	Dysthymic	PE↑
	757	Urine	Dysthymic	PE↑
Timolol	271	Plasma	Healthy normal	NA↑ (basal, $p < 0.05$); ↓ (exercize, p, < 0.05); DA (N.S.)
Tranyl cypromine	211	Plasma	Not classified	PE↑ (significant)
	211	Urine	Not classified	PE↑ (N.S.)

Yohimbine	591	Plasma	Healthy normal	MHPG↑ ($p < 0.001$)
	591	Plasma	Agoraphobia	MHPG↑ ($p < 0.001$)
	752	Plasma	Healthy normal	MHPG↑ ($p < 0.05$)
	604	Plasma	Not classified	DHPG↑; (+ desimipramine: DHPG↓)
	755	Plasma	Panic	MHPG↑
Zimelidine	668	CSF	Not classified	5-HIAA↓ ($p < 0.05$); IAA↑ ($p < 0.05$); MHPG↓ ($p < 0.05$); HVA↓ (N.S.)
	703	CSF	Unipolar + bipolar	MHPG↑ (N.S.)
	703	Urine	Unipolar + bipolar	MHPG↓ ($p < 0.01$)
	648	Urine	Unipolar + bipolar	MHPG↓ ($p < 0.05$); NA↓ (N.S.); NMN↓ (N.S.); VMA↓ (N.S.)
	658	Urine	Unipolar + bipolar	HVA↓ (N.S.); DA↑ (N.S.)
	753	CSF	Not classified	HVA↑ (N.S.); 5-HIAA↓ (significant)
	690	CSF	Not classified	MHPG↓ ($p < 0.02$); 5-HIAA↓ ($p < 0.01$); HVA (N.S.)
Electroconvulsive therapy	671	CSF	Endogenous	5-HIAA (N.S.); HVA (N.S.)
	676	CSF	Psychotic depression	5-HIAA↑ ($p < 0.01$); HVA ↑($p < 0.01$)
	677	CSF	Endogenous	5-HIAA↑ (N.S.); HVA↑ (N.S.);
	758	CSF	Psychotic depression	MHPG↓ (significant); 5-HIAA (N.S.); HVA (N.S.)
	658	Urine	Unipolar + bipolar	DA↓ ($p < 0.05$); DOPAC↑ (N.S.); HVA↓ (N.S.)
	708	Plasma	Not classified	MHPG↑ (N.S.); HVA↓ (N.S.); 5-HIAA↓ (N.S.)
	689	CSF	Unipolar + bipolar	5-HIAA↑ (N.S.); HVA↑ (N.S.)
	736	CSF	Endogenous	5-HIAA↑ (N.S.); HVA↑ (N.S.); MHPG↑ (N.S.)

[a] For abbreviations, see text; also: A = adrenaline; 3MT = 3-methoxytyramine. N.S. = not significant. ↑, Concentration increases during drug treatment; ↓, concentration decreases during drug treatment.

was observed that CSF monoaminergic metabolite concentrations in responders correlated well with each other, but did not correlate in non-responders[693]. This suggests that interactions between monoamine systems in non-responders may be disrupted.

3.1.10.3. Correlation of changes in metabolite levels with therapeutic response. If a dysfunction in noradrenergic (or other monoaminergic) activity is associated with depression, as has been postulated, a clinical recovery after drug treatment might be expected to be correlated with changes in amine metabolite concentrations. Several studies have been devoted to establishing such correlations, but most have not demonstrated significant correlations. In CSF, after ECT[671,677] or treatment with lithium[485], amitriptyline[630,688], desimipramine[685], femoxetine[685] or imipramine[688], changes in 5-HIAA concentrations were found not to be correlated with clinical improvement. Similarly for HVA in CSF, no correlation of changes in concentration with changes in psychometric ratings were reported after treatment of depressed patients with ECT[671,677], amitriptyline[630,688], desimipramine[685], femoxetine[685] or imipramine[688]. Changes in the MHPG concentration in CSF after imipramine[688], amitriptyline[688], desimipramine[685] or femoxetine[685] treatment also did not reflect improvements in clinical condition. However, manic scores have been reported to increase after lithium treatment of manic patients in a manner which parallels the increase in the CSF concentrations of 5-HIAA and HVA[675]. Changes in the plasma concentrations of MHPG[28,708] after treatment with desimipramine[28] or ECT[708] and of noradrenaline and serotonin after amitriptyline or lithium carbonate[659] do not correspond with clinical improvement. Plasma HVA and 5-HIAA concentrations also do not change in accord with clinical improvement following ECT[708], and patients improved after treatment with bupropion do not show any comparable change in plasma HVA levels[694]. However, in subjects suffering from panic attacks, a reduction in the frequency and severity of the attacks following clonidine or imipramine treatment is reflected in a corresponding reduction in plasma MHPG[894], whereas treatment with yohimbine, an adrenergic receptor antagonist which increases noradrenergic function, produces an increase in the frequency of panic attacks and a corresponding increase in plasma MHPG[591]. Changes in urinary MHPG excretion have been shown to be correlated with clinical response after treatment with imipramine[118], but not after treatment with desimipramine[648], zimelidine[648], L-deprenyl[635] and D-amphetamine[698]. However, Beckmann *et al.*[698] have shown that patients on amphetamine who exibited markedly increased psychomotor activity and behaviourally rated hypomania also exhibited elevations of MHPG excretion, whereas those without such responses exhibited reductions of MHPG. Acute lithium treatment was not associated with a change in urinary MHPG excretion which correlated with psychometric ratings, but chronic treatment was so correlated[25]. It was concluded that change in the clinical state is the most important variable contributing to MHPG changes. In a study of the effects of amitriptyline and imipramine on the noradrenergic system, a differential effect between responders and non-responders and between unipolar and bipolar patients on changes in the urinary concentrations of the metabolites of noradrenaline was noted[650]. Other workers have reported changes in urinary VMA concentration which are correlated with depression rating scale score changes following treatment with imipramine[118,665] or which are not correlated when treatment is with desimipramine[648]. Normetanephrine excretion behaves similarly to VMA in response to treatment with imipramine[665,754] and desipramine[648].

Noradrenaline excretion does not change with changes in clinical condition after alprazolam treatment[651]. Treatment with iproniazid has been claimed to produce a clinical recovery which is synchronous with an elevation of 5-HIAA excretion[366]. After administration of L-deprenyl, the improved clinical condition is not correlated with a corresponding change in phenylethylamine excretion[635].

3.2. Schizophrenia

3.2.1. Introduction

A hypothesis claiming hyperactivity of dopaminergic pathways in schizophrenia has been postulated in recent years and is supported by evidence that high doses of dopamine-releasing drugs may precipitate a schizophrenia-like psychosis even in normal subjects, and that neuroleptics which ameliorate schizophrenic symptoms are known to block dopamine receptors with a potency that parallels their therapeutic efficacy[762,870]. In contradiction, a recent report has claimed low dopamine activity in chronic schizophrenia[893].

Noradrenaline and serotonin[881] hypotheses of schizophrenia have been presented, but in a review Rodnight[762] has demonstrated that in the research carried out up to 1983 no consistent abnormalities have emerged. One of the earliest models of schizophrenia concerned the indoleamines and a transmethylation hypothesis in which the product would be the psychedelic N,N-dimethyltryptamine[763–765] of bufotenin[969]. Inadequate specificity and sensitivity of the isolation and quantification procedures initially seemed to lend support to this theory, but more recent work using more sophisticated methodology has shown at best only a trend to elevated urinary dimethyltryptamine excretion in schizophrenics[343,763–765]. However, a highly significant elevation in the urinary excretion of bufotenin in psychotics has recently been reported, although there was no correlation between the level of bufotenin excretion and severity of the disorder[969]. Another methylated product, 3,4-dimethoxyphenylethylamine, has been claimed to be found in greater amounts in schizophrenics than in normal controls[180,183,771], but the incidence has proved to be low and excretion inconsistent[179,180]. A third hypothesis stems from the close structural similarity between phenylethylamine and amphetamine, the similar behavior in rats induced by both compounds and the similarity with paranoid schizophrenic symptoms exhibited by drug abusers overdosed on amphetamine[624,625]. The weighted means of the results of studies on schizophrenia are presented in Tables 39–46. The subjects have not been differentiated according to clinical sub-type of schizophrenia.

3.2.2. Tables of values for schizophrenic subjects (all sub-types combined)

In aggregate, the studies reported in Tables 39–46 do not provide firm support for any of the amine hypotheses of schizophrenia. Dopamine concentration, although apparently markedly higher in the urine of schizophrenics, is lower in plasma and in CSF is no different from controls. HVA is higher in schizophrenic plasma, but not different from controls in urine or CSF. The noradrenaline concentration is elevated in the plasma and CSF of schizophrenics, as is the MHPG concentration in plasma, but this may not be of aetiological significance as it has been suggested that the high state of arousal often observed in schizophrenics may account for noradrenergic abnormalities in plasma and CSF[143,762]. No serotonergic abnormality is apparent

TABLE 39

UNCONJUGATED BIOGENIC AMINES IN URINE

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*	
			Schizophrenics	*Normals*[a]
Phenylethylalmine	89, 100, 101, 102, 105	157	11.3 ± 2.6	8.1 ± 1.8
m-Tyramine	102	23	44 ± 11	83 ± 10
p-Tyramine	102, 113	51	481 ± 97	579 ± 75
Tryptamine	117, 766, 767	59	115 ± 7	103 ± 19
Noradrenaline	131, 143, 148, 652, 768	113	46 ± 6	40 ± 4
Adrenaline	131, 143, 148, 652	128	13.2 ± 2.6	10.1 ± 1.2
Dopamine	143, 148, 768, 893	58	446 ± 61	263 ± 34
5-Hydroxytryptamine	769	22	51	120 ± 23
Normetanephrine	770, 893	42	242, 110	194 ± 17
Metanephrine	770	22	9.6	95 ± 9
3,4-Dimethoxy-phenylethylamine	179, 180, 183, 771	41	1.6 ± 0.4	0.35
N,N-Dimethyl-tryptamine	656	26	1.2	0.38 ± 0.12
N-Methyltryptamine	656	26	0.7	0.86 ± 0.22
Bufotenin	186	26	1.1	1.71
	969	75	1.88	0.37
			μg/g creatinine	μg/g creatinine

[a] Weighted normal means taken from Table 1.

TABLE 40

CONJUGATED BIOGENIC AMINES IN URINE

Amine	*Reference*	*Total No. of subjects*	*Weighted mean ± S.E.M. (μg per 24 h)*	
			Schizophrenics	*Normals*[a]
Noradrenaline	770	22	6.5	139 ± 37
Dopamine	770	22	446	785 ± 167
Normetanephrine	770	22	154	183 ± 23
Metanephrine	770	22	55	13.4 ± 2.7

[a] Weighted normal means taken from Table 2.

from these tables. Although phenylethylamine excretion is higher in schizophrenics, its concentration in plasma appears to be lower. No consistent trends for the major metabolite, phenylacetic acid, are observed.

A study of each of the references cited in the tables for comparisons of metabolite levels in controls and schizophrenics reveals no consistent differences, except for an elevation of noradrenaline in urine, plasma and CSF. As schizophrenia is not a single disease entity but a biologically heterogeneous collection of possibly distinct sub-types, this may explain some of the discrepancies reported for metabolite concentrations. Tables 47–50 summarize data obtained from several laboratories for different clinical types of schizophrenia.

3.2.3. Comparison of chronic and acute schizophrenic subjects

Comparisons of chronic and acute schizophrenic subjects are presented in Tables 47 and 48.

3.2.4. Comparison of paranoid and non-paranoid schizophrenic subjects

Comparisons of paranoid and non-paranoid schizophrenic subjects are presented in Tables 49 and 50.

3.2.5. Metabolite values as predictor of severity of disorder

Although an early study on the relationship between metabolite concentrations in the CSF of schizophrenic patients and diagnostic assessment of the severity of the disorder found no correlation[724], recent investigations have revealed a high positive correlation of plasma HVA (before treatment) with the global severity of the illness in schizophrenic patients[19,443,445,784,799,801]. However, it has also been claimed that plasma MHPG gives a better correlation with rated psychosis than does plasma HVA[802]. A positive correlation between the CSF concentration of 5-HIAA and the score on the Brief Psychiatric Rating Scale for schizophrenic behaviour has been reported[515]. MHPG excretion is not related to the severity of an acutely schizophrenic illness[807], but the sulfate conjugate has been reported to be strongly negative-

TABLE 41

UNCONJUGATED BIOGENIC AMINES IN PLASMA

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*	
			Schizophrenics	*Normals*[a]
Phenylethylamine	212	14	74 ± 24	124 ± 27
Noradrenaline	143, 148, 254, 280, 772, 773, 774	223	458 ± 56	275 ± 32
Adrenaline	143, 280	74	55 ± 4	63 ± 11
Dopamine	143, 280	74	49 ± 5	86 ± 15
5-Hydroxytryptamine	364, 359	200	74 ± 5[b] (ng/ml)	387 ± 84[c] (pg/ml)

[a] Weighted normal means taken from Table 3.
[b] Whole blood.
[c] Ultrafiltrate.

TABLE 42

UNCONJUGATED AND CONJUGATED BIOGENIC AMINES IN CEREBROSPINAL FLUID

Amine	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (pg/ml)*	
			Schizophrenics	*Normals*[a]
Phenylethylamine	455	15	45 ± 2	600 ± 100
Noradrenaline	21, 143, 326, 327, 328, 329, 331, 334, 345, 661, 734, 775, 777	327	162 ± 17	119 ± 16
Adrenaline	143	8	14.2 ± 1.8	47 ± 23
Dopamine	143	8	55 ± 1.4	48 ± 14
Dopamine (conjugated)	345	46	673 ± 47	594 ± 108
Normetanephrine	775	13	360 ± 200	1800 ± 420
3-Methoxytyramine	775	13	n.d.	635 ± 184

[a] Weighted normal means taken from Tables 5 and 6.

TABLE 43

UNCONJUGATED AND CONJUGATED ACID METABOLITES IN URINE

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (mg per 24 h)*	
			Schizophrenics	*Normals*[a]
Phenylacetic	352	23	11.0 ± 2.8	8.5 ± 1.7
Phenylacetic (conjugated)	105, 352	62	113 ± 16	143 ± 16
Indoleacetic	354, 766, 767	26	8.6 ± 0.8	10.2 ± 1.3
5-Hydroxyindole-acetic	131, 354, 358, 359, 360, 364 365, 767, 778, 779, 893	370	5.4 ± 0.7	4.5 ± 0.4
Homovanillic	354, 778, 779, 780, 893	64	3.9 ± 0.4	4.8 ± 0.3
Vanilmandelic	131, 407, 768, 778, 779, 780, 893	168	3.7 ± 0.3	4.1 ± 0.2
3,4-Dihydroxyphenylacetic	893	20	0.9 ± 0.1	1.9 ± 0.2
3,4-Dimethoxyphenylacetic	180, 781	23	0-27 μg/g creatinine	(trace)

[a] Weighted normal means taken from Tables 7 and 8.

TABLE 44

UNCONJUGATED AND CONJUGATED ACID METABOLITES IN PLASMA

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Schizophrenics	*Normals*[a]
Phenylacetic	425	42	149 ± 17	124 ± 14
Phenylacetic (conjugated)	425	42	423 ± 46	345 ± 35
m-Hydroxyphenylacetic	425	42	7.7 ± 1.2	13.4 ± 2.2
m-Hydroxyphenyl-acetic (conjugated)	425	42	0.9 ± 0.4	2.1 ± 1.1
p-Hydroxyphenylacetic	425	42	57.0 ± 6.8	69.0 ± 8.8
p-Hydroxyphenyl-acetic (conjugated)	425	42	37.6 ± 8.0	13.6 ± 4.3
Homovanillic	438, 442, 443, 445, 782, 783, 784, 801, 802, 803, 804, 805	198	13.2 ± 1.3	9.7 ± 0.9
3,4-Dihydroxyphenylacetic	801	15	2.4 ± 0.4	3.5 ± 0.5

[a] Weighted normal means taken from Tables 9 and 10.

TABLE 45

UNCONJUGATED AND CONJUGATED ACID METABOLITES IN CEREBROSPINAL FLUID

Acid metabolite	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (ng/ml)*	
			Schizophrenics	*Normals*[a]
Phenylacetic	336, 455, 456, 785	59	18.4 ± 2.6	24.0 ± 3.1
Phenylacetic (conjugated)	336, 455	26	20.2 ± 5.6	24.5 ± 4.5
p-Hydroxyphenylacetic	458,786	22	5.8 ± 0.7	8.8 ± 1.4
Indoleacetic	461	17	4.1 ± 0.1	4.4 ± 0.6
p-Hydroxymandelic	786	12	3.0 ± 0.4	1.3 ± 0.4
5-Hydroxyindoleacetic	18, 131, 328, 334, 345, 485, 503, 507, 508, 512, 515, 518, 626, 661, 669, 676, 687, 732, 758, 776, 777, 787, 788, 789, 790, 791, 791,792	619	24.5 ± 2.8	26.2 ± 2.2
Homovanillic	18, 131, 328, 345, 485, 503, 507, 508, 512, 515, 518, 534, 661, 676, 687, 695, 732, 758, 773, 775, 776, 777, 786, 787, 788, 789, 790, 791, 794, 795, 796, 797, 798, 799, 801	803	39.1 ± 4.2	40.7 ± 4.3
Homovanillic (conjugated)	503	7	0.20 ± 0.40	0.25 ± 0.58
Vanilmandelic	541, 775, 786	37	1.64 ± 0.37	0.98 ± 0.16
3,4-Dihydroxy-phenylacetic	331, 345, 503, 513, 775, 777, 786 (see also 801)	132	0.49 ± 0.06	0.49 ± 0.04
3,4-Dihydroxyphenylacetic (conjugated)	503	7	0.10 ± 0.10	0.22 ± 0.10

[a] Weighted normal means taken from Tables 11 and 12.

TABLE 46

UNCONJUGATED AND CONJUGATED ALCOHOL AND GLYCOL METABOLITES IN URINE, PLASMA AND CEREBROSPINAL FLUID

Glycol or alcohol	*References*	*Total No. of subjects*	*Weighted mean ± S.E.M. (normals[a])*
3-Methoxy-4-hydroxyphenyl glycol (unconjugated)	442, 588, 782, 801, 802, 803	207	4.18 ± 0.26 (ng/ml plasma) (3.55 ± 0.31)
3-Methoxy-4-hydroxyphenyl glycol (conjugated)	10, 131, 407, 565, 704, 893	179	1448 ± 315 (μg per 24 h urine) (1866 ± 165)
3-Methoxy-4-hydroxy-phenylethanol	775	13	340 ± 230 (pg/ml CSF) (5.7 ± 0.9) (ng/ml)
3-Methoxy-4-hydroxyphenyl glycol (unconjugated)	131, 345, 503, 507, 508, 515, 518, 538, 661, 758, 773, 775, 776, 789, 790, 791, 797, 800, 801	464	9.91 ± 1.04 (ng/ml CSF) (10.6 ± 1.2)
3-Methoxy-4-hydroxyphenyl glycol (conjugated)	503, 800	26	2.3 ± 0.4 (ng/ml CSF) (0.51 ± 0.10)

[a] Weighted normal means in parentheses taken from Tables 14, 15, 17 and 18.

TABLE 47

BIOGENIC AMINES

Amine	*Reference*	*Biological fluid*	*Chronic*	*Acute*	*Control*
Noradrenaline	143	Urine	38.7 ± 3.0 μg per 24 h	35.4 ± 1.4 μg per 24 h	37.2 ± 0.7 μg per 24 h
	143	Plasma	260 ± 18 pg/ml	265 ± 14* pg/ml	230 ± 10 pg/ml
	143	CSF	147 ± 9	159 ± 18	125 ± 6
	328	CSF	259 ± 148*	216 ± 16**	207 ± 11
Adrenaline	143	Urine	12.5 ± 1.0 μg per 24 h	11.7 ± 0.5 μg per 24 h	11.2 ± 0.5 μg per 24 h
	143	Plasma	38.1 ± 3.2 pg/ml	35.9 ± 1.9 pg/ml	37.0 ± 2.0 pg/ml
	143	CSF	15.4 ± 2.8	12.1 ± 0.6	12.2 ± 0.8
Dopamine	143	Urine	320 ± 16 μg per 24 h	292 ± 7 μg per 24 h	292 ± 11 μg per 24 h
	143	Plasma	59.3 ± 6.1 pg/ml	50.2 ± 3.3 pg/ml	55.2 ± 3.7 pg/ml
	143	CSF	54.3 ± 1.8	55.9 ± 2.6	53.5 ± 2.0

* $p < 0.05$ *vs.* control.
** $p < 0.05$ *vs.* chronic.

TABLE 48

ACID AND GLYCOL METABOLITES

Acid or glycol metabolite	*Reference*	*Biological fluid*	*Chronic*	*Acute*	*Control*
5-Hydroxy-indoleacetic acid	358	Urine	5.3 ± 0.4 mg per 24 h	5.5 ± 0.9 mg per 24 h	5.2 ± 0.3 mg per 24 h
	131	Urine	3.8 ± 0.4	2.6 ± 0.4	4.4 ± 0.9
	328	CSF	34.8 ± 3.1 ng/ml	31.4 ± 1.6 ng/ml	35.5 ± 2.1 ng/ml
	131	CSF	28.4 ± 2.2	20.6 ± 2.8	40.6 ± 4.2
	626	CSF	16.4 ± 1.1	10.9 ± 0.9*	17.4 ± 2.0
Homovanillic acid	131	CSF	42.4 ± 4.8	41.4 ± 4.2	42.4 ± 3.8
	328		ng/ml	ng/ml	ng/ml
		CSF	33.4 ± 3.0	28.8 ± 2.8	33.1 ± 2.3
Vanilmandelic acid	131	Urine	4.2 ± 0.4 mg per 24 h	3.6 ± 0.4 mg per 24 h	4.8 ± 0.8 mg per 24 h
3-Methoxy-4-hydroxyphenyl glycol	10	Urine	1530 ± 252 μg per 24 h	1097 ± 110 μg per 24 h	1352 ± 164 μg per 24 h
	131	Urine	1400 ± 400	1100 ± 400	2090 ± 300
	131	CSF	15.2 ± 3.6 ng/ml	12.6 ± 1.2 ng/ml	21.4 ± 3.2 ng/ml

* $p < 0.01$ *vs.* control.

ly correlated with severity in chronically ill subjects[407]. Repeated sampling in schizophrenics demonstrated that plasma MHPG appeared to be altered by changes in clinical state and may reflect psychosis-related changes in norepinephrine function in schizophrenia[892]. In addition, patients with idiosyncratic behaviour exhibit elevated MHPG excretion[704]. High levels of normetanephrine and metanephrine excretion have been associated with agitated behavior in both schizophrenic and depressed patients, with lower levels being reported during periods of calm[808]. An elevation in the excretion of the catecholamines and their metabolites during periods of catatonia returns nearly to normal during remission[809]. Conjugated phenylacetic acid concentrations in CSF have been shown to be highly correlated with hallucinatory behavior and unusual thought content[455]. Patients with the highest phenylethylamine concentration in CSF also had the highest scores on the Brief Psychiatric Rating Scale[455]. Urinary tryptamine excretion has been claimed to be positively correlated with the severity of psychotic activity and with changes in the severity of psychotic activity[767,810,821], as has urinary indoleacetic acid[821]. High urinary tryptamine, which corresponds to marked psychotic activity, has been shown to be associated with low platelet monoamine oxidase activity[117]. Platelet monoamine oxidase has been demonstrated to be significantly lower in chronic schizophrenics and lower in chronic paranoid schizophrenics than in chronic non-paranoid schizophrenics[824]. On this basis it has been suggested that chronic paranoid schizophrenia may be a different disorder from other chronic forms of schizophrenia.

Computed tomography scans of chronic schizophrenics revealed significantly larger ventricular brain ratios and structural changes consistent with cortical atrophy, and these results were significantly correlated inversely with the CSF concentration of HVA[508,798,799,812] and with 5-HIAA[508,811]. The results for MHPG also showed a negative correlation, but this was not statistically significant[508]. These results suggest that enlargements of the brain ventricles found in some schizophrenic patients may be associated with deficiencies in central monoamine transmission mechanisms. During measurements of regional cerebral blood flow in schizophrenic patients, the levels of HVA and 5-HIAA (but not MHPG) in the CSF were found to be correlated with blood flow during the Wisconsin Card Sorting Test but not during number-matching tests or at rest[813]. These results have been claimed to indicate a reduction in dopaminergic and serotonergic projections to the pre-frontal cortex as a possible neurochemical mechanism for pre-frontal dysfunction in schizophrenics[813].

3.2.6. Effects of drugs on metabolite concentrations

3.2.6.1. Effects on anti-psychotic drugs on metabolite concentrations. The subjects participating in the studies reported in Table 51 had been drug-free for at least 1 week and in most cases more than 2 weeks.

3.2.6.2. Metabolite values as predictor of response to drugs. High pretreatment excretion rates of the catecholamines and MHPG have been observed to be associated with a positive therapeutic response to propranolol, whereas low rates were associated with no improvement[818]. Early neuroleptic response has been reported to be significantly correlated to pretreatment plasma HVA in both sexes and to plasma MHPG in women[802,825]. Subjects who responded well to haloperidol treatment were found to exhibit significantly higher pretreatment plasma HVA and a trend to higher MHPG concentrations than those who did not respond[782]. No relationship between

TABLE 49

BIOGENIC AMINES

Amine	*Reference*	*Biological fluid*	*Paranoid*	*Non-paranoid*	*Control*
Phenylethylamine	100	Urine	19.0 ± 4.0* μg per 24 h	7.0 ± 1.5† μg per 24 h	8.0 ± 2.0 μg per 24 h
	102	Urine	17.4 ± 5.5*	7.9 ± 2.4†	5.3 ± 0.5
	101	Urine	8.3 ± 1.4**	5.0 ± 1.5†	4.1 ± 0.5
	105	Urine	16.6 ± 18.4*	10.2 ± 12.9†	6.5 ± 3.7
	806	Urine	18.1*	8.6	5.6
	212	Plasma	65 ± 5* pg/ml	–	99 ± 10 pg/ml
m-Tyramine	102	Urine	37.8 ± 11.6 μg per 24 h	49.8 ± 9.6 μg per 24 h	54.2 ± 6.3 μg per 24 h
p-Tyramine	102	Urine	603 ± 287	310 ± 68	377 ± 56
Noradrenaline	148	Urine	105 ± 19** ng/min	–	19 ± 3 ng/min
	143	Urine	35.0 ± 1.2 μg per 24 h	38.6 ± 4.8[a] μg per 24 h	37.2 ± 0.7 μg per 24 h
	143	Plasma	265 ± 13* pg/ml	249 ± 14[a] pg/ml	230 ± 11 pg/ml

	280	Plasma	426 ± 55	302 ± 65[a]	210 ± 6
	254	Plasma	238 ± 42	197 ± 20	201 ± 24
	329	CSF	163 ± 12	–	133 ± 10
	143	CSF	160 ± 11*	138 ± 7[a]	125 ± 6
Adrenaline	148	Urine	27.9 ± 8.6 ng/min	–	9.4 ± 4.1 ng/min
	143	Urine	11.9 ± 0.5 μg per 24 h	12.5 ± 1.7 μg per 24 h	11.2 ± 0.5 μg per 24 h
	280	Plasma	90 ± 72 pg/ml	59 ± 49[a] pg/ml	70 ± 40 pg/ml
	143	Plasma	36.6 ± 1.9	37.7 ± 4.3[a]	37.0 ± 2.0
	143	CSF	13.3 ± 1.1	15.6 ± 5.0	12.2 ± 0.8
Dopamine	143	Urine	283 ± 10 μg per 24 h	318 ± 25 μg per 24 h	292 ± 11 μg per 24 h
	148	Urine	524 ± 49** ng/min	–	210 ± 36 ng/min
	143	Plasma	54 ± 4 pg/ml	54 ± 7 pg/ml	55 ± 4 pg/ml
	280	Plasma	42 ± 10	24 ± 2	18 ± 9
	143	CSF	56 ± 2	54 ± 2	54 ± 2
					87
N,N-Dimethyl-tryptamine	656	Urine	1223* ng per 24 h	–	386 ng per 24 h
N-Methyl-tryptamine	656	Urine	678	–	856

* $p < 0.05$ *vs.* control.
** $p < 0.01$ *vs.* control.
† $p < 0.05$ *vs.* paranoid.
[a] Hebephrenic patients.

TABLE 50

ACID METABOLITES

Acid metabolite	*Reference*	*Biological fluid*	*Paranoid*	*Non-paranoid*	*Control*
Phenylacetic (unconjugated)	352	Urine	14 ± 5** mg per 24 h	8.2 ± 3* mg per 24 h	9.8 ± 1.5 mg per 24 h
	336	CSF	11.5 ± 1.6 ng/ml	–	22.2 ± 3.0 ng/ml
	455	CSF	11.6 ± 1.4	–	21.6 ± 3.1
Phenylacetic (total or conjugated)	105	Urine	125 ± 23 mg per 24 h	150 ± 19 mg per 24 h	141 ± 16 mg per 24 h
	806	Urine	90*	69*	156
	352	Urine	83 ± 11*	64 ± 18*	150 ± 19
	455	CSF	20.0 ± 5.5 ng/ml	–	22.6 ± 5.4 ng/ml
	336	CSF	20.4 ± 5.5	–	23.6 ± 5.2
5-Hydroxy-indoleacetic	18	CSF	32.2 ± 3.3 ng/ml	29.3 ± 3.0 ng/ml	–
Homovanillic	18	CSF	30.1 ± 2.8	22.7 ± 2.7†	–

* $p < 0.05$ *vs.* control.
** $p < 0.01$ *vs.* control.
† $p < 0.01$ *vs.* paranoid.

pretreatment CSF concentrations of HVA or MHPG and clinical response was observed for treatment with chlorpromazine, thiothixene, melperone, sulpiride or clozapine[797]. Also, pretreatment excretion rates of 5-HIAA or HVA proved to be unsuitable in predicting clinical response to reserpine treatment[778,779].

3.2.6.3. Correlation of changes in metabolite levels with therapeutic response. The changes in metabolite concentrations produced by treatment of schizophrenics with anti-psychotic drugs mostly did not correlate with clinical improvement. Thus, changes in urinary 5-HIAA concentration following chlorpromazine or reserpine treatment were not related to therapeutic response[778,779]. Changes in urinary HVA concentration after treatment with chlorpromazine or reserpine[778,779] and flupenthixol[807], in plasma HVA after treatment with apomorphine[820], chlorpromazine[815], fluphenazine[815], haloperidol[815], perphenazine[815], thioridazine[815] and thiothixene[807], trifluoroperazine[815] and verapamil[819] and in CSF HVA after treatment with chlorpromazine[695,797], haloperidol[695], melperone[797], thioridazine[695] and thiothixene[797] could not be demonstrated to be correlated with improvements in clinical condition. However, the acute effect of neuroleptics may be reflected by changes in plasma HVA concentration[804,805]. Decreases in plasma HVA following treatment with fluphenazine[445,784] and in CSF noradrenaline after treatment with pimozide proved to be significantly correlated with therapeutic response. Decreases in urinary MHPG after treatment with flupenthixol[807], in plasma MHPG after treatment with verapamil[819] and in CSF MHPG after treatment with chlorpromazine[797], melperone[797], thiothixene[797] and verapamil[819] were likewise not correlated with therapeutic response. It has been claimed that a decline in the excretion of 3,4-dimethoxyphenylethylamine during long-term treatment with chlorpromazine is highly significantly correlated with clinical improvement[183].

3.2.6.4. Tardive dyskinesia. Until recently, it has been thought that tardive dyskinesia was caused by excessive dopaminergic activity in nigrostriatal pathways secondary to chronic neuroleptic treatment. A statistically significant positive correlation between plasma HVA[816] and CSF HVA[334] and the severity of the dyskinesia has been reported. However, recent investigations have demonstrated no significant differences in either plasma HVA[822] or CSF HVA[790,513] between normals or control schizophrenics and subjects suffering from tardive dyskinesia. The CSF concentrations of MHPG[513,790], 5-HIAA[334,513,790] and DOPAC[513] are also not significantly different in tardive dyskinesia compared with controls. However, urinary MHPG may be lower in schizophrenics with tardive dyskinesia than in those free from the disorder[823]. Serum[772] and CSF[334] concentrations of noradrenaline have been reported to be elevated in schizophrenic subjects with tardive dyskinesia. The measurement of HVA and 5-HIAA in the CSF of tardive dyskinesic patients before and after treatment with probenecid gave no indication of reduced function of the dopaminergic and serotonergic systems[826].

TABLE 51

EFFECTS OF ANTI-PSYCHOTIC DRUGS ON METABOLITE CONCENTRATIONS

Drug	*Reference*	*Biological fluid*	*Category schizophrenia*	*Effect on amine or metabolite*[a]
Apomorphine	820	Serum	Chronic	HVA↓ ($p < 0.01$)
Chlorpromazine	778	Urine	Not classified	5-HIAA↑ (N.S.); HVA↑ ($p < 0.01$); VMA↓ ($p < 0.02$))
	779	Urine	Not classified	5-HIAA↑; HVA↑; VMA↓
	814	CSF	Acute	5-HIAA (N.S.); HVA↑ ($p < 0.005$)
	795	CSF	Acute	HVA↑ ($p < 0.001$)
	695	CSF	Acute	HVA↑ ($p < 0.01$)
	807	Urine	Acute	MHPG (N.S.)
	183	Urine	Acute	DMPEA↓ ($p < 0.05$)
	797	CSF	Not classified	HVA↑ ($p < 0.01$); MHPG↓ (< 0.001)
	815	Plasma	Psychotic	HVA↑ (longitudinal study; ↓ long-term)
	777	CSF	Chronic	DA↓ ($p < 0.05$); DOPAC↓ ($p < 0.01$); HVA↓ ($p < 0.05$); NA↓ (< 0.05) (effect of withdrawal of drug after long-term use)
Debrisoquin sulfate	801	Urine	Acute	MHPG↓ ($p < 0.01$); HVA↓ ($p < 0.001$); DOPAC↓ ($p < 0.005$)
	801	Plasma	Acute	MHPG↓ ($p < 0.001$); HVA↓ ($p < 0.001$); DOPAC↓ ($p < 0.001$)
	801	CSF	Acute	MHPG↓ ($p < 0.001$); HVA↓ (N.S.); DOPAC↓ (N.S.)
β-Flupenthixol	807	Urine	Acute	MHPG↓ ($p < 0.025$)
Fluphenazine	815	Plasma	Not classified	HVA↑ (longitudinal study; ↓ long-term)
	445	Plasma	Chronic	HVA↓ ($p < 0.01$)
	784	Plasma	Not classified	HVA↓ ($p < 0.001$)
	803	Plasma	Chronic	MHPG (N.S. with alprazolam augmentation)
Haloperidol	780	Urine	Acute	HVA↑ ($p < 0.02$); DA↑ (N.S.); VMA↑ (N.S.)
	695	CSF	Acute	HVA↑ ($p < 0.01$)
	515	CSF	Paranoid	MHPG↓ (N.S.); HVA↑ (N.S.); 5-HIAA↓ (N.S.)
	329	CSF	Paranoid	DA↑ ($p < 0.01$); NA↑ ($p < 0.01$)
	782	Plasma	Acute	HVA↑ (longer term↓); MHPG↑ (longer term↓);

	815	Plasma	Psychotic	HVA↑ (longitudinal: long-term↓)
	777	CSF	Chronic	DA↓ ($p < 0.05$); DOPAC↓ ($p < 0.01$); HVA↓ ($p < 0.05$); NA↓ ($p < 0.05$) (effect of withdrawal of drug after long-term use)
	19	Plasma	Not classified	HVA (N.S.)
	817	Plasma	Not classified	HVA↑
	804	Plasma	Not classified	HVA↑ ($p < 0.001$)
Melperone	789	CSF	Acute	HVA↑ ($p < 0.001$); (↓ $p < 0.025$ after 4 weeks); 5-HIAA (N.S.); MHPG (N.S.) (↓ $p < 0.001$ after 4 weeks)
	797	CSF	Not classified	HVA↑ ($p < 0.001$); MHPG↓ ($p < 0.001$)
α-Methyldopa	767	Urine	Chronic	5-HIAA↓ ($p < 0.01$); TRA (N.S.); IAA↓ (N.S.)
Oxypertine	139	Urine	Chronic	NA↑ ($p < 0.01$); MN↑ ($p < 0.02$); NMN↑ ($p < 0.01$); VMA↑ ($p < 0.02$) (after chronic chlorpromazine use)
Perphenazine	815	Plasma	Psychotic	HVA↑ (longitudinal study: ↓ long-term)
Pimozide	327	CSF	Undifferentiated, paranoid, schizoaffective	NA↓ ($p < 0.01$)
Reserpine	778	Urine	Not classified	5-HIAA↑ ($p < 0.05$); HVA↑ ($p < 0.001$); VMA↓ ($p < 0.001$)
	779	Urine	Not classified	5-HIAA↑ ($p < 0.05$); HVA↑ ($p < 0.001$); VMA↓ ($p < 0.001$)
	821	Urine	Not classified	IAA (N.S.); TRA (N.S.)
Sulpiride	797	CSF	Psychotic	HVA↑ ($p < 0.01$); MHPG (N.S.)
Thioridazine	695	CSF	Acute	HVA↑ ($p < 0.05$)
	815	Plasma	Psychotic	HVA↑ (longitudinal study: ↓ longer-term)
	777	CSF	Chronic	DA↓ ($p < 0.05$); DOPAC↓ ($p < 0.01$); HVA↓ ($p < 0.05$); NA↓ ($p < 0.05$); 5-HIAA (N.S.) (effect of withdrawal of drug after long-term use)
Thiothixene	789	CSF	Acute	HVA↑; 5-HIAA (N.S); MHPG (N.S.)
	797	CSF	Not classified	HVA↑ ($p < 0.001$); MHPG (N.S.)
	815	Plasma	Psychotic	HVA↑ (longitudinal study: ↓ longer-term)
Trifluoperazine	815	Plasma	Psychotic	HVA↑ (longitudinal study: ↓ longer-term)
	814	CSF	Acute	HVA↑ ($p < 0.005$); 5-HIAA (N.S.)
Verapamil	819	Plasma	Chronic	HVA↑ ($p < 0.01$); MHPG↓ ($p < 0.05$)
	819	CSF	Chronic	HVA↑ ($p < 0.01$); MHPG↓ (N.S.); 5-HIAA (N.S.)

[a] For abbreviations, see text and Table 38. MN = metanephrine; DMPEA = 3,4-dimethoxyphenylethylamine. ↑, Concentration increases during treatment; ↓, concentration decreases during treatment

TABLE 52

DOPAMINERGIC SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Parkinson's disease*	*Control*
Dopamine	156	Urine	67.5 ± 3.2** μg per 24 h (unconjugated)	126 ± 9 μg per 24 h
	156	Urine	230 ± 12 (conjugated)	246 ± 23
	111	Urine	150 ± 8* μg/g creatinine	127 ± 8 μg/g creatinine
	43	Urine	241 ± 22 μg per 24 h	316 ± 15 μg per 24 h
3,4-Dimethoxy-phenylethylamine	827	Urine	4.4 ± 0.3	6.8 ± 1.2
Homovanillic acid	828	Urine	4.31 mg/g creatinine	3.72 mg/g creatinine
	156	Urine	2.53 ± 0.16 mg per 24 h	2.17 ± 0.17 mg per 24 h
	497	CSF	20 ± 3.5 ng/ml	38 ± 8.5 ng/ml
	527	CSF	17 ± 2	47 ± 8
	472	CSF	12 ± 2	40 ± 7
	495	CSF	15.8 ± 1.1**	34.4 ± 1.9
	491	CSF	14.4 ± 0.8**	34.6 ± 1.9
	490	CSF	15 ± 7	53 ± 6
	529	CSF	82.1 ± 7.6 (low clinical score)	87.2 ± 9.2
	529	CSF	30.5 ± 8.6** (high clinical score)	87.2 ± 9.2
	535	CSF	15 ± 3.9	28 ± 6.8
	469	CSF	12 ± 9**	39 ± 13
	468	CSF	16 ± 5***	45 ± 5
	829	CSF	< 10 ± 1	23 ± 2
	486	CSF	19 ± 2***	53 ± 11
	533	CSF	8.5 ± 1.7**	31 ± 5
	476	CSF	20 ± 7	60 ± 8
	475	CSF	20 ± 2 ng/ml	50 ± 5 ng/ml
	532	CSF	54.6 ± 6.5***	85.9 ± 7.7
	830	CSF	63 ± 15	93 ± 7
	831	CSF (ventricular)	154 ± 19	216
3,4-Dihydroxy-phenylacetic acid	156	Urine	1.27 ± 0.08*** mg per 24 h	1.80 ± 0.18 mg per 24 h
	486	CSF	6 ± 1*** ng/ml	9 ± 1 ng/ml

* $p < 0.05$ *vs.* control
** $p < 0.001$ *vs.* control.
*** $p < 0.01$ *vs.* control.

4. NEUROLOGICAL DISORDERS

4.1. Parkinson's disease

4.1.1. Introduction

A central dopamine deficiency is considered to be an important factor responsible for the pathogenesis of Parkinson's disease. A discussion of dopamine and serotonin disturbances in Parkinsonism can be found in a review by Mendlewicz *et al.*[684]. *Post-mortem* examination of the brain of a Parkinsonian patient revealed reduced concentrations of dopamine and its metabolites in the striatum, putamen and caudate nucleus[333]. Serotonin and 5-HIAA were also lower in some regions. That the brains of patients suffering from Parkinson's disease synthesize less dopamine than normal subjects has also been shown by significantly decreased dopamine excretion and lower CSF concentrations of HVA (Table 52).

No correlation between the HVA concentration in the CSF of Parkinsonian patients and the presence or absence or rigidity or akinesia could be demonstrated[831], nor could a correlation between pretreatment severity and pretreatment HVA or 5-HIAA concentration be demonstrated[469,535,829,830,836–838].

Alterations in central serotonin metabolism have been reported to attend Parkinson's disease (Table 53), although it appears that this may be a secondary derangement having no effect on the severity of the extrapyramidal signs characteristic of the disorder[535,829,839]. Pretreatment concentrations of 5-HIAA in the CSF are generally lower in Parkinsonism (Table 53) than in control subjects but the differences have not been consistently statistically significant. The noradrenergic system appears to be normal in Parkinsonism (Table 54).

4.1.2. Metabolite concentrations in Parkinsonian patients and controls

Metabolite concentrations in Parkinsonian patients and controls are given in Tables 52–55. All concentrations given are for medication-free subjects.

4.1.3. Effect of L-*DOPA treatment*

Although the concentration of HVA in the CSF of Parkinsonian patients increases roughly in proportion to the dose of L-DOPA[468,486,495,497,529,533,828,829,836,837,840], this increase has generally not been found to correlate with clinical improvement[468,486,495,497,529,533,829,836,838]. The single exception to this finding has not been explained[830]. The DOPAC concentration in CSF also increases after ingestion of L-DOPA, but the increase is also not correlated with clinical improvement[486,840]. Furthermore, pretreatment values for HVA in CSF do not correlate with the responsiveness of the subject to L-DOPA treatment[472,535,829,838]. Some investigators have reported that those patients most responsive to L-DOPA therapy start treatment with much lower concentrations of HVA[529], whereas others have found that patients with high pretreatment HVA do as well on L-DOPA as those with low levels[838]. It has also been suggested that the change in the HVA concentration in CSF during L-DOPA treatment might be of value in predicting the response to L-DOPA[468].

A high baseline concentration of 5-HIAA in CSF has been reported to predict a good response to L-DOPA therapy[497]. Agreement on the effect of L-DOPA on the

TABLE 53

SEROTONERGIC SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Parkinson's disease*	*Control*
5-Hydroxytryptamine	523	CSF	7.75 ± 2.3 ng/ml	12.7 ± 2.6 ng/ml
5-Hydroxy-	832	Urine	8.73 ± 4.1 mg/g creatinine	6.6 ± 2.0 mg/g creatinine
indoleacetic	523	CSF	21.6 ± 3.6 ng/ml	25.5 ± 2.4 ng/ml
acid	475	CSF	20 ± 12	40 ± 1.5
	476	CSF	10 ± 2*	40 ± 2
	533	CSF	29 ± 3	30 ± 3
	486	CSF	13 ± 1**	19 ± 1
	829	CSF	20 ± 2	30 ± 2
	468	CSF	21 ± 2.6	27 ± 3
	469	CSF	17 ± 3.5	23 ± 3
	535	CSF	24 ± 3	28 ± 3
	490	CSF	24 ± 9	31 ± 3
	491	CSF	26.3 ± 1.5*	36.7 ± 3.8
	495	CSF	24.0 ± 1.3**	33.9 ± 3.3
	472	CSF	15 ± 3	27 ± 4
	497	CSF	14 ± 1	20 ± 2
	830	CSF	75 ± 12	57 ± 4

* $p < 0.05$ *vs.* control.
** $p < 0.01$ *vs.* control.

TABLE 54

NORADRENERGIC SYSTEM

Amine or metabolite	*Reference*	*Biological fluid*	*Parkinson's disease*	*Control*
Noradrenaline	43	Urine	40 ± 5 μg per 24 h	42 ± 3 μg per 24 h
Adrenaline	43	Urine	15 ± 0.4	17 ± 1.1
3-Methoxy-4-hydroxyphenol glycol	500	CSF	12.5 ± 1.3 ng/ml	15.1 ± 2.6 ng/ml
	833	CSF	18	16
	558	CSF	12.8 ± 1.5 (unconjugated)	11.0 ± 1.0 (unconjugated)
	558	CSF	32.0 ± 2.0 (total)	26.4 ± 3.5 (total)
	497	CSF	8 ± 1	7 ± 2
3-Methoxy-4-hydroxyethanol	558	CSF	2.8 ± 0.8 (unconjugated)	5.7 ± 0.9 (unconjugated)
	558	CSF	8.5 ± 0.5 (total)	9.0 ± 1.0 (total)

TABLE 55

OTHER AMINES AND METABOLITES

Amine or metabolite	*Reference*	*Biological fluid*	*Parkinson's disease*	*Control*
p-Tyramine	111	Urine	505 ± 33* μg/g creatinine	400 ± 34 μg/g creatinine
	834	Urine	500 ± 137	312 ± 29
Tryptamine	111	Urine	80 ± 8*	60 ± 5
	834		72 ± 5	83 ± 9
p-Hydroxyphenylacetic acid	102	Urine	7.4 ± 1.5 mg per 24 h	13.7 ± 3.5 mg per 24 h
m-Hydroxyphenyl acetic acid	102	Urine	1.7 ± 0.5 mg per 24 h	< 1 mg per 24 h
Phenylacetic acid	40	Plasma (U)[a]	105.7 ± 34.5 ng/ml	161.8 ± 22.2 ng/ml
	40	Plasma (C)[a]	425.5 ± 138.5	412.5 ± 68.7
	40	CSF (U)	25.6 ± 6.1	31.6 ± 6.5
	40	CSF (C)	20.2 ± 2.7	29.7 ± 4.0

* $p < 0.05$ *vs.* control.

[a] U = unconjugated; C = conjugated.

concentration of 5-HIAA in CSF has been poor, some workers claiming it to be reduced[495,793,829] and others finding no change[497]. No change in the CSF or urinary concentrations of MHPG[833,840] or the urinary excretion of VMA[840] was observed after L-DOPA administration.

p-Tyramine has been reported to be reduced in Parkinsonian patients on L-DOPA[103,834], but to be near normal in untreated subjects[107,111]. Tryptamine and indoleacetic acid are not affected by L-DOPA ingestion[793,834].

Although no pretreatment clinical distinction between Parkinsonian patients with low or high concentrations of HVA in CSF could be made, those with low pretreatment values responded well to treatment with amantidine[469]. With probenecid pretreated Parkinsonian patients, the administration of L-tyrosine results in a statistically significant increase in the CSF concentration of HVA, indicating an increase in dopamine turnover in these patients[846].

4.1.4. Effect of probenecid

As L-DOPA therapy can have serious side effects and because treatment should be continued for several months prior to a final assessment, it would be useful to be able to predict the results of therapy in advance. It has been suggested that the determination of HVA and 5-HIAA in CSF after treatment with probenecid may be a better diagnostic tool for Parkinson's disease than the pre-treatment values[475]. A significant ($p < 0.03$) negative correlation between the post-probenecid HVA concentration in CSF and clinical improvement scores after L-DOPA therapy, and a significant negative correlation between HVA accumulation and degree of improvement, have been demonstrated[835]. Thus, a single lumbar puncture and HVA determination after probenecid administration permits the prediction of the results of L-DOPA therapy. Several studies have shown that Parkinsonian patients treated with probenecid exhibit a significantly smaller increase in the CSF HVA concentration than normal or neurological controls, indicating a decreased dopamine turnover for the Parkinsonian patients[475,491,495,527,841]. Parkinsonian patients with markedly decreased dopamine metabolism as shown by the probenecid test seem to benefit more from L-DOPA therapy[835]. The ratio of the CSF concentrations of 5-HIAA and HVA is significantly greater in Parkinson's disease than in controls after probenecid administration, again indicating a lower dopamine turnover in Parkinsonism[830]. A substantial diminution in the response of CSF 5-HIAA to probenecid administration in Parkinson's disesase compared with controls has been reported, indicating that central serotonin turnover may also be deficient[475,495,535]. However, unlike the CSF HVA concentrations, no correlation of 5-HIAA concentrations or changes in 5-HIAA concentrations after probenecid treatment with the degree of clinical improvement after L-DOPA administration could be demonstrated[835].

4.2. Alzheimer's disease

A *post-mortem* analysis of the putamen and caudate nucleus from patients with dementia of the Alzheimer type revealed reduced concentrations of noradrenaline, serotonin and their metabolites[333]. Some differences and similarities in the pattern of reduced concentrations between patients with Alzheimer's and Parkinson's diseases were noted. The data summarized in Table 56 for metabolite concentrations

TABLE 56

METABOLITE CONCENTRATIONS IN ALZHEIMER PATIENTS AND CONTROLS

Metabolite	*Reference*	*Biological fluid*	*Alzheimer's disease*	*Control*
Homovanillic acid	526	CSF	41.7 ± 4.0 ng/ml	41.5 ± 3.8 ng/ml
	775	CSF	33.7 ± 5.1*	70.4 ± 7.4 (schizophrenic)
	333	CSF	32 ± 32	35 ± 21
	522	CSF	29.1 ± 5.9**	42.3 ± 3.4
	521	CSF	26.9 ± 0.4	26.3 ± 0.7
	507	CSF	46.4 ± 7.6	52.1 ± 4.7
	17	CSF	27.5 ± 4.2*	67.5 ± 3.7
	464	CSF	52 ± 6***	78 ± 7
	845	CSF	29 ± 5.4	33 ± 4.6
	472	CSF	38 ± 6	40 ± 6
	844	CSF	28 ± 5.4**	51 ± 7.3
	479	CSF	23 ± 4.1***	60 ± 7.2
	476	CSF	30 ± 6.3	60 ± 7.8
	339	CSF	14.8 ± 2.4*	42.2 ± 4.5
3,4-Dihydroxy-phenylacetic acid	339	CSF	0.41 ± 0.04**	0.74 ± 0.10
Noradrenaline	333	CSF	340 ± 90 pg/ml	580 ± 20 pg/ml
	243	CSF	411 ± 25**	245 ± 33
	243	Plasma	677 ± 64***	253 ± 37
3-Methoxy-4-hydroxyphenyl glycol	526	CSF	9.6 ± 0.8 ng/ml	10.1 ± 0.9 ng/ml
	775	CSF	8.5 ± 0.5	9.4 ± 0.7 (schizophrenic)
	333	CSF	5.5 ± 1.7	5.9 ± 0.4
	522	CSF	8.3 ± 0.4	7.2 ± 0.5
	507	CSF	8.8 ± 1.1	7.2 ± 0.6
	610	CSF	10.6 ± 0.1	9.8 ± 0.1
	339	CSF	9.9 ± 0.8	8.2 ± 0.4
	243	CSF	10.8 ± 0.9	7.6 ± 0.8
	243	Plasma	5.4 ± 0.6	3.4 ± 0.3
5-Hydroxy-tryptamine	337	CSF	110 ± 10 pg/ml**	400 ± 140 pg/ml (caudal)
	523	CSF	12.1 ± 3.9 ng/ml	12.7 ± 2.6 ng/ml
	339	CSF	470 ± 90 pg/ml	370 ± 100 pg/ml
5-Hydroxy indoleacetic acid	476	CSF	20 ± 3*** ng/ml	40 ± 2 ng/ml
	479	CSF	35 ± 3.4	40 ± 2.4
	844	CSF	20 ± 2.5***	29 ± 2.4
	472	CSF	31 ± 3	27 ± 4
	464	CSF	35 ± 2***	42 ± 3
	17	CSF	29.5 ± 4.5	32.5 ± 2.0
	507	CSF	22.9 ± 2.3	22.5 ± 2.7
	521	CSF	14.4 ± 0.2	10.8 ± 0.4
	522	CSF	16.0 ± 1.5**	21.4 ± 1.8
	333	CSF	17 ± 2	23 ± 3
	337	CSF	12.9 ± 1.3**	20.2 ± 1.8
	523	CSF	27.9 ± 2.9	25.5 ± 2.4
	775	CSF	22.0 ± 1.5	28.7 ± 2.3 (schizophrenic)
	526	CSF	20.6 ± 1.9	20.8 ± 2.1
	339	CSF	8.1 ± 1.9**	14.3 ± 1.3

* $p < 0.001$ vs. control.
** $p < 0.05$ vs. control.
*** $p < 0.01$ vs. control.

in CSF and in some reviews[684,842,843], although partly contradictory, indicate reduced levels of HVA and 5-HIAA in Alzheimer's disease. Significantly higher levels of noradrenaline in both plasma and CSF have been interpreted to be compatible with an increased turnover of noradrenaline in Alzheimer's disease, although the MHPG concentrations generally fall in the normal range[243]. Pre-senile dementia has been associated with lower CSF concentrations of both 5-HIAA and HVA than senile dementia[476,522,844], whereas the MHPG concentrations are not different. The more severe symptoms of the disorder are associated with lower concentrations of 5-HIAA and HVA in CSF[17,464,841], and with higher concentrations of plasma[243] and CSF[243,841] MHPG and noradrenaline. In Alzheimer patients, serotonin and 5-HIAA are negatively correlated, whereas in the same CSF fraction from Parkinsonian patients they are positively correlated, indicating a differential involvement of the serotonergic system in the two disorders[523]. Various anti-depressant drugs have been tested in Alzheimer patients and the effects on the CSF concentrations of HVA and 5-HIAA determined[526,753]. The HVA/5-HIAA ratio was able to discriminate more powerfully between the effects of different drugs than either metabolite separately.

5. OTHER DISORDERS

5.1. Aggression and violence

5.1.1. Introduction

The concentrations of 5-HIAA in the CSF of violent individuals have been consistently reported to be lower than those in non-violent persons. Brown *et al.*[636] have shown that more impulsive aggressive individuals exhibit significantly reduced 5-HIAA concentrations in their CSF compared with less compulsive individuals having a significantly lower aggression score. The MHPG and HVA concentrations did not differ between the two groups. Similarly, the 5-HIAA concentration in the CSF of murderers who have committed more than one violent crime is significantly less than that in murderers who have committed only one violent crime[734]. Both homicidal and suicidal killers have lower 5-HIAA levels in CSF than normal controls[733]. A negative correlation of the 5-HIAA concentration in CSF with the incidence of criminal acts has been demonstrated[847]. These results suggest that low levels of 5-HIAA in CSF reflect a disorder of serotonin turnover which can make an individual more prone to violence in states of emotional turmoil. Support for this hypothesis has been provided by a study of subjects exhibiting the 47, XYY syndrome[481,848] in which probenecid tests showed a much smaller CSF accumulation of 5-HIAA for the aggressive subjects than for controls, indicating a decreased serotonin turnover in the former. A dramatic increase in the CSF levels of 5-HIAA was observed for these subjects following treatment with L-tryptophan[848]. The clinical improvement in these patients was equivalent to that obtained by treatment with conventional neuroleptics.

Sandler *et al.*[423] have proposed that phenylethylamine overproduction may represent a compensatory response to curb aggressive tendencies arising as a result of some unknown functional derangement. In support of this theory they found significantly elevated levels of total and unconjugated phenylacetic acid in the plasma of aggressive prison inmates. More recently, the results of investigations by Boulton *et al.*[426] and Yu *et al.*[428] failed to confirm this finding, demonstrating a trend to a

reduced plasma concentration of phenylacetic acid in aggressive subjects. These contradictions may reflect inadequate definitions of aggression and violence, and also confusion between violent and agressive behaviour which may not be synonymous.

5.1.2. Metabolite concentrations in aggressive subjects and controls

The metabolite concentrations obtained in experiments on violent and aggressive subjects are summarized in Table 57.

5.2. Hyperkinesis and attention deficit disorder

5.2.1. Introduction

The data indicate that a dysfunction of the noradrenergic system may be involved in the aetiology of hyperkinesis and attention deficit disorder. The other monoaminergic systems do not appear to be involved, although a trend to elevated plasma indoleacetic acid in hyperkinetic children has been reported[428]. Behavioural changes in boys with attention deficit disorder with hyperactivity treated with methylphenidate were not correlated with the urinary excretion of noradrenaline or its metabolites[849]. However, treatment with D-amphetamine produced a decrease in urinary MHPG[850,851] that was related to the response to the drug. Subjects with low pretreatment urinary HVA tended to respond better to D-amphetamine than did those with normal HVA levels, and their clinical improvement corresponded with an increase in HVA excretion[851,852]. D-Amphetamine caused a reduction in the CSF concentration of HVA which was closely correlated with clinical improvement[853]. It was suggested that these results support the view that an alteration in central dopamine-mediated synaptic function may occur in hyperactive children[853].

5.2.2. Metabolite concentrations in hyperkinetic children and controls

Metabolite concentrations in hyperkinetic children and controls are given in Table 58.

5.3. Migraine

Dysfunctions of the serotonin, dopamine, noradrenaline and tyramine systems have all been implicated in migraine aetiology. A conjugation defect in dietary and tyramine-sensitive migraine has been postulated; support for this theory has been obtained by challenging patients and controls with oral tyramine and observing significantly less excretion of conjugated tyramine in the migraine patients[108,109]. However, in a more recent study in which the migraine sufferers were challenged with deuterium-labelled tyramine, these findings could not be confirmed, although a conjugating enzyme, phenolsulfotransferase, proved to be significantly less active in the migraine subjects[97].

Significant changes in serotonin and dopamine metabolism during different stages of migraine attacks have been reported[489]. Urinary 5-HIAA[367,489,859] and VMA[367] excretion have been shown to increase during a migraine attack, although some studies have shown no change in VMA excretion during an attack[489,859]. Significantly higher plasma VMA and urinary MHPG have been found in migraine patients even in the absence of an attack[97]. The relationship between the serotonergic system and migraine has been reviewed[860].

TABLE 57

METABOLITE CONCENTRATIONS IN AGGRESSIVE SUBJECTS AND CONTROLS

Metabolite		*Reference*	*Biological fluid*	*Aggression-violence*	*Control*
5-Hydroxyindoleacetic acid		481	CSF	21.6 ± 1.9 ng/ml	21 ± 3 ng/ml
		733	CSF	17.4 ± 1.4	17.4 ± 0.9
Homovanillic acid		428	Plasma	9.0 ± 0.8[c]*	5.3 ± 0.6[d]
		481	CSF	29.5 ± 11.4	47 ± 8
		733	CSF	47.3 ± 1.1	42.0 ± 0.5
3-Methoxy-4-hydroxyphenyl glycol		733	CSF	8.8 ± 0.5	9.7 ± 0.3
Vanilmandelic acid		428	Plasma	4.9 ± 0.2[a]	5.7 ± 0.6[b]
		428	Plasma	4.8 ± 0.3[c]	4.9 ± 0.6[d]
Phenylacetic acid	-U[e]	423	Plasma	214.7 ± 40.8**	107.2 ± 10.7
	-T[e]	423	Plasma	661.4 ± 113.1**	386.0 ± 50.7
	-U[e]	426	Plasma	135.5 ± 12.1[g]	125.9 ± 9.7[f]
	-U[e]	426	Plasma	136.1 ± 18.0[g]	126.9 ± 9.5[f]
	-U[e]	426	Plasma	136.1 ± 18.0[g]	79.5 ± 9.9[h]
	-C[e]	426	Plasma	308.8 ± 24.8[g]	362.6 ± 32.3[f]
	-C[e]	426	Plasma	270.4 ± 18.3[g]	361.3 ± 32.4[f]
	-C[e]	426	Plasma	270.4 ± 18.3[g]	296.4 ± 30.9[h]
	-U	428	Plasma	102.2 ± 10.0[g]	94.9 ± 9.7[f]
	-U	428	Plasma	70.6 ± 5.6[a]**	116.9 ± 13.1[b]
	-U	428	Plasma	77.9 ± 9.6[c]**	126.5 ± 21.1[d]
	-C	428	Plasma	246.4 ± 17.5[g]	263.5 ± 26.3[f]
	-C	428	Plasma	212.8 ± 19.6[a]**	275.5 ± 21.5[b]
	-C	428	Plasma	214.3 ± 29.0[c]**	325.5 ± 35.0[d]
	-T[e]	428	Plasma	343.7 ± 24.8[g]	363.5 ± 35.7[f]

	-T[e]	428	Plasma	283.1 ± 23.7[a]**	391.2 ± 31.4[b]
	-T[e]	428	Plasma	282.2 ± 33.7[c]*	452.0 ± 55.1[d]
p-Hydroxyphenylacetic acid		426	Plasma	65.9 ± 6.6[g]**	99.4 ± 13.1[f]
		426	Plasma	64.5 ± 6.8[g]**	99.5 ± 17.9[f]
		426	Plasma	64.5 ± 6.8[g]	66.6 ± 15.9[h]
		428	Plasma	44.5 ± 4.1[a]	65.6 ± 8.7[b]
		428	Plasma	47.0 ± 7.3[c]	44.9 ± 11.0[d]
m-Hydroxyphenylacetic acid		426	Plasma	10.3 ± 2.4[g]	7.8 ± 1.3[f]
		426	Plasma	9.6 ± 1.8[g]	9.5 ± 1.6[f]
		426	Plasma	9.6 ± 1.8[g]	16.6 ± 2.7[h]
		428	Plasma	4.6 ± 0.8[c]	6.2 ± 1.2[d]
		428	Plasma	6.2 ± 1.1[a]	6.1 ± 0.6[b]
Indoleacetic acid		428	Plasma	220 ± 14[a]	369 ± 62[b]
		428	Plasma	230 ± 24[c]	213 ± 45[d]
p-Hydroxymandelic acid		428	Plasma	9.5 ± 1.2[a]	8.7 ± 0.4[b]
		428	Plasma	8.3 ± 0.8[c]	7.0 ± 1.7[d]

* $p < 0.01$ *vs.* controls.
** $p < 0.05$ *vs.* controls.
[a] = Aggressive.
[b] = Non-aggressive.
[c] = Violent-aggressive.
[d] = non-violent, non-agressive institutional control
[e] = U = unconjugated; C = conjugated; T = total.
[f] = Non-violent institutional control.
[g] = Violent.
[h] = Non-institutional control.

TABLE 58

METABOLITE CONCENTRATIONS IN HYPERKINETIC CHILDREN AND CONTROLS

Metabolite	*Reference*	*Biological fluid*	*Hyperkinesis*	*Control*
Noradrenaline	854	Urine	31.2 μg per 24h***	17.2 μg per 24 h
Adrenaline	854	Urine	14.5	12.2
Normetanephrine	855	Urine	125 ± 15*	94 ± 7
	850	Urine	113 ± 8	93 ± 6
	856	Urine	157.7	109.9
Metanephrine	855	Urine	85 ± 9.4	74 ± 5.5
	850	Urine	73 ± 4.6	74 ± 5.0
	856	Urine	101	68
3-Methoxy-4-hydroxyphenyl glycol[a]	855	Urine	806 ± 58*	1044 ± 76
	854	Urine	1180	960
	857	Urine	761*	1078
	850	Urine	740 ± 64**	1042 ± 67
	856	Urine	830*	649
	851	Urine	666 ± 85	872 ± 95
	852	Urine	658 ± 81 (non responders)[b]	929 ± 53 μg/m² per 24 h
	852	Urine	723 ± 69 (responders)[b]	929 ± 53

	858	Urine(M)	810 ± 29* μg per 24 h (sulfate)	1029 ± 38 μg per 24 h (sulfate)
	858	Urine(F)	680 ± 57* (sulfate)	848 ± 37 (sulfate)
Dopamine	854	Urine	211.7	151.9
Homovanillic	855	Urine	2.83 ± 0.18 mg per 24 h	3.21 ± 0.22 mg per 24 h
acid	854	Urine	4.17	3.73
	851	Urine	2.34 ± 0.24	2.88 ± 0.21
	852	Urine	3.32 ± 0.22 $\mu g/m^2$ per 24 h (non-responders)[b]	3.07 ± 0.13 $\mu g/m^2$ per 24 h
	852	Urine	2.50 ± 0.16 (responders)[b]	3.07 ± 0.13
	853	CSF	78 ± 11 ng/ml	62 ± 7.8 ng/ml
	511	CSF	36.5 ± 3.4[c]	37.1 ± 3.3[c]
5-Hydroxy-	853	CSF	37.0 ± 6.8	37.0 ± 2.8
indoleacetic	511	CSF	23.2 ± 3.1[c]	24.0 ± 3.9[c]
acid				

* $p < 0.05$ *vs.* controls.
** $p < 0.01$ *vs.* controls.
*** $p < 0.003$ *vs.* controls.
[a] Total or conjugated MHPG.
[b] Responders or non-responders to amphetamine.
[c] Adults.

TABLE 59

EFFECTS OF AGE ON THE CONCENTRATIONS OF BIOGENIC AMINES AND METABOLITES

↑, Concentrations increase with increasing age; ↓, concentrations decrease with increasing age; N.C., concentrations not correlated with age; ▼, concentrations increase with age to middle age, then decrease; ▲, concentrations decrease with age to middle age, then increase.

Amine or metabolite	*Urine*	*Plasma*	*CSF*
Noradrenaline	▼: 124[a] ↑: 861[a] ($p < 0.02$); 9[a] (N.S.); 864[f]	↑: 263[a](F); 659[b](F); 228[a]; 248[a]; 251[a]; 862[a] ($p < 0.05$); 253[a]; 863[a]; 250[a,f]	N.C.: 327[d]; 634[b]; 329[d]
Adrenaline	▼: 124[a]; 861[a] N.C.: 9[a] ↑: 864[f]	↓: 228[a]	N.C.: 634[b]
Dopamine			N.C.: 329[d]
Histamine	↓: 80[a] (N.S.)		
Phenylethylamine	N.C.: 101[d]		
Phenylacetic acid	N.C.: 72[a,b]	↑: 424[a] (N.S.)	▲: 456[a] ($p < 0.01$)
Indoleacetic acid	↑: 865[a,d]		↑: 461[a] ($p < 0.02$) N.C.: 667[b]; 460[a]
p-Hydroxyphenylacetic acid	↓: 866[a]		
5-Hydroxyindoleacetic acid			↑: 534[a]; 667[b]; 170[b](F) ($p < 0.01$); 691[b](F); 867[a] ($p < 0.05$); 736[b]; 518[a,b]; 692[a] ($p < 0.01$), 170[c] (F) ($p < 0.03$); 510[b] ($p < 0.001$) ↓: 170[c](F); 524[a] (N.S.) ▲: 868[a] ($p < 0.01$) N.C.: 736[a]; 488[a]; 497[a]; 632[a,b]; 683[a,b]; 525[a]; 627[a]; 742[b]; 689[b]; 682[b]

Homovanillic acid	↑: 387[f]; 389[a,f] ↓: 869[a]	↑: 822[d] ($p < 0.01$); 822[a] (N.S.); 712[b] ($p < 0.004$) N.C.: 440[a](F); 816[d]; 784[d]	↑: 534[a]; 497[a]; 170[a]; 513[a]; 682[b]; 691[b](F); 518[b]; 525[a] ($p < 0.05$); 510[b] ($p < 0.05$) ↓: 170[c]; 524[a] (N.S.); 518[a]; ▼: 868[a] N.C.: 867[a]; 488[a]; 683[a,b]; 742[b]; 689[b];
3-Methoxy-4-hydroxyphenyl glycol	↑: 572[b] ($p < 0.05$); 713[b] ($p < 0.01$); 744[b] ($p < 0.01$); 32[b](F); 566[b]; 683[a,b] N.C.: 702[b]; 544[a]; 704[b,c,d]; 28[b]; 572[a]; 706[b]	↑: 607[a,b]; 28[b]; 712[b] ($p < 0.004$) N.C.: 26[b]; 599[b]	↑: 736[b]; 526[e](N.S.) 170[a,b]; 518[a,b] ↓: 682[b] ($p < 0.05$) N.C.: 608[a]; 742[b]; 736[a]; 683[a,b]
3,4-Dihydroxyphenyl glycol	N.C.: 706[b]	↑: 603[b](U, N.S.) ↓: 603[a](C) N.C.: 713[b]	
Vanilmandelic acid 3,4-Dihydroxyphenylacetic acid	↓: 869[a]		N.C.: 608[a] ↓: 524[a] (N.S.)

[a] Controls.
[b] Depressed.
[c] Manic.
[d] Schizophrenic.
[e] Alzheimer's disease.
[f] Children.

6. FACTORS AFFECTING THE CONCENTRATIONS OF THE BIOGENIC MONOAMINES AND THEIR METABOLITES IN BIOLOGICAL FLUIDS

6.1. Subjects variables

6.1.1. Age

That the concentrations of some biogenic amines and their metabolites may be age-dependent was established more than 30 years ago by Karki[124]. The results of numerous studies on metabolite concentration–age correlations are summarized in Table 59.

Some inconsistencies are evident, but a trend to higher concentrations with increasing age appears to occur.

6.1.2. Sex

In general, male urinary excretion of biogenic amines and their metabolites is greater than female excretion, although these differences often disappear if the results are expressed in μg/g creatinine rather than in μg per 24 h[11,126,557,563]. As women, on average, weigh less than men and also excrete less creatinine than men[9], differences in the total daily excretion of amine metabolites may be related, at least in part, to weight differences between the sexes which will be corrected for if the excretion is expressed in μg/g creatinine. Tables 60–63 also show that metabolite concentrations in the CSF of women are generally higher than those for men. As men, on average, are taller than women this difference may be at least partially accounted for by an inverse correlation between body height and CSF metabolite concentrations[882].

6.1.3. Weight

In human CSF, 5-HIAA levels in women have been reported to be affected by body size whereas those of men are not[691]. In mixed populations 5-HIAA, HVA[170,526,632] and IAA[461] concentrations in CSF appear to be independent of body weight. No correlation between the urinary catecholamines and their metabolites with weight have been found[9,170], although a trend to increasing noradrenaline excretion with increasing weight has been noted[9].

6.1.4. Height

The concentrations of HVA and 5-HIAA in CSF are widely reported to be negatively correlated with height[170,510,512,518,524,525,632,691,736,882,883], although in some instances this has been true only for female subjects[170,691]. MHPG concentrations show no correlation with height[170,736,882], except in depressed populations in which there is a modest negative correlation[170,882]. Indoleacetic acid levels in CSF are independent of subject height[461]. It has been suggested that because 5-HIAA and HVA appear in higher concentrations in the cisternal than in the lumbar CSF and show a decrease as the fluid travels towards the lumbar sac, it is not surprising that some investigators have reported a negative correlation of these metabolites with height[524].

6.1.5. CSF gradients

Because of the pronounced cisternal–lumbar and lumbar–ventricular metabo-

lite concentration gradients, it is exceedingly important that the site of puncture be standardized within a given study and also preferably between studies if meaningful comparisons are to be made[345,501,504,524,883,886,887]. The gradients for DOPAC, HVA, 5-HIAA and MHPG have been shown to be statistically significant[345]. Considerable intra-individual variations in the concentrations of noradrenaline, 5-HIAA, MHPG and HVA in the CSF from one lumbar puncture to the next have been indicated, although the mean concentrations were similar[661]. The results of spinal cord transection and spinal fluid block suggest that the spinal cord contributes to the concentration of MHPG, possibly to 5-HIAA but little to HVA[884].

A marked increase in the concentrations of amine metabolites, particurlarly HVA and 5-HIAA, as more and more CSF is removed has been demonstrated[463,501,504,530,786,886,887,889,890]. However, there is disagreement on whether a gradient is present in both recumbent and sitting subjects[886,890]. 5-HIAA and HVA, but not MHPG, were found to be significantly lower in caudal than in rostral samples[890,891] and the lumbar concentrations of several metabolites have been demonstrated to be substantially lower than in cisternal[460] or ventricular[457,473,500,581] samples. No correlation between the differences in ventricular and lumbar metabolite concentrations and body height was found, suggesting that body height may not be an accurate measure of the rostrocaudal gradient[736].

The concentrations of HVA and 5-HIAA are significantly reduced where there is evidence of a restricted flow of CSF from the ventricles to the lumbar sac[467], a fact which should be remembered when interpreting low concentrations of metabolites in the CSF. CSF sampling by air encephalography was shown to be inferior to lumbar puncture in that the former produced much higher values for metabolite concentrations[626].

6.1.6. Genetics and race

Studies of schizophrenia in twin cohorts have indicated that there is probably a genetically controlled biological mechanism that predisposes one to the illness. Data for monozygotic twins suggest that some abnormality of catecholamine metabolism may be related to this genetic predisposition[895]. The urinary excretion levels of the catecholamines were higher than normal in both members of discordant pairs and showed significant intra-class correlations, which strongly suggested the possibility of genetic control[895]. However, within families, the urinary concentrations of the catecholamine metabolites did not demonstrate any changes that could be related to psychopathology[34]. For those subjects reporting increased psychiatric morbidity among relatives, there has been found an increased variance in MHPG excretion compared with those without such a history, suggesting that urinary MHPG may be a predictor of family vulnerability for psychiatric morbidity in healthy subjects[34]. In healthy monozygotic twins, a high concordance for both free and conjugated plasma MHPG suggests that plasma levels may reflect a heritable biological trait[585].

Studies on the family histories of schizophrenic subjects have shown higher concentrations of 5-HIAA and HVA in the CSF of those patients with a family history of schizophrenia than in those without such a history[512,791,896,897]. On the other hand, low 5-HIAA and HVA concentrations are related to an increased risk of depressive disorders in family members[897]. In a study of twins and unrelated individuals, only the MHPG concentration in CSF was found to be under any major genetic influence[898].

TABLE 60

MALE–FEMALE COMPARISON OF NORADRENERGIC METABOLITE CONCENTRATIONS

Amine or metabolite	*References*	*Biological fluid*	*Control disorder*	*Male*	*Female*
Noradrenaline	124	Urine	Normal	25.2 ± 1.6 μg per 24 h	24.9 ± 1.8 μg per 24 h
	126	Urine	Normal	32.9 ± 5.5	23.6 ± 1.5
	141	Urine	Normal	8.1 ± 0.5	6.9 ± 0.5
	217	Urine	Normal	Not significantly different[a]	
	193	Urine	Depressed	330 ± 64* (total)	240 ± 22 (total)
	862	Plasma	Normal	258 pg/ml	238 pg/ml
	250	Plasma	Normal	270 ± 42	307 ± 31
	265	Plasma	Normal	444 ± 39	550 ± 33
	263	Plasma	Normal	230 ± 20	300 ± 30
	253	Plasma	Normal	390 ± 40*	530 ± 50
	772	Plasma	Schizophrenic	770 ± 80	900 ± 80
	263	CSF	Normal	160 ± 30	166 ± 10
	661	CSF	Schizophrenic	123 ± 8	125 ± 16
	327	CSF	Schizophrenic	Not significantly different[a]	
Adrenaline	126	Urine	Normal	8.9 ± 0.8* μg per 24 h	6.5 ± 0.6 μg per 24 h
	141	Urine	Normal	3.0 ± 0.3*	2.1 ± 0.2
	124, 217	Urine	Normal	Not significantly different[a]	
	862	Plasma	Normal	45 pg/ml	58 pg/ml
	265	Plasma	Normal	124 ± 23	130 ± 27
	871	Plasma	Normal	1180 ± 44**	1460 ± 82
Normetanephrine	562	Urine	Normal	196 ± 17 μg per 24 h	233 ± 33 μg per 24 h
	170	Urine	Normal	195 ± 11	189 ± 11
	557	Urine	Normal	Not significantly different[a]	
	562	Urine	Depressed	172 ± 17	214 ± 17
	170	Urine	Depressed	280 ± 23	278 ± 22
	170	Urine	Manic	329 ± 49	246 ± 34
	557	Urine	Depressed	Not significantly different[a]	
Metanephrine	562	Urine	Normal	86 ± 9 μg per 24 h	83 ± 9 μg per 24 h
	170	Urine	Normal	107 ± 7	88 ± 5
	557	Urine	Normal	Not significantly different[a]	
	562	Urine	Depressed	114 ± 22	97 ± 6
	170	Urine	Depressed	149 ± 8**	122 ± 7
	170	Urine	Manic	157 ± 23	91 ± 11
	557	Urine	Depressed	Not significantly different[a]	

Vanilmandelic acid	8	Urine	Normal	3.7 ± 0.2** mg per 24 h	2.9 ± 0.2 mg per 24 h
	562	Urine	Normal	4.16 ± 0.65	4.61 ± 0.41
	384	Urine	Normal	3.3 ± 0.10	3.3 ± 0.14
	93	Urine	Normal	4.55 ± 0.40	4.65 ± 0.32
	170	Urine	Normal	2.72 ± 0.24	2.72 ± 0.22
	608	Urine	Normal	Not significantly different[a]	
	872	Urine	Normal	4.67* mg per 24 h	3.07 mg per 24 h
	562	Urine	Depressed	4.60 ± 0.32	4.38 ± 0.26
	93	Urine	Depressed	5.37 ± 0.57*	3.68 ± 0.36
	170	Urine	Depressed	3.54 ± 0.25	3.58 ± 0.34
	170	Urine	Manic	3.88 ± 0.50	2.99 ± 0.56
3,4-Dihydroxy-phenyl glycol	603	Plasma	Normal	784 ± 52 pg/ml(U)[b]	839 ± 73 pg/ml (U)[b]
	603	Plasma	Normal	1119 ± 140 (C)[c]	1161 ± 175 (C)[c]
	549	Plasma	Normal	1184 ± 346	1091 ± 163
	603	Plasma	Depressed	560 ± 48 (U)	546 ± 50 (U)
	603	Plasma	Depressed	807 ± 94 (C)	789 ± 88 (C)
	713	Plasma	Depressed	596 ± 51 (U)	556 ± 36 (U)
	713	Plasma	Depressed	966 ± 161 (C)	707 ± 75 (C)
	706	Plasma	Depressed	Not significantly different[a]	
3-Methoxy-4-hydroxyphenyl glycol (unconjugated)	11	Urine	Normal	150 ± 23 μg per 24 h	190 ± 45 μg per 24 h
	548	Urine	Normal	112 ± 11**	140 ± 45
	544	Urine	Normal	128 ± 11	139 ± 21
	545	Urine	Normal	110 ± 30	90 ± 30
(conjugated or total)	557	Urine	Normal	1660 ± 85*	1397 ± 63
	545	Urine	Normal	1490 ± 350	1230 ± 330
	554	Urine	Normal	923 ± 142	637 ± 200
	561	Urine	Normal	2084 ± 272 (sulfate)	1664 ± 146 (sulfate)
	11	Urine	Normal	1360 ± 165 (sulfate)	1260 ± 190 (sulfate)
	11	Urine	Normal	910 ± 50 (glucuronide)	1000 ± 115 (glucuronide)
	562	Urine	Normal	1674 ± 117	1348 ± 65
	563	Urine	Normal	2105 ± 255**	1618 ± 212
	70	Urine	Normal	2253 ± 296	1591 ± 71
	477	Urine	Normal	2190 ± 230**	1370 ± 80
	566	Urine	Normal	1440 ± 210	1230 ± 120
	576	Urine	Normal	2750	2200
	32	Urine	Normal	3478 ± 280*	2558 ± 280
	548	Urine	Normal	1340 ± 105 (sulfate)	809 ± 117 (sulfate)

(*Continued on p. 180*)

TABLE 60 (*continued*)

Amine or metabolite	*References*	*Biological fluid*	*Control disorder*	*Male*	*Female*
	548	Urine	Normal	1470 ± 150 (glucuronide)	751 ± 117 (glucuronide)
	544	Urine	Normal	776 ± 80 (sulfate)	662 ± 64 (sulfate)
	544	Urine	Normal	1031 ± 122 (glucuronide)	853 ± 84 (glucuronide)
	568	Urine	Normal	2021 ± 176	1820 ± 157
	170	Urine	Normal	2267 ± 143**	1660 ± 121
	873	Urine	Normal	600 ± 50 μg/g creatinine	690 ± 52 μg/g creatinine (sulfate)
	574	Urine	Normal	1945 ± 304 μg per 24 h	1626 ± 175 μg per 24 h
	141	Urine	Normal	2200 ± 185**	1510 ± 105
	578	Urine	Normal	2040 ± 88	1410 ± 59
	557	Urine	Depressed	1337 ± 106*	892 ± 146
	562	Urine	Depressed	1394 ± 89	1155 ± 58
	563	Urine	Depressed	2019 ± 168**	1357 ± 147
	477	Urine	Depressed	1730 ± 220	1480 ± 130
	566	Urine	Depressed	1680 ± 200	1500 ± 100
	683	Urine	Unipolar	2282 ± 202	2208 ± 221
	32	Urine	Unipolar	3606 ± 241*	3054 ± 222
	32	Urine	Bipolar	3772 ± 631*	2502 ± 205
	568	Urine	Unipolar	2378 ± 133	1853 ± 146
	568	Urine	Bipolar	1410 ± 186	1336 ± 127
	170	Urine	Depressed	2273 ± 144	1968 ± 136
	170	Urine	Manic	2740 ± 512	1909 ± 751
	572	Urine	Unipolar	1890 ± 150*	1670 ± 150
	572	Urine	Depressed	1600 ± 140*	1370 ± 130
	572	Urine	Manic	2510 ± 200*	1630 ± 240
	193	Urine	Depressed	1748 ± 140**	1049 ± 92
	743	Urine	Depressed	1725***	1282
	713	Urine	Depressed	1080 ± 133 μg/g creatinine	1070 ± 97 μg/g creatinine
	744	Urine	Depressed	1100 ± 124 μg/g creatinine	1040 ± 89 μg/g creatinine

	701, 704, 706	Urine	Depressed	Not significantly different	
	807	Urine	Schizophrenic	2140 ± 170 μg/g creatinine	3610 ± 360 μg/g creatinine
(unconjugated)	442	Plasma	Normal	3.4 ± 0.9 ng/ml	3.3 ± 1.1 ng/ml
	440	Plasma	Normal	3.2 ± 0.5	3.5 ± 0.9
	597	Plasma	Normal	3.6 ± 0.2	3.4 ± 0.2
	26	Plasma	Normal	Not significantly different	
	442	Plasma	Depressed	4.0 ± 1.3	4.4 ± 1.7
	440	Plasma	Depressed	3.3 ± 0.2	4.1 ± 0.4
	26, 593	Plasma	Panic Depressed	Not significantly different	
3-Methoxy-4-hydroxyphenyl glycol (total)	874	Plasma	Normal	18.8 ± 4.5 ng/ml	15.6 ± 3.1 ng/ml
	607	Plasma	Normal	14.9 ± 0.9	13.8 ± 0.9
	606	Plasma	Normal	16.3 ± 1.4	12.6 ± 1.4
	607	Plasma	Depressed	16.9 ± 1.8	16.6 ± 1.5
(unconjugated)	509	CSF	Normal	7.8 ± 0.3	8.1 ± 0.3
	505	CSF	Normal	8.8 ± 0.3	9.7 ± 0.6
	614	CSF	Normal	7.3 ± 0.4	8.1 ± 0.6
	170	CSF	Normal	7.8 ± 0.3	8.1 ± 0.3
	608, 524	CSF	Normal	Not significantly different	
	683	CSF	Depressed	8.0 ± 0.3***	9.4 ± 0.5
	505	CSF	Depressed	8.9 ± 0.7	9.7 ± 0.5
	505	CSF	Suicidal	7.9 ± 0.5	8.4 ± 0.6
	685	CSF	Depressed	10.7 ± 0.9	11.6 ± 1.1
	686	CSF	Depressed	8.1 ± 0.3*	9.2 ± 0.4
	170	CSF	Depressed	8.5 ± 0.3	9.1 ± 0.3
	170	CSF	Manic	10.6 ± 1.3	11.7 ± 1.5
	688	CSF	Depressed	8.5 ± 0.4	9.1 ± 0.4
	509	CSF	Manic	10.6 ± 1.3	11.7 ± 1.5
	661	CSF	Schizophrenic	5.6 ± 0.5*	8.0 ± 1.0
(conjugated)	614	CSF	Normal	0.43 ± 0.03	0.40 ± 0.03

* $p < 0.05$ *vs.* females.
** $p < 0.01$ *vs.* females.
*** $p < 0.001$ *vs.* females.
[a] Separate values for males and females are not reported.
[b] Unconjugated.
[c] Conjugated

TABLE 61

MALE–FEMALE COMPARISON OF SEROTONERGIC METABOLITE CONCENTRATIONS

Amine or metabolite	*References*	*Biological fluid*	*Control disorder*	*Male*	*Female*
5-Hydroxy-	769	Urine	Normal	72 μg per 24 h	55 μg per 24 h
tryptamine	769	Urine	Depressed	40	54
	769	Urine	Schizophrenic	54	47
	359	Blood	Normal	190 ± 20 ng/ml	150 ± 10 ng/ml
	304	Plasma	Normal	2.7 ± 0.2	1.830 ± 0.3
	302	Serum	Normal	91.5 ± 5.2*	122.3 ± 6.1
	359	Blood	Schizophrenic	130 ± 20	70 ± 20
Melatonin	342	Plasma	Normal	70 ± 5 pg/ml (day)	45 ± 5 pg/ml (day)
	342	Plasma	Normal	140 ± 11 (night)	130 ± 7 (night)
	342	CSF	Normal	59 ± 33	57 ± 28
6-Hydroxy-	875	Urine	Normal	21 μg per 24 h	16 μg per 24 h
melatonin	190	Urine	Normal	9.1 ± 0.8	10.2 ± 2.7
sulfate	876	Urine	Normal	12.4 (total)	11.9 (total)
5-Hydroxy-	359	Urine	Normal	4.87 ± 0.31 mg per 24 h	5.14 ± 1.37 mgper24h
indoleacetic	141	Urine	Normal	5.31 ± 0.69*	3.11 ± 0.36
acid	872	Urine	Normal	4.41	4.34
	359	Urine	Schizophrenic	8.0 ± 2.1	6.6 ± 3.4
	627	CSF	Normal	42.7 ± 3.8 ng/ml	41.0 ± 6.4 ng/ml
	460	CSF	Normal	19.2 ± 1.2**	25.3 ± 2.3
	460	CSF (cisternal)	Normal	25.7 ± 1.5**	32.4 ± 2.4
	170	CSF	Normal	20.6 ± 0.8	21.8 ± 1.3
	505	CSF	Normal	17.0 ± 1.1	23.1 ± 1.9
	511	CSF	Normal	15.7 ± 3.4**	33.8 ± 5.3
	525	CSF	Normal	19.3 ± 1.2**	22.8 ± 1.0
	518	CSF	Normal	20.6 ± 2.9	23.8 ± 2.1

488, 524, 534, 868	CSF	Normal	Not significantly different	
491	CSF	Normal	M > F ($p < 0.01$)	
627	CSF	Depressed	21.4 ± 2.0 ng/ml	18.5 ± 2.3 mg/ml
627	CSF	Manic	20.3 ± 2.3	18.7 ± 2.3
667	CSF	Depressed	15.8 ± 2.1	21.0 ± 1.4
675	CSF	Manic	19.7 ± 2.1	20.2 ± 2.9
683	CSF	Depressed	18.7 ± 0.7***	24.1 ± 1.0
505	CSF	Depressed	16.9 ± 1.8	20.0 ± 1.9
505	CSF	Suicidal	10.1 ± 1.8	15.4 ± 1.3
685	CSF	Depressed	18.7 ± 2.7	16.8 ± 1.6
686	CSF	Depressed	19.3 ± 1.0***	25.3 ± 1.3
170	CSF	Unipolar	19.7 ± 0.9***	25.4 ± 1.2
170	CSF	Bipolar	20.4 ± 1.4	23.3 ± 1.7
170	CSF	Manic	22.5 ± 2.8	30.8 ± 3.5
170	CSF	Depressed	20.1 ± 0.8	24.8 ± 1.0
688	CSF	Depressed	19.7 ± 1.0**	25.2 ± 1.4
518	CSF	Depressed	19.2 ± 1.8**	25.8 ± 2.2
509	CSF	Manic	22.5 ± 2.8	30.8 ± 3.5
691	CSF	Depressed	16.0 ± 1.3	17.0 ± 1.1
661	CSF	Schizophrenic	23.9 ± 2.1	25.4 ± 3.1
511	CSF	Attention deficit	15.7 ± 9.0 (S.D.)**	33.8 ± 13.0 (S.D.)
491	CSF	Parkinson's disease	25.3 ± 2.6	26.8 ± 1.9
486	CSF	Parkinson's disease	Not significantly different	

* $p < 0.01$ *vs.* females.
** $p < 0.05$ *vs.* females.
*** $p < 0.001$ *vs.* females.

TABLE 62

MALE–FEMALE COMPARISON OF DOPAMINERGIC METABOLITE CONCENTRATIONS

Amine or metabolite	*References*	*Biological fluid*	*Control disorder*	*Male*	*Female*
Dopamine	156	Urine	Normal	161 ± 15* μg per 24 h	115 ± 14 μg per 24 h
	156	Urine	Normal	275 ± 46 (conjugated)	250 ± 79 (conjugated)
	335	CSF	Normal	1440 ± 85* pg/ml	1180 ± 96 pg/ml
	263	CSF	Normal	2920 ± 390	2810 ± 230
	156	Urine	Parkinson's disease	97.8 ± 7.0** μg per 24 h	73.3 ± 5.5 μg per 24 h
	156	Urine	Parkinson's disease	281 ± 20 (conjugated)	299 ± 38 (conjugated)
3,4-Dihydroxy-phenylacetic acid	156	Urine	Normal	2300 ± 340 μg per 24 h	1830 ± 391 μg per 24 h
	872	Urine	Normal	1210	1310
	263	CSF	Normal	3120 ± 680 pg/ml	1910 ± 140 pg/ml
	524	CSF	Normal	Not significantly different	
	156	Urine	Parkinson's disease	1586 ± 145	1813 ± 200
	486	CSF	Parkinson's disease	Not significantly different	
Homovanillic acid	156	Urine	Normal	3.53 ± 0.28*** mg per 24 h	1.36 ± 0.23 mg per 24 h
	877	Urine	Normal	3.26 ± 0.26 mg/g creatinine	3.33 ± 0.39 mg/g creatinine
	384	Urine	Normal	4.9 ± 0.1 mg per 24 h	4.9 ± 0.1 mg per 24 h
	93	Urine	Normal	4.59 ± 0.40	3.75 ± 0.25
	141	Urine	Normal	5.53 ± 0.55**	3.51 ± 0.24
	872	Urine	Normal	5.19**	3.73
	93	Urine	Depressed	3.93 ± 0.47	3.28 ± 0.18
	156	Urine	Parkinson's disease	2.4 ± 0.2	3.4 ± 0.5
	442	Plasma	Normal	13.3 ± 0.6 ng/ml	14.0 ± 0.5 ng/ml
	438	Plasma	Normal	13.2 ± 0.8	12.5 ± 1.7
	822	Plasma	Normal	12.5	14.1
	440	Plasma	Normal	13.1 ± 1.1	12.3 ± 0.7
	440	Plasma	Depressed	11.7 ± 1.0*	17.9 ± 1.8
	442	Plasma	Psychotic	14.3 ± 0.7	19.2 ± 8.7

438	Plasma	Psychotic	11.7 ± 1.2*	20.9 ± 3.4
822	Plasma	Schizophrenic	12.3	16.3
816	Plasma	Schizophrenic	14.1	14.6
783	Plasma	Schizophrenic	7.1 ± 1.2	8.4 ± 1.1
784	Plasma	Schizophrenic	Not significantly different	
505	CSF	Normal	39.1 ± 3.8 ng/ml	47.8 ± 3.5 ng/ml
263	CSF	Normal	22.9 ± 4.1	19.9 ± 1.3
170	CSF	Normal	40.0 ± 2.3	43.7 ± 2.3
525	CSF	Normal	35.3 ± 2.5	39.8 ± 2.9
509	CSF	Normal	40.0 ± 1.2	43.7 ± 2.6
486, 488, 491, 511 524, 534, 868	CSF	Normal	Not significantly different	
675	CSF	Manic	45.7 ± 6.7	43.0 ± 4.6
683	CSF	Depressed	28.6 ± 2.4	34.4 ± 2.5
505	CSF	Depressed	35.0 ± 4.4	39.8 ± 3.5
505	CSF	Suicidal	27.5 ± 3.4	36.6 ± 4.2
685	CSF	Depressed	40.2 ± 6.2	40.8 ± 4.6
686	CSF	Depressed	30.3 ± 1.7*	37.1 ± 2.6
170	CSF	Depressed	33.1 ± 1.9	36.9 ± 3.8
170, 509	CSF	Manic	43.7 ± 5.4	59.0 ± 10.4
688	CSF	Depressed	30.1 ± 1.7*	35.9 ± 2.7
691	CSF	Depressed	28.0 ± 3.0	33.6 ± 2.5
488	CSF	Depressed	Not significantly different	
795	CSF	Schizophrenic	26.0 ± 5.8*	44.2 ± 3.5
695	CSF	Schizophrenic	26.2 ± 3.1**	38.8 ± 3.1
661	CSF	Schizophrenic	32.4 ± 2.5	37.9 ± 5.3
534	CSF	Schizophrenic	Not significantly different	
491	CSF	Parkinson's disease	13.7 ± 1.1	15.0 ± 1.2
486	CSF	Parkinson's disease	Not significantly different	
511	CSF	Attention deficit	Not significantly different	

* $p < 0.05$ *vs.* females.
** $p < 0.01$ *vs.* females.
*** $p < 0.001$ *vs.* females.

TABLE 63

MALE–FEMALE COMPARISON OF TRACE AMINE METABOLITE CONCENTRATIONS

Amine or metabolite	*Reference*	*Biological fluid*	*Control disorder*	*Male*	*Female*
Phenylethyl-amine	93	Urine	Normal	4.7 ± 0.7 μg per 24 h	5.0 ± 0.8 μg per 24 h
	878	Urine	Normal	7.3 ± 2.9 μg/g creatinine	14.5 ± 3.1 μg/g creatinine
	101	Urine	Normal	Not significantly different	
	93	Urine	Depressed	6.24 ± 1.58 μg per 24 h	4.74 ± 0.94 μg per 24 h
	101	Urine	Schizophrenic	Not significantly different	
	878	Plasma	Normal	4.3 ± 0.4 ng/ml	5.6 ± 0.8 ng/ml
Phenylacetic acid (total)	93	Urine	Normal	121.9 ± 18.0 mg per 24 h	119.8 ± 20.9 mg per 24 h
	105	Urine	Normal	138.1 ± 10.4	160.9 ± 26.3
	72	Urine	Normal	129.0 ± 74.2 (S.D.)	156.6 ± 18.0 (S.D.)
	418	Urine	Normal	Not significantly different	
	93	Urine	Depressed	123.9 ± 9.0	109.9 ± 19.0
	72	Urine	Depressed	164.4 ± 14 (S.D.)	132.0 ± 102.0 (S.D.)
(unconjugated)	424	Plasma	Normal	79.5 ± 13.5* ng/ml	119.5 ± 19.1 ng/ml
(conjugated)	424	Plasma	Normal	296.4 ± 42.9**	365.6 ± 59.2
(unconjugated)	39	CSF[a]	Normal	26.7 ± 2.7	30.8 ± 5.2
	39	CSF	Depressed	13.8 ± 1.9**	23.6 ± 2.3
o-Hydroxy-phenylacetic acid	93	Urine	Normal	1.3 ± 0.2 mg per 24 h	0.8 ± 0.1 mg per 24 h
	93	Urine	Depressed	0.9 ± 0.1	0.8 ± 0.1

m-Hydroxy-	93	Urine	Normal	5.3 ± 1.3	6.2 ± 0.9
phenylacetic	93	Urine	Depressed	4.1 ± 0.9	4.7 ± 0.9
acid	424	Plasma	Normal	16.6 ± 3.7	22.6 ± 8.3
p-Hydroxy-	93	Urine	Normal	19.6 ± 3.6	22.3 ± 2.4
phenylacetic	93	Urine	Depressed	12.2 ± 2.2	13.8 ± 1.5
acid	424	Plasma	Normal	66.6 ± 22.0 ng/ml	59.6 ± 13.0 ng/ml
p-Hydroxy-	93	Urine	Normal	2.03 ± 0.32 mg per 24 h	1.59 ± 0.22 mg per 24 h
mandelic acid	93	Urine	Depressed	1.47 ± 0.31	0.96 ± 0.15
Tryptamine	769	Urine	Normal	64 μg per 24 h	56 μg per 24 h
	769	Urine	Depressed	39	30
	769	Urine	Schizophrenic	52	72
Indoleacetic	460	CSF[a]	Epileptic	3.4 ± 0.4 ng/ml	4.3 ± 0.6 ng/ml
acid	460	CSF[b]	Epileptic	2.8 ± 0.4***	4.2 ± 0.5
	667	CSF	Depressed	6.4 ± 1.4	6.4 ± 0.8
	461	CSF	Depressed	4.46 ± 0.30	4.95 ± 0.44
Histamine	879	Urine	Normal	42 μg per 24 h	49 μg per 24 h
	880	Urine	Normal	33.3 μg/l	44.4 μg/l
	80	Urine	Normal	11.5 ± 1.0 μg per 24 h	14.3 ± 1.7 μg per 24 h
	199	Plasma	Normal	790 ± 180 pg/ml	760 ± 82 pg/ml
N-Methylhistamine	880	Urine	Normal	250 μg per 24 h	163 μg per 24 h

* $p < 0.05$ *vs.* females.
** $p < 0.01$ *vs.* females.
*** $p < 0.001$ *vs.* females.
[a] Lumbar CSF.
[b] Cisternal CSF.

There was no evidence of cultural or environmental contributions to MHPG levels, but for HVA and 5-HIAA a familial influence was found where cultural heritability was higher than the genetic[898]. Urinary phenylethylamine excretion is lower in both schizophrenic and non-schizophrenic East Indian subjects than in similar North American subjects[101]. Whether this is due to dietary, cultural or other environmental factors or to genetics was not established. White males have been shown to exhibit higher plasma noradrenaline levels than black males, although females were not different[253].

6.1.7. Physical-organic diseases

Highly significant increases in the urinary excretion of VMA[387,394,401,900–904], HVA[381,387,904], MHPG conjugates[11,387,575], normetanephrine[902,905] and 5-HIAA[370,906,907] have been observed in cancer patients, particularly those suffering from pheochromocytoma. 5-HIAA and serotonin urinary excretions[907] and plasma[908] levels are elevated in patients with functioning carcinoid tumors but not in those with non-functioning or non-carcinoid tumors.

Plasma[220,228,264] and urinary[861] catecholamines and plasma[586] and urinary[151,566] MHPG have been shown to be positively correlated with blood pressure. Further, the CSF[220] and plasma[158,228,909] concentrations of noradrenaline, adrenaline and dopamine have been reported to be significantly elevated in subjects with essential hypertension, although these results were not confirmed by other investigators[252,253]. Urinary HVA and VMA excretion are also elevated in hypertensive patients[392].

In poorly controlled diabetics, increases in plasma noradrenaline have been demonstrated[258]. In Huntington's chorea, both the CSF concentration and turnover of HVA have been shown to be significantly reduced[513]; in an early study, however, the urinary excretion of HVA was normal[382]. Phenylethylamine excretion in phenylketonuria is highly significantly elevated[899]. A dysfunction of the blood–CSF barrier has been reported to cause significantly increased CSF concentrations of noradrenaline and adrenaline[262].

6.2. Environmental variables

6.2.1. Diet

Fasting has been employed as a means of determining the degree to which diet contributes to biogenic amine and metabolite levels in plasma and urine. Plasma HVA[783], phenylethylamine and *p*-tyramine[99,102] and urinary HVA[390], 3,4-dimethoxyphenylethylamine[73], VMA[390,406,409] and MHPG[70,563] have been reported to be unaffected by fasting. However, contradictory results have shown that fasting does eliminate dietary effects on plasma HVA[550,913] and urinary VMA[911]. Fasting or a diet restricted in foods known to contain caffeine[910] or protein[156] reduces the excretion of catecholamines[156,910] and their metabolites, free and conjugated histamine and its metabolites[78,912] and free plasma phenylacetic acid[424].

The ingestion of bananas, pineapple and some other foods such as plums, tomatoes and walnuts produces markedly elevated levels of urinary 5-HIAA[116,362,369,872,915]. Bananas have also been reported to result in increased urinary excretion of VMA, DOPAC and HVA[872] and plasma dopamine[240,322,916] and

noradrenaline[240,916], particularly the sulfate conjugates. Eating oranges has been reported to increase the excretion of *p*-hydroxymandelic acid[355]. Caffeine intake appears to affect primarily the noradrenergic system, increasing plasma noradrenaline and adrenaline concentrations[172,590], plasma MHPG[590] and urinary normetanephrine and metanephrine[173,910]. The ingestion of chocolate, which contains phenylethylamine, *p*-tyramine and other biogenic amines, produced no significant changes in the urinary excretion of these amines[99,100], whereas the ingestion of cheese, which also contains some biogenic amines, has been shown to produce a small increase in the excretion of conjugated *p*-tyramine and unconjugated *p*-hydroxyphenylacetic acid[107]. Urinary excretion rates of phenylethylamine[806,914] and phenylacetic acid[806] exhibited no dietary influences.

6.2.2. Smoking

Cryer *et al.*[293] have reported significantly elevated plasma noradrenaline and adrenaline concentrations in smokers. Other investigators have found an increase in the urinary excretion of adrenaline[132,350] and 1,4- and 1,5-methylimidazoleacetic acid[350,888] in smokers. No effect of smoking on VMA, 5-HIAA or histamine excretion was observed, however. It was concluded[350] that regular smokers metabolize certain endogenous amines differently from non-smokers and that this difference may be at least partly responsible for the withdrawal symptoms sometimes seen on stopping smoking. Davidson *et al.*[550] have demonstrated that smoking two cigarettes has no effect on the HVA concentration in plasma. Cyanide and formaldehyde in the saliva of cigarette smokers has been shown to react with phenylethylamine and *p*-tyramine to produce N-cyanomethyl derivatives[917].

6.2.3. Alcohol consumption

The catecholamines have been reported to be elevated in the urine[918,919] and plasma[920,921] of intoxicated subjects, although delay in the increase in plasma noradrenaline concentration following alcohol consumption has been observed[921]. An increase in plasma levels of normetanephrine and metanephrine, a significant decline in VMA excretion and a concomitant elevation in MHPG excretion have been reported to occur while subjects were drinking[920,922]. The MHPG concentration in CSF was found to be correlated with blood alcohol and to be significantly elevated during intoxication of alcoholic patients and also in healthy volunteers after ingesting 80 g of alcohol[923]. These data indicate that alcohol ingestion stimulates noradrenaline and adrenaline metabolism in the central nervous system and may be associated with an alteration in the pathways of catecholamine metabolism. Stress-induced adrenaline release (as measured by urinary excretion) has been found to be lower in non-alcoholic adoptees with alcoholic biologic relatives than in similar controls with no alcoholic relatives, suggesting that familial alcoholism may be associated with a trait of globally decreased adrenaline responsiveness[919].

The HVA concentrations in the CSF of alcoholics do not seem to differ significantly from those of controls[733,867,923], although there may be a sub-group of alcoholics who are non-suppressors in the dexamethasone suppression test and exhibit a low CSF HVA concentration[687]. 5-HIAA concentrations in the CSF of alcoholics, although tending to be lower, are not significantly different from the concentrations in non-alcoholics[463,923]. However, after 4–9 weeks of abstinence or in the immediate

post-intoxication phase, a significant decline in the CSF concentration of 5-HIAA has been reported[867]. However, alcoholic murderers proved to have significantly more 5-HIAA in their CSF than did non-alcoholic murderers[733]. Serum indoleacetic acid in alcoholics is not different from that in controls[429].

6.2.4. Stress and posture

Plasma and urinary noradrenaline can be altered by posture changes, exercize, emotional stress, various environmental factors and the method of blood sampling. Mild physical stress, as in changing posture from recumbent to standing, produces a statistically significant increase in plasma free noradrenaline (Table 64) but not in conjugated noradrenaline or free or conjugated adrenaline. However, the urinary excretion of both catecholamines has been reported to be significantly elevated in standing compared with supine subjects[129,924]. Both schizophrenic[254] and depressive[285,288,723] subjects exhibited a greater increase in plasma noradrenaline levels during stress than did controls. Furthermore, dexamethasone-resistant depressed patients showed higher noradrenaline and adrenaline plasma levels in both the lying and standing positions than did non-resistant depressed subjects, suggesting that an inefficient hyperactivity to physiological stress characterizes a noradrenergic dysregulation in depression[761]. Posture appears to have no influence on the CSF concentrations of amine metabolites[890].

More vigorous physical stress, such as walking, bicycling, handgrip contractions and knee-bends, increases urinary[124] and plasma noradrenaline concentrations (Table 65); the increases in plasma concentrations have been found to be correlated with oxygen consumption[929]. Increases in plasma adrenaline[234,299] and dopamine[234] levels in response to physical activity have also been reported. Depending on the type and duration of the exercize, MHPG excretion may be either increased[924,930] or unchanged[544,931]. Similarly, plasma MHPG[931] and DHPG[235,244,289,604] may be increased[244,289] or unchanged[235,604,931], depending on the stressor. Plasma HVA has been reported to be unchanged during exercize[550,783], although Kendler *et al.*[913] claimed an increase in plasma HVA during moderate exercise. Plasma histamine[932] and urinary phenylacetic acid[351] are unaffected by physical stressors.

Exposure to cold is mainly associated with increases in urinary[134,925] and plasma[231,238,239,288,299,927] concentrations of noradrenaline, although urinary[134] and plasma[239,299] adrenaline, plasma dopamine[239] and urinary MHPG[23] have also been reported to be increased by cold stress. The effect of cold immersion on noradrenaline is significantly greater in depressed than in normal subjects[288], showing that the noradrenergic system is dysregulated in depression.

Mental stress involving exhilarating or aggressive reactions has been associated with elevated noradrenaline excretion, whereas emotional stress involving apprehension, anxiety, pain or general discomfort is regularly accompanied by an increase in adrenaline excretion[925,933]. MHPG excretion has been reported to be significantly increased in aviators during carrier landings[934], as has phenylethylamine excretion in parachutists[914]. On the other hand, trainee pilots showed no significant changes in phenylethylamine or phenylacetic acid excretion after training flights[96]. Venipuncture generally results in an increase in plasma noradrenaline[229,251,926], although Kopin *et al.*[266] found a decrease in such stressful situations as venipuncture and hospitalization. Hospitalization has been claimed to result in increased CSF concen-

TABLE 64

PLASMA FREE CATECHOLAMINES IN NORMAL RECUMBENT AND STANDING SUBJECTS

Reference	*Noradrenaline*[a]		*Adrenaline*[a]		*Dopamine*[a]	
	Recumbent	*Standing*	*Recumbent*	*Standing*	*Recumbent*	*Standing*
248	292 ± 16	538 ± 44				
227	160 ± 20	270 ± 40	160 ± 50	210 ± 80		
228	248 ± 56	559 ± 126	41 ± 5	52 ± 9	46 ± 6	50 ± 9
265	444 ± 39	2210 ± 473				
260	182 ± 53	403 ± 87	87 ± 25	115 ± 41	33 ± 11	51 ± 18
251	279 ± 20	659 ± 83				
252	403 ± 61	639 ± 81				
274	200 ± 40	323 ± 33	58 ± 12	54 ± 13	48 ± 10	48 ± 8
235	215 ± 28	400 ± 36	83 ± 12	126 ± 11		
236	196 ± 17	336 ± 22	36 ± 4	59 ± 7	62 ± 5	56 ± 6
237	217 ± 58	351 ± 21				
275	297 ± 30	500 ± 40				
277	166 ± 14	372 ± 35	59 ± 11	51 ± 9		
254	201 ± 24	365 ± 40				
238	252 ± 34	461 ± 41	48 ± 11	57 ± 11		
245	208 ± 45	440 ± 65				
284	147 ± 10	291 ± 18				
289	149 ± 25	450 ± 18				
Weighted mean:	235 ± 26	502 ± 61	71 ± 15	89 ± 20	49 ± 8	51 ± 9
Total no. of subjects:	360	323	76	72	36	36

[a] Mean ± standard error of the mean, pg/ml.

TABLE 65

EFFECT OF PHYSICAL EXERCIZE ON PLASMA CATECHOLAMINE CONCENTRATIONS

Reference	*Noradrenaline*[a]			*Adrenaline*[a]		
	Resting	*Exercize*	*Type of exercize*	*Resting*	*Exercize*	*Type of exercize*
216	155 ± 13	373 ± 39		48 ± 3	51 ± 22	
936	–	2200 ± 100	Heavy			
219	200 ± 30	1010 ± 70	Heavy	60 ± 10	150 ± 20	Heavy
928	140	240		40	60	
937	950 ± 8	2400	Handgrip	420 ± 9	1000	Handgrip
221	108 ± 9	1000 ± 78		329 ± 21	359 ± 28	
248	292 ± 16	778 ± 80	Handgrip			
938	740 ± 160	1070 ± 200		690 ± 150	710 ± 120	
232	210 ± 11	443 ± 47	Walking	29 ± 9	42 ± 12	Walking
235	215 ± 28	259 ± 35	Handgrip	83 ± 12	152 ± 30	Handgrip
267	583 ± 39	1726 ± 264		117 ± 17	164 ± 13	
268	426 ± 81	2000 ± 380	Bicycling			
277	166 ± 44	1285 ± 425	Bicycling	59 ± 11	445 ± 137	Bicycling
144	365 ± 41	1740 ± 99		< 100	230 ± 100	
238	252 ± 34	1624 ± 316	Bicycling	48 ± 11	210 ± 70	Bicycling
244	265 ± 18	620 ± 70				
271	355 ± 58	4235 ± 1031	Maximal	71 ± 17	821 ± 235	Maximal

[a] Mean ± standard error of the mean, pg/ml.

trations of 5-HIAA and HVA[524], but another study showed no effect[170]. Plasma adrenaline shows a greater increase to cognitive stressors than to other stressors[926]. A combination of submaximal work and mental tasks induced a significant increase in the excretion of adrenaline, metanephrine and MHPG[924]. Examination stress, public speaking and mental arithmetic have been shown to increase plasma adrenaline[231,267,299,935], noradrenaline[279] or both[289,926] with the noradrenaline increase being less pronounced than that of adrenaline. Urinary MHPG and HVA (but not 5-HIAA) increased significantly during mental stress[141]. Stress and anxiety have been associated with elevated MHPG excretion, but it proved not to be possible to predict that an individual with high state anxiety will necessarily have a high urinary MHPG excretion or *vice versa*[731]. Alleviation of mental stress by transcendental meditation did not result in a lowering of plasma catecholamine levels[226].

6.2.5. Circadian, seasonal and menstrual cycles

Many physiological variables display circadian rhythms – a waxing and waning apparent only if frequent measurements are taken across the 24-h day[885]. Because the rhythms may not be synchronized in all individuals, a single measurement, even if taken at the same time for all subjects, may produce misleading results. The most dramatic day–night cycle is that of melatonin in plasma or serum[314,315,317–319,342,660,940], CSF[319,342] and urine[187,316,317], in which the night-time values are significantly higher than during the day. The male and female cycles appear to be the same[342,941]. During depression, the circadian rhythm of melatonin is disturbed but returns to normal on recovery[660]. 6-Hydroxymelatonin and its sulfate conjugate also exhibit a marked (up to 10-fold) diurnal variation in urinary excretion[188,317,876,940], which can be abolished by treatment with atenolol[317].

Noradrenaline and adrenaline excretion peaks during the day and declines at night[124,129,942–945], although the diurnal variation is lost during excercize. In contrast, dopamine excretion tends to increase during sleep[943,944,946]. Normetanephrine and metanephrine excretion show no diurnal variation[195]. Plasma noradrenaline and adrenaline have been reported to be significantly elevated during the day[947,948], although a later study revealed no diurnal variation[863]. These contradictions may be explained by another study which demonstrated that recumbent subjects exhibited a fluctuation in plasma noradrenaline levels with a median period of 107 min regardless of the time of day. The amplitude of the fluctuations was at times greater than that due to postural change[949]. Unconjugated urinary histamine is significantly higher during the day[79]. Urinary phenylethylamine excretion does not appear to show a diurnal variation[91,806,914]; however, one study has demonstrated phenylethylamine excretion to be highest between 4 and 12 p.m.[89].

Plasma MHPG[37,38,573,950] and HVA[950] exhibit a circadian rhythm with peak levels occuring during the day. In subjects who follow a constant routine, the MHPG, but not the HVA, variation is abolished[950]. It was concluded that diurnal variations for MHPG are evoked by changes in physical activity, posture and related factors, whereas the major component of HVA diurnal variation is regulated by a circadian oscillator independent of sleep or activity. In depressed patients the circadian rhythms of MHPG[573,951] and VMA[951] become desynchronized. Daily fluctuations in urinary MHPG excretion peak during the day, necessitating 24-h collections[36,70,563,952]. However, it has been demonstrated that afternoon plasma MHPG

values correlate well with 24-h excretion values[607]. In depressed patients, the urinary MHPG excretion cycle is about 3 h earlier than in normal subjects[952]. During cold exposure, the circadian rhythm of MHPG excretion, but not of VMA excretion, is abolished[23]. VMA excretion may be as much as twice as high during the day as at night[12,23,378,403]. Neither free nor conjugated plasma 3,4-dihydroxyphenyl glycol exhibit a circadian rhythm[953], although the sulfate showed a slight elevation in the afternoon. Both urinary[378] and plasma[297] 3,4-dihydroxyphenylacetic acid exhibit a marked daily periodicity, with the low point occuring at night. Urinary HVA and 3,4-dihydroxymandelic acid show a small diurnal variation[378,403]. The concentration of 5-HIAA in CSF increases progressively during the day, reaching its highest value around midnight and then decreasing by half by morning[954]. No diurnal variation in the urinary[806] or CSF[39] concentrations of phenylacetic acid were observed.

Cycles longer than the circadian rhythms are more difficult to measure. A trend towards elevated dopamine and serotonin metabolite levels in CSF in the late autumn have been reported, with the lowest levels occuring in late spring[883,955], but this could not be confirmed in later studies[524,736]. CSF samples from schizophrenic and Alzheimer patients during October to March had significantly higher concentrations of HVA and 5-HIAA (but not MHPG) than samples taken during April to September[956]. Urinary MHPG excretion is reported to be above normal in winter and below normal in summer[951].

The menstrual cycle has been reported to be the cause of variations in the excretion of histamine and its metabolites, the levels depending on the place in the cycle at the time of sampling[888]. Plasma HVA has been reported to decline after ovulation[783].

6.2.6. Sample stability and storage conditions

It has been claimed that catecholamines in plasma[959] or urine[960] are stable for periods of up to 240 days if they are frozen with antioxidants; however, other investigators have shown that storage of plasma samples with antioxidants at low temperatures was not sufficient to prevent the decomposition of unconjugated catecholamines[961,962]. Autoxidation appears to be particularly rapid when the catecholamines are placed in buffer solutions[962]. Some workers have reported that small delays in freezing samples of plasma catecholamines result in major losses[244,961], whereas others claimed that specimens stored at room temperature for periods of up to 3 weeks show no significant decline in catecholamine concentrations[963]. The contradictory nature of these results clearly indicates that caution should prevail and that the catecholamines should be analyzed as soon as possible after sample collection.

For serotonin, the best results are obtained if samples are stored at pH 6.5 and -20°C[964,965]; at acidic pH and room temperature appreciable degradation occurs[958,965,966]. Degradation of both 5-HIAA and serotonin has been reported to accelerate in the summer months, suggesting sensitivity to light[958]. In quantifying plasma serotonin, the plasma fraction must not only be platelet- and cell-free, but should also be subjected to ultrafiltration to remove protein-bound serotonin before a true plasma serotonin value can be obtained[306,307]. An albumin–sucrose "cushion" has been demonstrated to prevent platelet damage during differential centrifugation[301]. In addition, the use of prostacyclin during blood collection minimizes platelet aggregation during which platelets may be activated and release serotonin. These

procedural modifications resulted in consistent values for serotonin which were much smaller than previously published results[301]. Sulfatoxymelatonin in plasma or urine has been shown to be stable without preservative for at least 2 years at −20°C and for 5 days at +20°C[190], suggesting that the instability of the serotonin moiety resides at the free hydroxyl group. Like serotonin, 5-HIAA is stable for up to 1 month if stored at pH 6.5 and −20°C[520,957,965]. Decomposition is facilitated by light[520,958,965–967], high or room temperature[966,967] and acidic conditions[957,958,965,966].

MHPG in urine[564,567,610] and CSF[520,610,957] is stable if stored at neutral pH and −20°C (or colder) for periods of at least 2 months and for up to 30 months[564]. At acidic pH, urinary conjugated MHPG has been reported to hydrolyze almost completely within 2 weeks even at −14°C[546]. Homovanillic acid in CSF appears to be stable for long periods of storage[520,957]. 3,4-Dihydroxyphenyl glycol and 3,4-dihydroxyphenylethanol concentrations in plasma and CSF have been reported to increase after freeze–thaw cycles, although this does not appear to be due to a breakdown of the sulfate conjugate[602].

A recent study of the effect of long-term (up to 9 months) storage of untreated frozen (*i.e.*, at physiological pH and −20°C) plasma and urine samples on the concentrations of several biogenic amine metabolites demonstrated significant declines in the urinary concentrations of *m*-hydroxyphenylacetic acid, *p*-hydroxymandelic acid IAA, 5-HIAA, HVA, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenyl glycol; only the phenylacetic acid, mandelic acid and VMA concentrations remained unchanged[968]. The concentration of *p*-hydroxyphenylacetic acid appeared to increase over the 9-month period, possibly owing to breakdown of the sulfate conjugate. On the other hand, the plasma concentrations remained stable except for 5-HIAA acid and *m*-hydroxyphenylacetic acid, which declined significantly.

Clearly, failure to standardize storage time and conditions within and between studies may invalidate the results and could account, in part, for many of the inconsistencies in the results reported in the literature.

7. CONCLUDING REMARKS

A generation of scientists have devoted their efforts to finding biological markers for psychiatric and neurological disorders among the biogenic amines and their metabolites, partly in the hope of opening a window on the underlying mechanisms of the disorders and partly, from the practical side, in anticipation of providing useful tools for diagnosis and treatment. Overall, the results have been disappointing, inconsistent and often contradictory. In the early studies problems with the specificity and sensitivity of the analytical procedures were responsible for the lack of agreement in the results of different groups. With modern, sensitive methodology such as GC–MS and HPLC, however, these worries are behind us and new difficulties have become apparent. The fact that, in spite of sophisticated analytical methods, no single variable or group of variables have yet been unequivocally identified as markers may lie in the multifactorial nature of psychiatric disorders and, importantly, in persisting differences in the diagnostic criteria employed by different groups of researchers. In addition, because factors such as age, sex, height and weight of the subject, together with environmental factors such as diet, smoking, drinking and drug therapy, are now known to affect the concentrations of the biogenic amines and their metabolites,

it is clear that it is imperative that controls be matched with patients for these variables, difficult as this may be. Further, the small numbers of subjects in most previous studies has undermined the potential statistical power of even the best research designs.

Clearly, future studies in the field of markers for psychiatric disorders will prove to be no more consistent than past studies unless age-, sex- and height-matched groups of large numbers of subjects and widely accepted, well defined diagnostic criteria are employed.

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9. SUMMARY

The biogenic monoamines and their metabolites have been isolated, identified and quantified in human body fluids over the past forty years using a wide variety of chromatographic separation and detection techniques. This review summarizes the results of those studies on normal, psychiatric and neurological subjects. Tables of normal values and the methods used to obtain them should prove to be useful as a reference source for benchmark amine and metabolite concentrations and for successful analytical procedures for their chromatographic separation, detection and quantification. Summaries of the often contradictory results of the application of these methods to psychiatric and neurological problems are presented and may assist in the assessment of the validity of the results of experiments in this field. Finally, the individual, environmental and the methodological factors affecting the concentrations of the amines and their metabolites are discussed.

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NATURE OF TEMPERATURE GRADIENTS IN CAPILLARY ZONE ELECTROPHORESIS

ALLAN E. JONES
Engineering Department, E.I. du Pont de Nemours, Wilmington, DE 19898 (U.S.A.)
and
ELI GRUSHKA*[a]
Central Research and Development, E.I. du Pont de Nemours, Wilmington, DE 19898 (U.S.A.)
(Received September 5th, 1988)

SUMMARY

An expression for the radial temperature profile in capillary zone electrophoresis was derived, taking into account the temperature dependence of the buffer electrical conductivity and the polyimide coating of the quartz capillary. Calculations show that in typical capillary zone electrophoresis experiments; *i.e.*, capillaries with 50–100 μm I.D., 375 μm O.D., and up to 5 W power input, the temperature profile rigorously derived is nearly identical to a parabolic profile. At high input powers, the parabolic approximation underestimates the temperature at the capillary center.

INTRODUCTION

Capillary zone electrophoresis (CZE) is a separation technique the plate height efficiency of which can be very high. In practice, however, the theoretically calculated efficiencies are not observed. One possible reason for the lower than theoretical efficiencies can be traced to heating effect due to the passage of current through the capillary[1]. This heating effect causes a temperature difference between the center of the capillary and the wall, which, in turn, causes differences in the viscosity of the buffer electrolyte within the column. The resulting radial viscosity gradient produces a velocity difference between the center of the capillary and the wall. This velocity difference gives rise to mass transfer effects which lower the system efficiency. Grushka *et al.*[2] have studied the effect of temperature differences on the efficiency of CZE. In that study the assumed temperature profile produced a parabolic velocity profile across the capillary. In the present communication, this parabolic temperature profile assumption is justified.

[a] Permanent address: Department of Inorganic and Analytical Chemistry, The Hebrew University, Jerusalem, Israel.

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THEORY

Derivation of temperature profile

Modern CZE separations are carried out in quartz capillaries that are coated with a thin layer of polyimide. In this analysis, R_1 symbolizes the internal radius, R_2 the quartz radius and R_c the total radius of the capillary. The temperature dependence of the thermal and electrical conductivities should be taken into account in the heat balance equations in order to obtain the correct temperature profile. Using an approach similar to that of Coxon and Binder[3] and by Brown and Hinckle[4], the heat balance equation for CZE can be written as

$$\frac{1}{r}\frac{\mathrm{d}}{\mathrm{d}r}\left(r\frac{\mathrm{d}T}{\mathrm{d}r}\right) = -\frac{G}{k_1} \tag{1}$$

where T is the temperature, r is the radial position, G is the heat generation per unit volume and k_1 is the termal conductivity of the buffer solution. The boundary condition is

$$-R_1k_1\frac{\mathrm{d}T}{\mathrm{d}r} = UR_1(T_1 - T_s) \qquad \text{at } r = R_1$$

where R_1 is the internal radius of the capillary, T_1 and T_s are the temperature at the glass wall and the capillary surroundings (*i.e.*, a thermostated bath) respectively, (at R_1), and k_1 is the thermal conductivity of the buffer. The quantity UR_1 is related to the heat dissipation through the capillary wall, and it is given by

$$\frac{1}{R_1U} = \frac{1}{k_2}\ln\left(\frac{R_2}{R_1}\right) + \frac{1}{k_c}\ln\left(\frac{R_c}{R_2}\right) + \frac{1}{R_ch} \tag{2}$$

where R and k indicate radius and thermal conductivity and subscripts 2 and c indicate quantities relating to the quartz glass and polyimide coating, respectively. Because of the electrical conductivity dependence on temperature, the rate of heat generation will be written as

$$G = G_0\left[1 + \alpha\left(\frac{T - T_s}{T_s}\right)\right] \tag{3}$$

where α is the temperature coefficient of electrical conductivity of the buffer, G_0 is the rate of heat generation in the absence of temperature dependence of the electrical conductivity and T_s is the temperature of the capillary surroundings. When this temperature dependence of the heat generation is taken into account, the equation describing the heat balance can be written as[3]

$$\frac{1}{y}\frac{\partial}{\partial y}\left(y\frac{\partial\theta}{\partial y}\right) = -S(1 + \alpha\theta) \tag{4}$$

where

$$y = \frac{r}{R_c}$$

$$S = \frac{G_0 R_c^2}{k_1 T_s}$$

$$\theta = \frac{T - T_s}{T_s}$$

The boundary condition is

$$-k_1 \frac{d\theta}{dy} = UR_c\theta_1 \qquad \text{at } y = y_1$$

The solution of eqn. 4 is

$$\theta = AJ_0(\beta y) - \frac{1}{\alpha} \tag{5}$$

where $\beta^2 = \alpha S$ and A is a constant of integration. Using the boundary conditions it can be shown that

$$A = \frac{1}{\alpha[J_0(\beta y_1) - (\beta/\gamma) J_1(\beta y_1)]} \tag{6}$$

where

$$\gamma = \frac{UR_c}{k_1}$$

The expression for the temperature profile is, therefore:

$$\theta = \frac{1}{\alpha}\left\{\frac{J_0(\beta y)}{[J_0(\beta y_1) - (\beta/\gamma) J_1(\beta y_1)]} - 1\right\} \tag{7}$$

or

$$T = \frac{T_s}{\alpha}\left\{\frac{J_0(\beta y)}{[J_0(\beta y_1) - (\beta/\gamma) J_1(\beta y_1)]} - 1\right\} + T_s \tag{8}$$

Evaluation of the temperature profile

The temperature expression in eqn. 8 is similar to the expression obtained by Coxon and Binder[3]. However, eqn. 8 accounts for the fact that the quartz capillary tubing used in CZE is coated with polyimide. To evaluate the temperature profile, it is

essential to obtain G_0, which is needed to calculate β. Coxon and Binder[3] did not describe the manner by which G_0 is obtained. Since the correct evaluation of G_0 is crucial to the proper determination of the temperature, we will elaborate here on this point.

Since heat dissipation in the capillary is from the center to the wall, we will re-write the expression for G in terms of a differential equation in the radial direction

$$dG = 2\pi rLG_0(1 + \alpha\theta)dr$$

Or, in terms of reduced radius

$$dG = 2\pi LR_c^2G_0(1 + \alpha\theta)ydy \tag{9}$$

Substituting for θ from eqn. 7 and rearranging, we get

$$dG = 2\pi LR_c^2G_0[\alpha AJ_0(\beta y)]ydy \tag{10}$$

Since the electrical conductivity of the buffer varies radially with the temperature, we integrate over y. Thus, a substitution for A and integration between 0 and y_1 yield

$$G = \frac{2\pi LR_c^2G_0y_1J_1(\beta y_1)}{\beta[J_0(\beta y_1) - (\beta/\gamma)J_1(\beta y_1)]} \tag{11}$$

Eqn. 11 allows us to obtain the value of G_0 by iteration (note that β is also a function of G_0). However, due to the transcendental nature of eqn. 11, numerical procedures must be used.

Calculations

A computer program was written to compute G_0 iteratively for a given input power. The iteration of eqn. 11 should be done with caution since the equation can have many roots. The only physically significant solution is the smallest root. Therefore, the iteration should start with a low initial estimate of G_0 to insure the proper convergence.

Once the value of G_0 is determined, the temperature profile is calculated using eqn. 8, as well as the following parabolic profile

$$T = T_s + \frac{GR_c}{2U} + \frac{GR_1^2}{4k_1}\left(1 - \frac{r^2}{R_1^2}\right) \tag{12}$$

The values of the various parameters used in the calculation are given in the sub-titles of Tables I and II. The radii of the capillary used in the calculations are typical of the sizes used in practice; *i.e.*, inner diameter of 50 μm, quartz capillary outer diameter of 345 μm and polyimide coating thickness of 15 μm (giving a total outside diameter of 375 μm). Assuming that an 1-degree change will cause a 2% change in the conductivity, the value of α is then 7.75.

Table I gives the temperature at the capillary center, at the inner wall and ΔT as

TABLE I

BUFFER TEMPERATURES AT THE CENTER AND AT THE WALL OF THE CAPILLARY

Parameters used: $L = 1$ m; $R_2 = 1.725 \times 10^{-4}$ m; $R_c = 1.875 \times 10^{-4}$ m; $T_a = 298$ K; $h = 10000$ W/m^{2}K; $k_1 = 0.605$ W/mK; $k_2 = 1.5$ W/mK; $k_c = 0.155$ W/mK; $\alpha = 7.75$.

Power (W)	*Eqn. 8*			*Parabolic*		
	Center (K)	*Wall (K)*	*ΔT (K)*	*Center (K)*	*Wall (K)*	*ΔT (K)*
Internal diameter = 50 μm						
2	299.214	298.951	0.263	299.214	298.951	0.263
3	299.722	299.326	0.396	299.721	299.326	0.395
5	300.738	300.077	0.661	300.735	300.077	0.658
Internal diameter = 100 μm						
2	299.067	298.804	0.263	299.067	298.804	0.263
3	299.501	299.106	0.395	299.500	299.106	0.394
5	300.370	299.709	0.661	300.367	299.709	0.658

found from the two methods of calculation. Shown in Table I are results for several capillary internal radii and power inputs. The results in Table I have several important implications: (a) typical CZE systems exhibit small temperature drops between the center and the wall —under the usual operating conditions, temperature effects are minimal[2]; (b) within the limits of the power input shown in the table, the temperature difference between the center and the inner wall of the capillary is independent of the inner radius. However, the actual temperatures are a function of the internal radius, showing a decrease with an increase in the diameter; (c) the temperatures calculated using eqn. 8 are identical, for all practical purposes, to those calculated from eqn. 12 over the whole cross section of the capillary. Fig. 1 shows the temperature profile within the capillary. The line depicting the temperature behavior is actually the superposition of two lines, one calculated using eqn. 8 and the other calculated using the parabolic profile, eqn. 12.

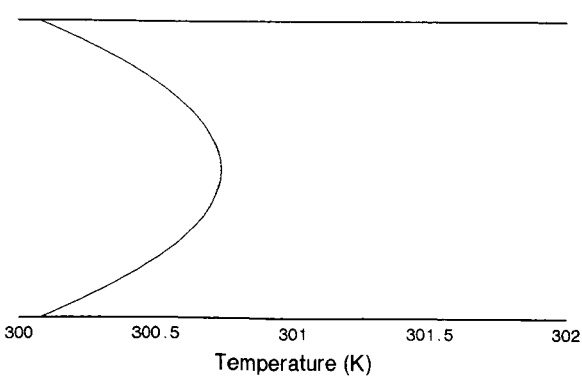

Fig. 1. Temperature profile as calculated from eqn. 8. Parabolic equation yields identical profile. Parameters used in the calculations are identical to those in Table I. Input power, 5 W; radius of capillary, 25 μm.

TABLE II

BUFFER TEMPERATURE AT THE CENTER AND AT THE WALL AT HIGH INPUT POWERS

Parameters used: $L = 1$ m; $R_2 = 1.725 \times 10^{-4}$ m; $R_c = 1.875 \times 10^{-4}$ m; $T_a = 298$ K; $h = 10000$ W/m^{2}K; $k_1 = 0.605$ W/mK; $k_2 = 1.5$ W/mK; $k_c = 0.155$ W/mK; $\alpha = 7.75$; internal diameter = 50 μm.

Power (W)	*Eqn. 8*			*Parabolic*		
	Center (K)	*Wall (K)*	*ΔT (K)*	*Center (K)*	*Wall (K)*	*ΔT (K)*
10	303.280	301.954	1.326	303.270	301.954	1.315
15	305.826	303.832	1.995	305.805	303.832	1.973
25	310.930	307.586	3.344	310.874	307.586	3.288

Table I describes the results for a capillary whose overall outer diameter is 375 μm. The conclusions drawn from the table are valid even if the capillary radius is changed, provided that the power input is the same. Eqn. 8 and the parabolic profile will yield similar results as long as the power input is relatively small. When the power is increased, the discrepancy between the two temperature profiles increases. Table II shows the results of the calculation for power inputs of 10, 15 and 25 W. If the CZE system is thermostated, such power inputs can be tolerated, as evidenced from the relatively low predicted temperatures. We see from Table II that as the power increases, eqn. 8 predicts a greater temperature difference between the center and the inner wall of the capillary. Both, eqns. 8 and 12 give a similar wall temperature. However, eqn. 8 calculates a higher center temperature than the parabolic equation. Fig. 2 shows the temperature profile as determined by both approaches. From Fig. 2 we can see that the greatest difference between eqn. 8 and the parabolic equation occurs in the center of the capillary. The difference between the two profiles is small even at very high power input values, which are not used in the conventional practice of CZE (*e.g.*, 0.06 degree difference at a power input of 25 W). At such high input powers, the temperature difference between center and wall is rather high (above 3 degrees) so that the contribution to the plate height is prohibitively high[2].

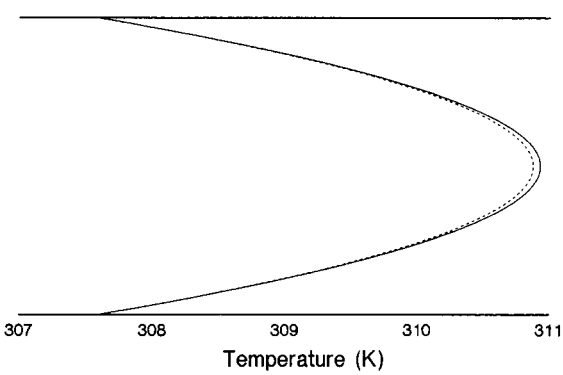

Fig. 2. Temperature profiles as calculated from eqn. 8 (——) and from the parabolic equation (– – – –). Parameters used in the calculations are identical to those in Table II. Input power, 25 W; radius of capillary, 25 μm.

With relatively wide tubes (several mm in diameter), the resulting current is quite high at voltages which yield reasonable analysis times. In such cases, the power dissipated in the tube is very high, and eqn. 8 will be more accurate than eqn. 12 in predicting the temperature profile. However, the use of very wide tubes for CZE is not recommended since the temperature difference between center-to-wall will be much too large to obtain efficient separations.

CONCLUSIONS

Under the normal operating conditions, the nearly identical behaviors of eqns. 8 and 12, justify the use of parabolic temperature profiles in determining the effect of temperature on the efficiencies of CZE separations[2].

SYMBOLS

A Integration constant
G heat generation rate (W/m^3)
G_0 heat generation in the absence of temperature dependence of the buffer (W/m^3)
h heat transfer coefficient (W/m^{2}K)
J_0 Bessel function of zero order and first kind
J_1 Bessel function of first order and first kind
k_1 thermal conductivity of the buffer (W/mK)
k_2 thermal conductivity of the capillary wall (W/mK)
k_c thermal conductivity of the polyimide coating (W/mK)
L capillary length (m)
R_1 inner radius of the capillary (m)
R_2 outer radius of the quartz wall (m)
R_c outer radius of the capillary; glass and polyimide (m)
S reduced coefficient of heat generation (see eqn. 4)
T_1 temperature at the inside wall of the capillary (K)
T_s temperature of the capillary surrounding (K)
U overall heat transfer coefficient (W/m^{2}K)
y_1 dimensionless radial position r/R_1
α coefficient of electrical conductivity of the buffer (dimensionless)
β $(\alpha S)^{1/2}$
γ reduced heat transfer coefficient (see eqn. 6)
θ reduced temperature (see eqn. 4)

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EFFECT OF THE CENTRAL ION OF OCTAHEDRAL TRANSITION METAL COMPLEXES ON THEIR THIN-LAYER CHROMATOGRAPHIC R_F VALUES

GORDANA VUČKOVIĆ and N. JURANIĆ
Institute of Chemistry, Faculty of Sciences, University of Beograd, P.O. Box 550, 11001 Beograd (Yugoslavia)
D. J. RADANOVIĆ
Department of Chemistry, Svetozar Marković University of Kragujevac, P.O. Box 60, 34001 Kragujevac (Yugoslavia)
and
M. B. ĆELAP*
Institute of Chemistry, Faculty of Sciences, University of Beograd, P.O. Box 550, 11001 Beograd (Yugoslavia)
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SUMMARY

The R_F values of twenty complexes of Cr^{III}, Co^{III}, Ru^{III}, Rh^{III}, Fe^{II}, Co^{II}, Ni^{II} and Zn^{II} containing ligands such as ethylenediamine-N,N′-diacetato-N,N′-di-3-propionato (eddadp), ethylenediaminetetra-3-propionato (edtp), 2,4-pentanedionato (acac), 1-phenyl-1,3-butanedionato ion (bzac) or α,α′-dipyridyl (dipy), were determined by thin-layer chromatography. Development was carried out with 22 single-component solvents. The results obtained for anionic and neutral complexes can best be explained by using the polarization power of the central ions of the complexes, assuming an adsorption separation mechanism.

INTRODUCTION

In previous studies we investigated the effect of various factors on the R_F values of transition metal complexes obtained by thin-layer chromatography (TLC) on silica gel and/or aluminium oxide. The effects of the geometric configuration of octahedral[1], square-planar[2] and facial-meridional isomers[3], of the chelate ring size[1], of the absolute configuration of a complex[4] and of the length of the coordinated ligand side-chain were studied[1,5].

In this work we examined the effect of the central ion. Many papers[6] have dealt with TLC separations on different adsorbents of various transition metal complexes containing the same ligands. However, the aim in almost all of those studies was simply to achieve the chromatographic separation, and not to establish the regularities in the chromatographic behaviour of the complexes. In most instances the complexes of various metals were not previously isolated in the solid state, the chromatographic separations being carried out using solutions containing simple salts and the corresponding ligands.

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In only two of the papers published so far were attempts made to correlate the nature of the central ion of the complexes with the R_F values[7,8]. Subbotina *et al.*[7] separated β-diketonato complexes of lanthanides on thin layers of aluminium oxide using multi-component solvent systems. On the basis of few examples it was concluded that the R_F value of a complex decreases with increasing radius of the central ion, although in some instances deviations from this rule were observed. Haworth and Hung[8] chromatographed mixtures of transition metal 2,4-pentanedionato complexes on thin layers of microcrystalline cellulose using multi-component solvent systems. The order of the R_F values of complexes containing different central ions varied, and this was ascribed to the competition of two factors essential for the separation, *viz.*, the size of the metal ion and the solubility of the complex in the solvent system used.

We decided to examine in more detail the effect of the central ion on the R_F values of complexes obtained on silica gel thin layers, as previous separations were performed on cellulose and aluminium oxide. For development we applied single-component solvents, resulting in an adsorption mechanism; previous separations were performed with multi-component solvent systems, which may involve a partition separation mechanism[1].

TABLE I

EFFECT OF THE CENTRAL ION OF TRANSITION METAL COMPLEXES ON THE R_F VALUES

No.	*Isomer*	*Complex*	*Metal (M)*	*Ref.*	*r (nm)*	$Z^*/r \cdot 10^{-10}$ (m^{-1})
1	*trans*(O_5)	$[M(eddadp)]^-$	Co	9	0.052	13.0
2			Rh	10	0.066	10.4
3			Cr	11	0.061	8.1
4		$[M(edtp)]^-$	Co	12, 13	0.052	13.0
5			Rh	14	0.066	10.4
6			Cr	15	0.061	8.4
7		$[M(acac)_3]$	Co	16	0.052	13.0
8			Rh	17	0.066	10.4
9			Ru	18	0.068	9.2
10			Cr	19	0.061	8.1
11	Facial	$[M(bzac)_3]$	Co	16	0.052	13.0
12			Rh	20	0.066	10.4
13			Cr	20	0.061	8.1
14	Meridional	$[M(bzac)_3]$	Co	16	0.052	13.0
15			Rh	20	0.066	10.4
16			Cr	20	0.061	8.1
17		$[M(dipy)_3]^{2+}$	Zn	21	0.074	11.4
18			Ni	21	0.070	10.3
19			Co	22	0.065	10.1
20			Fe	22	0.061	9.7

[a] Solvents 1–22 are given in Table II. Thin layers: K_1 = commercial silica gel SIL-G on an aluminium sheet; K_2 = commercial silica gel SIL-G on a plastic sheet; A = aluminium oxide; G = silica gel G; H = silica gel H.

EXPERIMENTAL

Thin-layer chromatography

The complexes were synthesized according to literature procedures (Table I). The purity of the products was checked by microanalysis. Chromatographic development was carried out by the ascending method on silica gel H (Merck, Darmstadt, F.R.G.) or G (Riedel-de Haën, Hannover, F.R.G.), aluminium oxide (Merck) and commercially prepared silica gel SIL-G (Machery, Nagel & Co., Düren, F.R.G.) plates.

The preparation of the plates and all the other experimental data were described in a previous paper[1]. Before development, the complexes were dissolved in the corresponding solvent systems. The solvents (analytical-reagent grade) were dried with anhydrous sodium sulphate, except benzene, which was treated with dehydrated zeolite.

The solvents used are listed in Table II. All the substances were coloured and hence could be detected visually on the plates.

$R_F \cdot 100^a$																					
1	*2*	*3*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*
K_1	*A*	K_2	*G*	K_2	*G*	*G*	K_2	*G*	K_1	*H*	*H*	*A*	*G*	*G*	*G*	*G*	*G*	K_1	K_1	*G*	*H*	*H*
–	–	–	–	–	73	14	–	15	–	18	8	85	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	77	17	–	36	–	22	15	88	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	79	35	–	44	–	43	18	91	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	48	18	48	13	78	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	51	24	59	24	80	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	57	32	52	15	86	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	20	10	–	–	–	–	–	–	–	–	71	71	43	70	89	68	79	86	53	50
–	–	–	22	11	–	–	–	–	–	–	–	–	74	72	44	69	95	74	85	94	58	59
–	–	–	27	14	–	–	–	–	–	–	–	–	75	77	48	71	98	77	87	96	60	60
–	–	–	23	17	–	–	–	–	–	–	–	–	79	80	57	75	95	74	84	96	59	58
4	–	44	–	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	–	50	–	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	46	–	15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	20	60	64	36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	26	67	70	54	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20	28	62	66	48	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	18	–	83	28	–	–	–	31	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	17	–	84	32	–	–	–	36	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	5	–	78	17	–	–	–	9	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	2	–	75	14	–	–	–	13	–	–	–	–	–	–	–	–	–	–

Solubility

The solubilities of the complexes were determined as described previously[5].

Calculation of Z^*/r values

The polarization power of an ion (ρ) is proportional to the ratio of the effective charge ($Z - S$) and the radius of the ion (r): $\rho \propto (Z - S)/r$, where Z is the atomic number, S Slaiter's shielding constant and r the ionic radius according to Shanon and Prewit[23].

RESULTS AND DISCUSSION

Table I gives the R_F values for 20 cationic, anionic and neutral complexes obtained with the 22 solvents listed in Table II.

For all the anionic and neutral complexes, the Co^{III} complex had a smaller R_F value than the corresponding Cr^{III}, Ru^{III} and Rh^{III} complexes. In addition, in most instances the following order of R_F values was observed: $Co^{III} < Rh^{III} < Ru^{III} < Cr^{III}$.

In order to interpret these results we first considered the correlation between the R_F values and the radii of the corresponding metal ions (Table I). This made it possible to explain why a Co^{III} complex always has the smallest R_F value, as its radius is much smaller than those of the other metal ions. However, it is not possible on this basis to explain the order of R_F values of complexes whose central ions have similar radii.

However, taking into account that the radius of the ion affects the distribution of the complex charge via the polarization power of the central ion, we thought that this polarization power might be a better parameter to provide an explanation of the order of the R_F values. We therefore compared the R_F values with the Z^*/r values (Z^* is the effective charge and r the radius of the central ion), which are proportional to the polarization powers of the central ions.

In this way we established that the order of the R_F values of the complexes containing central ions with a large difference in their radii can be well explained by

TABLE II

SOLVENTS USED

No.	*Solvent*	*Time of development (min)*	*No.*	*Solvent*	*Time of development (min)*
1	Benzene	15	12	Ethylene glycol	120
2	Carbon tetrachloride	20	13	*sec.*-Butanol	85
3	Chloroform	15	14	*n*-Butanol	65
4	Methylene chloride	10	15	*n*-Hexanol	80
5	Distilled water	15	16	*n*-Pentanol	120
6	Methanol	15	17	Acetylacetone	60
7	Formic acid	30	18	Methyl *n*-propyl ketone	15
8	1,2-Propanediol	16 h	19	Methyl ethyl ketone	12
9	Formamide	10	20	Cyclohexanone	60
10	1,3-Propanediol	10 h	21	Isobutanol	70
11	Ethylene glycol monomethyl ether	55	22	Cyclohexanol	6 h

means of the polarization power of the central ion (*e.g.*, Co^{III} and other metals). Moreover, by means of this parameter it is possible to explain in most instances the order of the R_F values of complexes whose central ions have similar radii. For example, in the series of $[M(eddadp)]^-$ and $[M(edtp)]^-$ complexes (Table I), on the basis of the central ion radii, the order of the R_F values of the complexes $Co^{III} < Cr^{III} < Rh^{III}$ would be expected, but this order was not in agreement with the experimental results. However, on the basis of the polarization power the order of the R_F values of these complexes should be $Co^{III} < Rh^{III} < Cr^{III}$, which agreed with the experimental results. Nevertheless, in some instances when the difference between the polarization powers of central ions was small, deviations from the expected order were observed, pointing to the existence of some other factors that affect the R_F values. Therefore, we determined the solubility of three complexes in three solvent systems (Table III). It can be seen that in some instances the chromatographic behaviour of the complexes is in accordance with their solubilities, whereas in others it is not, indicating that some other factors are operating.

TABLE III

SOLUBILITIES (s, mol dm^{-3}) OF SOME OF TRIS(ACETYLACETONATO) TRANSITION METAL COMPLEXES AND THE CORRESPONDING R_F AND Z^*/r VALUES

Complex	$Z^*/r \cdot 10^{-10}$ (m^{-1})	*Chloroform*		*n-Butanol*		*Acetylacetone*	
		s	$R_F \cdot 100$	s	$R_F \cdot 100$	s	$R_F \cdot 100$
$[Co(acac)_3]$	13.0	0.259	20	0.0418	71	0.0947	89
$[Ru(acac)_3]$	9.2	0.324	27	0.0449	77	0.177	98
$[Cr(acac)_3]$	8.1	0.304	23	0.0795	80	0.213	95

As regards the separation mechanism, we consider that with anionic and neutral complexes, the greater the polarization power of the central ion the more the ligand is polarized; hence the negative charge on the ligator is higher and stronger hydrogen bonds with silanol groups are formed.

With cationic complexes, in all instances the R_F value of the Zn^{II} complex was greater that those of the Co^{II} and Fe^{II} complexes, whereas there was no regularity in the order of the R_F values of Co^{II}, Fe^{II} and Ni^{II} complexes.

As the order of the R_F values of these complexes cannot be explained on the basis of either ionic radii or polarization power, we have assumed that in this instance, in addition to the adsorption mechanism, ion exchange may be also involved, as silica gel is known to behave also as a cation exchanger[24].

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CHEMICAL CHANGES OF ORGANIC COMPOUNDS IN CHLORINATED WATER

XVI. GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF REACTIONS OF TRICYCLIC AROMATIC HYDROCARBONS WITH HYPOCHLORITE IN DILUTE AQUEOUS SOLUTION

SUKEO ONODERA*, KIYOSHI IGARASHI, ATSUKO FUKUDA, JUNKO OUCHI and SHIZUO SUZUKI

Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162 (Japan)

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SUMMARY

The products of aqueous chlorination reactions of tricyclic aromatic hydrocarbons (fluorene, carbazole, dibenzofuran, anthracene, phenanthrene and some methyl derivatives) with hypochlorite have been determined by gas chromatography–mass spectrometry. They included chloro-substituted, oxygenated (quinones) and hydroxylated (phenols) compounds, and products of addition which were readily formed at ambient temperature. The extent of the reactions was shown to depend on the chlorine dose, the solution pH, the initial concentrations of both compounds and the structures. Monochlorinated compounds and quinones were shown to be present in chlorinated water under the conditions utilized for water treatment.

INTRODUCTION

The disinfection of water and waste-water with chlorine is a well established procedure. However, the increasing presence and variety of aquatic pollutants raises the question of the chemical fate of these contaminants when subjected to aqueous chlorination[1].

Even the numerous studies of phenol, which established the intermediate formation of mono-, di- and trichlorophenols[2–9], leave the question of the end-products of this reaction unresolved[10].

Several polynuclear aromatic hydrocarbons (PAHs) in aqueous solution are degraded by addition of chlorine[11,12], chlorine dioxide[13,16] or sodium hypochlorite[17–22]. These reactions may occur at the site of chlorine addition and through the water distribution system[23]. PAHs have been suggested as the precursors of at least a portion of the mutagens produced in some chlorination processes[23]. The levels of these ubiquitous compounds may be increased by the presence of coal tar coating inside pipes and water storage tanks[24,25].

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In order to provide further insight into the possible rôle of organic compounds in the formation of chlorine-substituted compounds and of chlorine-induced mutagens, this laboratory has continued the study of the aqueous chlorination chemistry of organic compounds[26]. The present report describes a detailed study of the product distributions of several tricyclic aromatic hydrocarbons (TCAHs) which were chosen because of their previous identification in unpurified water[27] and drinking water[28–30].

EXPERIMENTAL

Materials

The tricyclic aromatic hydrocarbons (TCAHs) were obtained from Wako Pure Chemical Industry (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan) and Nakarai Chemical (Kyoto, Japan). Chloro-substituted, oxygenated (quinones) and hydroxylated (phenols) compounds of TCAHs, which are expected to be formed during chlorination of TCAHs with aqueous chlorine, were commercially available. The chemical names and gas chromatographic–mass spectrometric (GC–MS) data for these compounds are summarized in Table I.

Hypochlorite solution was prepared by diluting sodium hypochlorite solution (*ca.* 10% available Cl, Nakarai Chemical) in 0.1 *M* disodium hydrogenphosphate–potassium dihydrogenphosphate buffer solution, pH 7. The hypochlorite concentrations were determined by iodometric titration.

TABLE I

GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC DATA FOR TRICYCLIC AROMATIC HYDROCARBONS

Compound	*Relative retention time*[a]	*Molecular ion, m/z*	*Compound*	*Relative retention time*[a]	*Molecular ion, m/z*
Fluorene	0.741	166	Anthraquinone	1.203	208
Fluorenone	0.935	180	1,4-Anthraquinone	1.339	208
9-Chlorofluorene	0.944	200	1-Chloroanthraquinone	1.454	242
Carbazole	1.055	167	2-Chloroanthraquinone	1.383	242
Dibenzofuran	0.663	168	1,5-Dichloroanthraquinone	1.680	276
2-Chlorodibenzofuran	0.861	202	1,8-Dichloroanthraquinone	1.663	276
2,7-Dichlorodibenzofuran	1.100	236	1-Hydroxyanthracene	1.328	194
Dibenzo-*p*-dioxin	0.668	184	2-Methylanthracene	1.148	192
1-Chlorodibenzo-*p*-dioxin	0.908	218	2-Methylanthraquinone	1.358	222
2-Chlorodibenzo-*p*-dioxin	0.895	218	9-Methylanthracene	1.199	192
2,3-Dichlorodibenzo-*p*-dioxin	1.150	252	9-Chloromethylanthracene	1.402	226
2,7-Dichlorodibenzo-*p*-dioxin	1.135	252	9,10-Dimethylanthracene	1.387	206
Anthracene	1.000[b]	178	Phenanthrene	1.000	178
1-Chloroanthracene	1.241	212	Phenanthrenequinone	1.517	208
2-Chloroanthracene	1.228	212	1-Methylphenanthrene	1.171	192
9,10-Dichloroanthracene	1.449	246	2-Methylphenanthrene	1.146	192

[a] GC conditions: column, 2% OV-1/Uniport HP (60–80 mesh), glass (2 m × 2 mm); column temperature, programmed from 120 to 260°C at 5°C/min; nitrogen gas flow-rate; 50 ml/min.

[b] The retention time of anthracene under these conditions was 15.78 min.

Treatment of aqueous TCAH solutions with hypochlorite and extraction of reaction mixture

A mixture of 100 ml of hypochlorite solution and each TCAH compound dissolved in 1 ml of methanol was stirred in a stoppered conical flask using a magnetic stirrer at 20°C for 24 h. After the desired reaction time, the residual chlorine was removed by addition of an equivalent volume of sodium thiosulphate solution. The reaction mixture was then acidified to pH 2 with 0.1 *M* hydrochloric acid before extraction with two 30-ml volumes of diethyl ether. The solvents were dried over anhydrous sodium sulphate and 2 ml of *n*-hexane–acetone (1:1, v/v) were added to prevent evaporation of the reaction products during concentration under vacuum at 40°C to suitable volumes for GC and GC–MS analyses.

Product resolution and characterization

A Shimadzu GC-6A gas chromatograph equipped with a flame ionization detector and 2 m × 2 mm I.D. glass column packed with 2% OV-1 on Uniport HP (60–80 mesh) was programmed from 120 to 260°C at 5°C/min. The nitrogen carrier gas flow-rate was 40 ml/min. A Shimadzu Chromatopac-1A data system was used to determine the retention times and peak areas on the chromatograms.

An Hitachi M-80 mass spectrometer–gas chromatograph equipped with an Hitachi M-008 data processing system was used for the qualitative analyses of samples under the following conditions: temperature of ion source, 250°C; trap current, 70 μA; electron energy, 70 eV. A glass column (2 m × 3 mm I.D.) packed with 2% OV-1 on Uniport HP (60–80 mesh) was used for the GC separation of the diethyl ether extracts. The oven temperature of the gas chromatograph was programmed from 120 to 240°C at a rate of 5°C/min. The products were identified by comparison of their retention times and mass spectra with those of authentic compounds.

RESULTS AND DISCUSSION

In a preliminary examination of the reaction of individual TCAHs with hypochlorite in dilute aqueous solution, the decrease in the concentration of the active chlorine during contact with each of these compounds was followed by iodometric titration. A much slower decrease occurred at low concentration, less than 1 mg/l, of TCAH compounds with two exceptions, in comparison with aqueous phenolic solutions[7,31–33]. In the present work, therefore, each TCAH (10^{-4} *M*) was allowed to react with hypochlorite (10^{-2} *M*) and the products identified.

GC–MS analysis of chlorination products of fluorene

A typical GC–MS (total ion current) trace of a diethyl ether extract of aqueous fluorene solution (0.3 mmol/l, pH 5) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at 20°C for 24 h is shown in Fig. 1. At least fifteen reaction products can be seen on the chromatogram. Some of the peaks were identified on the basis of their retention times and mass spectra as compared with those of authentic compounds. Compounds corresponding to other peaks were determined from the mass spectra of each peak (Fig. 2).

Fig. 2A shows the mass spectrum of the peak at scan 128 which is the main reaction product when fluorene was treated with less than 5 equivalents of chlorine per

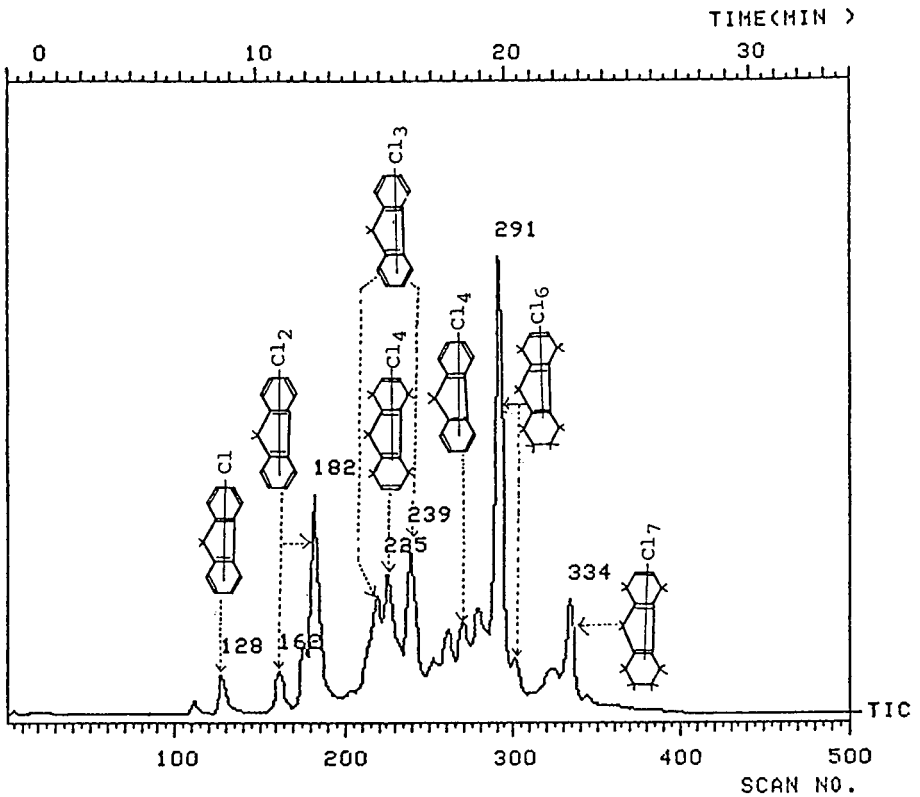


Fig. 1. Mass chromatogram (total ion current) of a diethyl ether extract of a fluorene solution (0.3 mmol/l) after treatment with hypochlorite (40 equiv. of chlorine per mol of compound) at 20°C and pH 5 for 24 h. Mass spectrum for each peak as in Fig. 2. The GC column temperature was raised from 120 to 250°C at 5°C/min. For other GC–MS conditions, see Experimental.

mol of compound for 24 h. The molecular ion (M^+) of the peak at scan 128 occurs at *m/z* 200, indicating one chlorine atom, and a fragment ion occurs at *m/z* 165, which arises by the loss of one chlorine atom from the molecular ion. The mass spectrum and retention time of this compound were in agreement with those of 9-chlorofluorene.

The compound corresponding to the peak at scan 182 in Fig. 1 gave a molecular ion at *m/z* 234 (Fig. 2B), indicating two chlorine atoms, and two fragment ions at *m/z* 199 ($M^+ - Cl$) and 163 ($M^+ - Cl - HCl$). The molecular ion and its fragmentation pattern indicate the occurrence of dichlorofluorene in the diethyl ether extract. The peak at scan 162 in Fig. 1 gave a similar fragmentation pattern, with two chlorine atoms, to that of the peak at scan 182, which also suggests the occurrence of an isomer of a dichlorofluorene in the extract.

Fig. 2C and D show the mass spectra of the peak at scans 225 and 291 in Fig. 1, respectively, which are the reaction products when fluorene was treated with hypochlorite at high molar ratios of chlorine per mol of compound (over 20 equivalents) for 24 h. The compound corresponding to the peak at scan 225 in Fig.

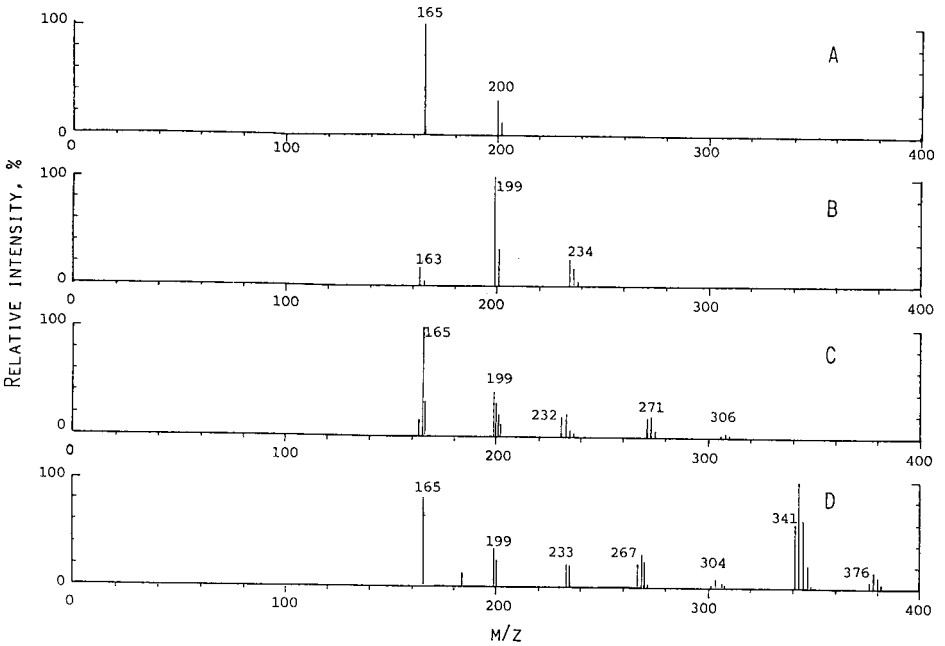


Fig. 2. Mass spectra of peaks of scans 128 (A), 182 (B), 225 (C) and 291 (D) in Fig. 1.

1 gave a molecular ion at *m/z* 306, having four chlorine atoms, and four fragment ions at *m/z* 271 ($M^+ - Cl$), 235 ($M^+ - Cl - HCl$), 199 ($M^+ - Cl - 2HCl$) and 165 ($M^+ - 3Cl - HCl$). The molecular ion of the peak at scan 291 occurs at *m/z* 376, having six chlorine atoms, and six fragment ions occur at *m/z* 341 ($M^+ - Cl$), 302 ($M^+ - 2HCl - 2H$), 267 ($M^+ - 3HCl - H$), 233 ($M^+ - Cl - 3HCl$), 199 ($M^+ - 3Cl - 2HCl$) and 165 ($M^+ - 6Cl - H$). These molecular ions and their mass fragmentation patterns indicate the compounds to be tetrahydrotetrachlorofluorene and hexahydrohexachlorofluorene, respectively, but their exact structures could not be determined.

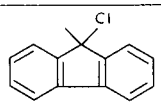
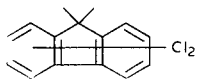
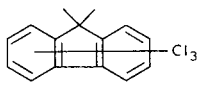
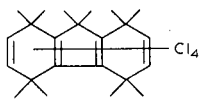
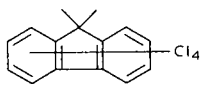
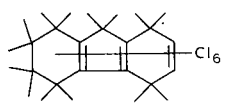
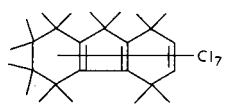
A summary of the chlorination products identified or determined from their relative retention times and mass spectra is presented in Table II. These findings lead to the conclusion that chlorination of aqueous fluorene solutions with hypochlorite produces not only products of substitution, but also those of addition.

The occurrence of mono- and dichlorofluorenes and fluorenone in chlorine-treated fluorene solution has been confirmed by Oyler *et al.*[20] by GC–MS analysis of a acetonitrile–dichloromethane extract, using C_{18} Porasil B and XAD-2 adsorption, from the reaction solution. Similar compounds have also been shown to be present in chlorinated leachate from a commercial coal tar[24,25] and in drinking water[30]. However, no products of addition have been reported in the literature[12,20,24,25,30], although it has been shown that chlorination of methylnaphthalene by molecular chlorine in acetic acid produces both products of substitution and addition[34].

TABLE II

PRODUCTS OF REACTION OF FLUORENE WITH HYPOCHLORITE IN ACIDIC AQUEOUS SOLUTION AT 20°C FOR 24 h[a]

MS data for each compound appearing in Tables II–V are available from the authors.

Proposed structure	*Scan no. in Fig. 1*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
1  [c]	128	0.945	200
2 	162	1.084	234
3 Isomer of compound 2	182	1.165	234
4 	219	1.321	268
5 	225	1.346	306
6 Isomer of compound 4	239	1.402	268
7 	271	1.545	302
8 	291	1.626	376
9 Isomer of compound 8	302	1.664	376
10 	334	1.844	410

[a] pH 5, $[\text{fluorene}]_0 = 0.3 \cdot 10^{-3}\ M$, $[\text{hypochlorous acid}]_0 = 6 \cdot 10^{-2}\ M$.

[b] Reference = anthracene.

[c] Relative retention time and mass spectrum are identical to those of the authentic compound.

Chlorination products of carbazole, dibenzofuran and dibenzo-p-dioxin

Typical GC–MS (total and mass fragment ion currents) traces of diethyl ether extracts from carbazole, dibenzofuran and dibenzo-*p*-dioxin solutions (pH 5) after treatment with an excess of hypochlorite at 20°C for 24 h are shown in Fig. 3. Some of the peaks were identified on the basis of GC retention times and mass spectra compared with those of authentic compounds. Compounds corresponding to other peaks were determined from the mass spectrum of each peak in the same manner as described for the fluorene products.

Fig. 3 shows that treatment of carbazole, dibenzofuran and dibenzo-*p*-dioxin with an excess of hypochlorite in aqueous solutions (pH 5) produces several chloro-substitution products. The reaction in low concentrations of both compounds (less than 1 mg/l) gave mono- and, occasionally, dichloro-substituted compounds, while higher chlorinated products were detected in the reactions at high concentrations.

A summary of the chlorination products identified or determined from the GC retention times and mass spectra is presented in Table III. Monochlorodibenzofuran has been identified to be present in chlorine-treated dibenzofuran solution[12,20], in chlorinated leachate from commercial coal tar[24,25] and in drinking water[30].

Chlorination products of anthracene, phenanthrene and their methyl derivatives

As can be seen in Fig. 4A, treatment of 2-methylanthracene with an excess of hypochlorite in the buffered solution of pH 5 produced 2-methylanthraquinone as the predominant product, minor products being chloro-2-methylanthraquinone and chloro-substituted derivatives. This product distribution was the similar to that of the anthracene–hypochlorite reaction (Table IV).

Fig. 4B shows that treatment of 9-methylanthracene with an excess of hypochlorite in the buffered solution of pH 5 produces several reaction products

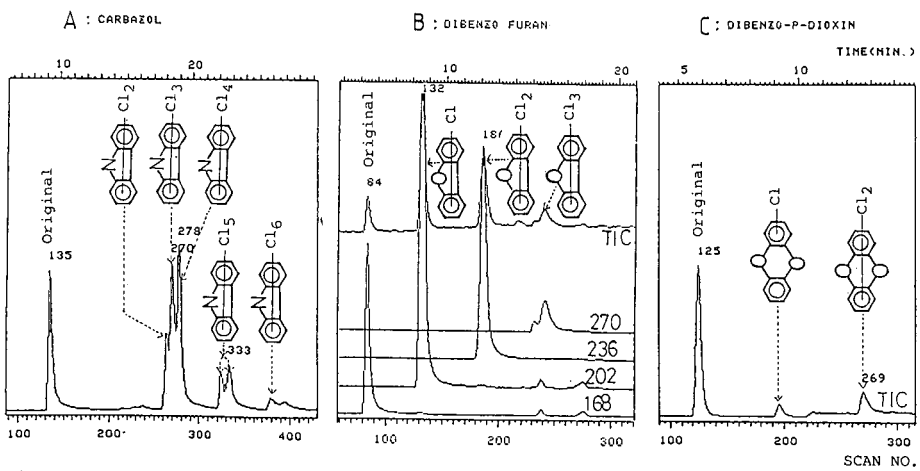
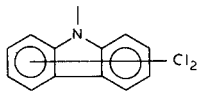
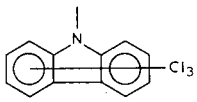
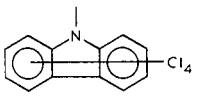
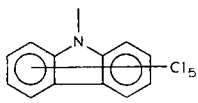
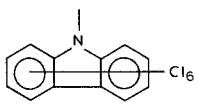
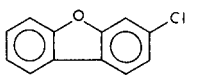
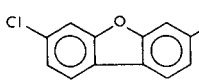
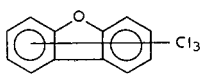
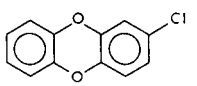
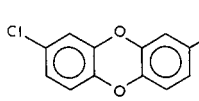


Fig. 3. Mass chromatograms (total or fragment ion current) of diethyl ether extracts from carbazole, dibenzofuran and dibenzo-*p*-dioxin solutions (0.3 mmol/l) after treatment with hypochlorite (40 equiv. of chlorine per mol of compound) at 20°C and pH 5 for 24 h. GC column temperature as in Fig. 1. For other GC–MS conditions, see Experimental. Mass spectral data for each peak as in Table III.

TABLE III

PRODUCTS OF REACTION OF CARBAZOLE, DIBENZOFURAN AND DIBENZO-*p*-DIOXIN WITH HYPOCHLORITE IN ACIDIC AQUEOUS SOLUTION AT 20°C FOR 24 h[a]

Proposed structure	*Scan no. in Fig. 3*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
Carbazole products			
1 	263	1.570	235
2 	270	1.610	269
3 	278	1.640	303
4 	325	1.830	337
5 Isomer of compound 4	333	1.870	337
6 	395	2.050	371
Dibenzofuran products			
1  [c]	132	0.858	202
2  [c]	186	1.102	236
3 	241	1.323	270
Dibenzo-p-dioxin products			
1  [c]	195	0.908	218
2  [c]	269	1.149	252

[a] pH 5, $[\text{compound}]_0 = 0.3 \cdot 10^{-3}$ *M*, $[\text{hypochlorous acid}]_0 = 6 \cdot 10^{-2}$ *M*.

[b] Reference = anthracene.

[c] Relative retention time and mass spectrum are identical to those of the authentic compound.

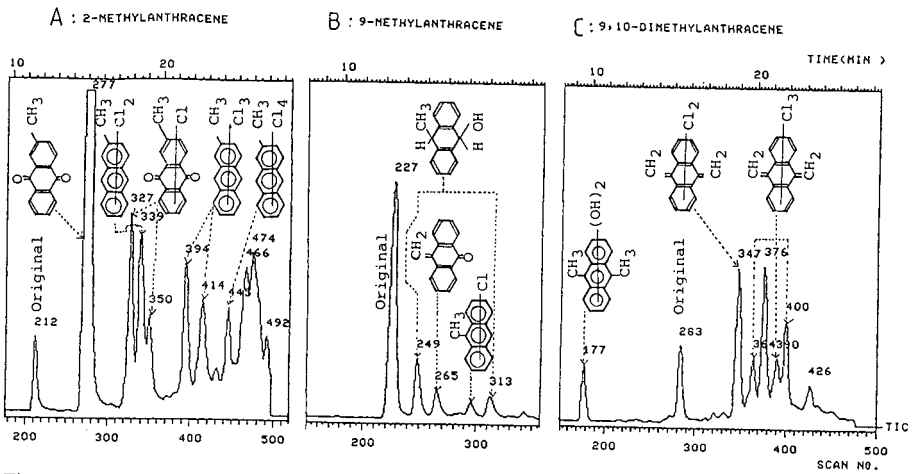


Fig. 4. Mass chromatograms (total ion current) of diethyl ether extracts from 2- and 9-methylanthracene and 9,10-dimethylanthracene solutions (0.3 mmol/l) after treatment with hypochlorite (40 equiv. of chlorine per mol of compound) at 20°C and pH 5 for 24 h. GC–MS conditions as in Fig. 1. Mass spectral data for each peak as in Table IV.

including hydroxylated (phenols), oxygenated (9-methyleneanthracenone) and chlorine-substituted compounds. 9-Chloromethylanthracene exhibits a mass spectrum which is nearly identical to that of its ring substituted isomer, chloro-9-methylanthracene. A difference in relative retention times (1.402 *versus* 1.437, respectively) allowed the conclusion that the latter compound occurs in the chlorinated 9-methylanthracene solution.

Treatment of 9,10-dimethylanthracene with an excess of hypochlorite in the buffered solution of pH 5 has been shown to produce hydroxylated (phenol) and chlorinated 9,10-dimethyleneanthracenes (Fig. 4C and Table IV). A summary of chlorination products identified or determined from the retention times and mass spectra is presented in Table IV.

Fig. 5 shows that treatment of 1- and 2-methylphenanthrenes with an excess of hypochlorite in the buffered solution of pH 5 at 20°C for 24 h produces several reaction products including chlorine-substituted derivatives and products of addition. The reactions in low concentrations of both compounds (less than 1 mg/l) gave only monochlorinated derivatives, while products of addition were detected in the high concentration reactions. A summary of the chlorination products identified or determined from retention times and mass spectra is shown in Table V.

Oxygenated (quinones), hydroxylated (phenols) and monochlorinated compounds have been identified in chlorine-treated anthracene or phenanthrene solutions[12,20], in a chlorinated leachate from commercial coal tar[24,25] and in drinking water[30].

Effect of the experimental conditions on the TCAH–hypochlorite reactions in aqueous solution

GC analysis of the diethyl ether extracts indicates that the reactions of TCAHs with hypochlorite in neutral aqueous solution are strongly dependent on structural

TABLE IV

PRODUCTS OF REACTION OF ANTHRACENE AND ITS METHYL DERIVATIVES WITH HYPOCHLORITE IN ACIDIC AQUEOUS SOLUTION AT 20°C FOR 24 h[a]

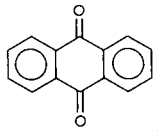
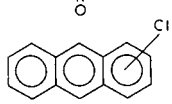
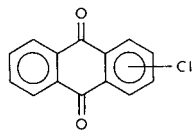
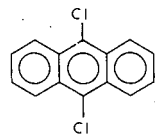
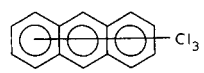
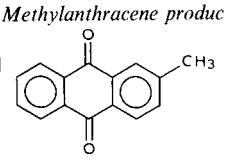
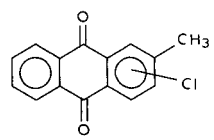
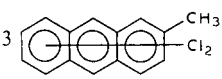
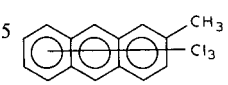
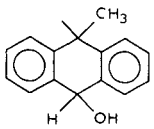
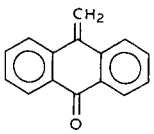
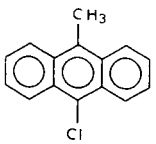
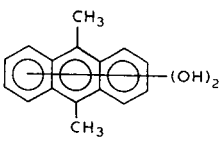
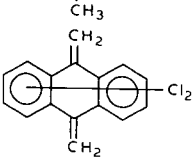
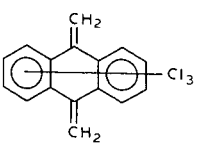
Proposed structure	*Scan no. in Fig. 4*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
Anthracene products			
1  [c]		1.203	208
2 		1.236	212
3 		1.405	242
4  [c]		1.450	246
5 		1.670	280
2-Methylanthracene products			
1 	270	1.358	222
	327	1.555	256
2 	329	1.600	260
3 			
4 Isomer of compound 2	350	1.630	256
5 	414	1.867	294

TABLE IV (*continued*)

Proposed structure	*Scan no. in Fig. 4*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
9-Methylanthracene products			
1 	249	1.301	210
2 	265	1.363	206
3 	296	1.437	226
4 Isomer of compound 1	313	1.536	210
9,10-Dimethylanthracene products			
1 	177	0.991	238
2 	347	1.656	272
3 	364	1.713	306
4 Unknown	376	1.756	–
5 Isomer of compound 3	400	1.855	306

[a] pH 5, $[\text{compound}]_0 = 0.3 \cdot 10^{-3}\ M$, $[\text{hypochlorous acid}]_0 = 6 \cdot 10^{-2}\ M$.
[b] Reference = anthracene.
[c] Relative retention time and mass spectrum are identical to those of the authentic compound.

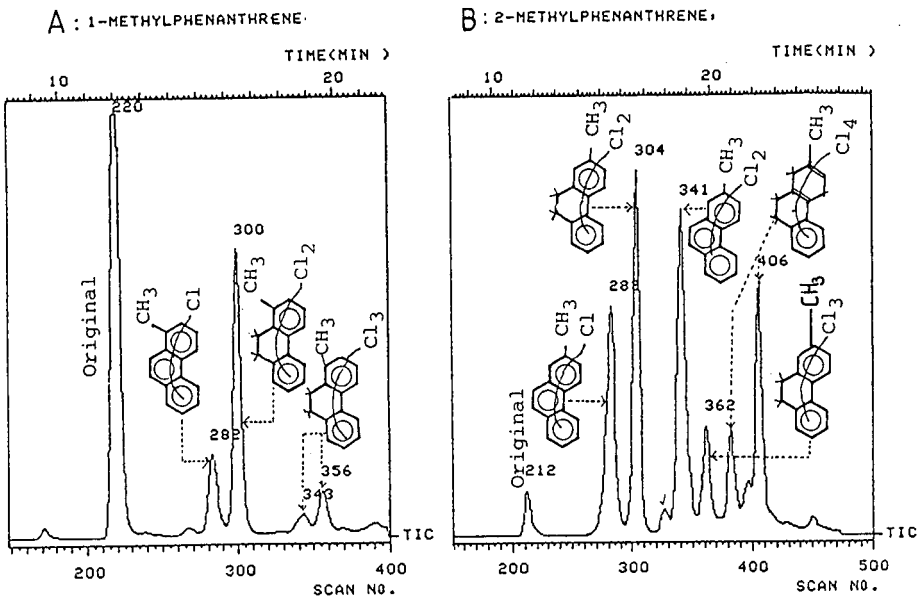


Fig. 5. Mass chromatograms (total ion current) of diethyl ether extracts from 1- and 2-methylphenanthrene solutions (0.3 mmol/l) after treatment with hypochlorite (40 equiv. of chlorine per mol of compound) at 20°C and pH 5 for 24 h. GC–MS conditions as in Fig. 1. Mass spectral data for each peak as in Table V.

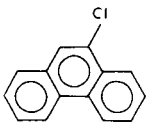
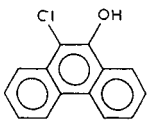
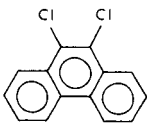
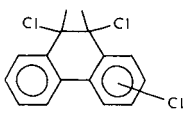
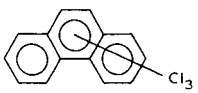
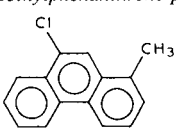
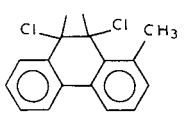
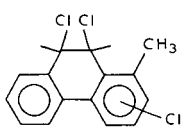
features (Fig. 6). Carbazole and 9-methylanthracene readily reacted with hypochlorous acid in water. A moderate reactivity was found for the anthracene, 2-methylanthracene and 9,10-dimethylanthracene solutions, and low reactivity for the fluorene, dibenzofuran, phenanthrene, 1-methyl- and 2-methylphenanthrene solutions.

Oxygenated (quinones) and hydroxylated (phenols) compounds were present at high concentrations in water when anthracene, 2-methyl- and 9-methylanthracenes were treated with hypochlorite at high molar ratios of hypochlorous acid to compound (Fig. 6B). Monochloro-9-methylanthracene and dichlorocarbazole were also detected at high concentrations in water when 9-methylanthracene and carbazole were treated with hypochlorite at moderate and high molar ratios of hypochlorous acid to compound (Fig. 6C). These reactions at high molar ratios of hypochlorous acid to compound may occur at the site of chlorine addition, as compared with those observed at a terminal water supplying system or in the presence of other substances reactive to chlorine in water.

Fig. 7 shows the results of GC determinations of diethyl ether extracts from aqueous solutions of TCAHs after treatment with an excess of hypochlorite at various pH values for 24 h. Oxygenated (quinones), hydroxylated (phenols) and chlorine-substituted compounds were detected at high concentrations under acidic and neutral conditions (Fig. 7B and C). Low concentrations of these oxygenated and chlorinated compounds were detected when TCAH compounds were treated with an excess of hypochlorite at pH 9. An exception was observed for the reaction of 9-methylanthracene with hypochlorite in water (Fig. 7).

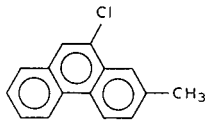
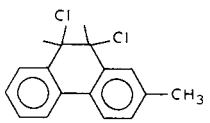
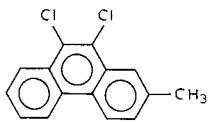
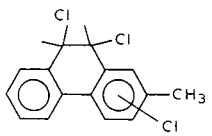
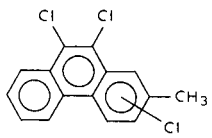
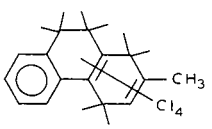
TABLE V

PRODUCTS OF REACTION OF PHENANTHRENE AND ITS METHYL DERIVATIVES WITH HYPOCHLORITE IN ACIDIC AQUEOUS SOLUTION AT 20°C FOR 24 h[a]

Proposed structure	*Scan no. in Fig. 5*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
Phenanthrene products			
1 		1.270	212
2 		1.380	228
3 		1.56	246
4 		1.700	282
5 		1.740	280
6 Isomer of compound 5		1.76	280
1-Methylphenanthrene products			
1 	282	1.446	226
2 	300	1.506	262
3 	343	1.652	296
4 Isomer of compound 3	356	1.711	296

(*Continued on p. 246*)

TABLE V (*continued*)

Proposed structure	*Scan no. in Fig. 5*	*Relative retention time (GC)*[b]	*Molecular ion, m/z*
2-Methylphenanthrene products			
	283	1.436	226
2 	304	1.516	262
3 	341	1.666	260
	362	1.735	296
5 	396	1.873	294
6 	406	1.896	332

[a] pH 5, $[\text{compound}]_0 = 0.3 \cdot 10^{-3}$ *M*, $[\text{hypochlorous acid}]_0 = 6 \cdot 10^{-2}$ *M*.
[b] Reference = anthracene.

At the typical ranges of pH found during the course of most water treatment processes (pH 5–9), the activated chlorine species can range from entirely hypochlorite ($^-$OCl) to entirely hypochlorous acid (HOCl, $pK_a = 7.5$ at 20°C). It has been observed that chlorine is more readily incorporated into aromatic systems at low pH values[22,35], a result which parallels the observation of increasing disinfection ability with decreasing pH[36]. Thus it seems that higher concentrations of chlorine-incorporated compounds would be formed when acidic industrial water effluents were treated with an excess of hypochlorite.

In order to confirm whether similar reactions take place under conditions

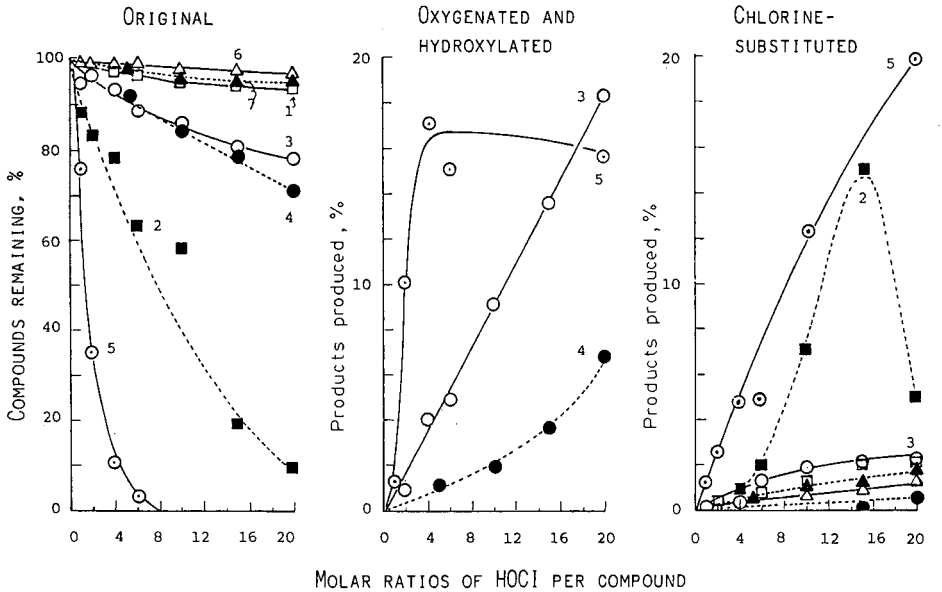


Fig. 6. Effect of the chlorine dose on the reactions of TCAHs (0.3 mmol/l) with hypochlorite in neutral aqueous solutions at 20°C for 24 h. The yields were derived from GC peak areas, relative to the area of each starting material. 1 (□), fluorene; 2 (■), carbazole; 3 (○), anthracene; 4 (●), 2-methylanthracene; 5 (⊙), 9-methylanthracene; 6 (½), phenanthrene and 7 (▲), 2-methylphenanthrene as the starting material.

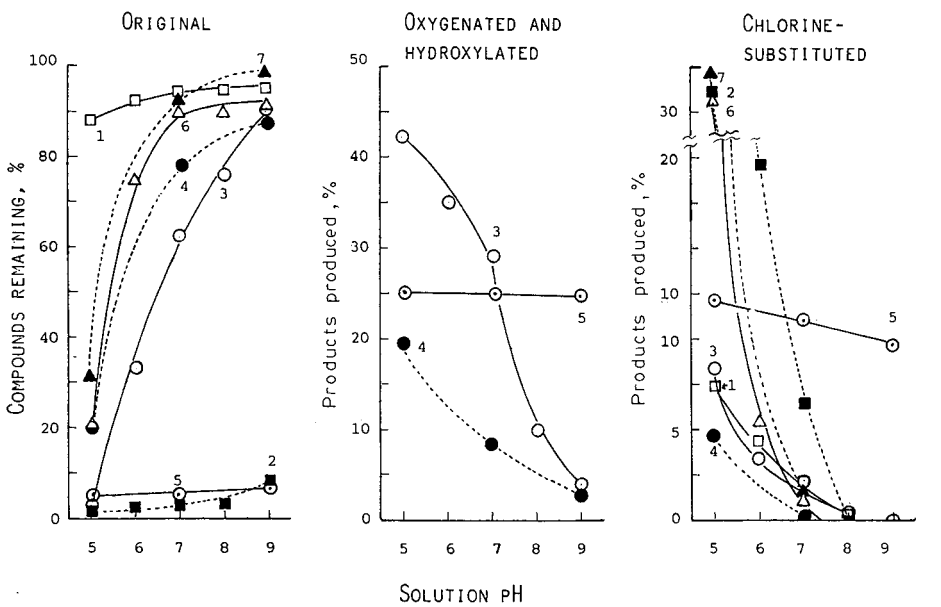


Fig. 7. Effect of the solution pH values on the reactions of TCAHs (0.3 mmol/l) with hypochlorite (6 mmol/l) in aqueous solutions at 20°C for 24 h. Other details as in Fig. 6.

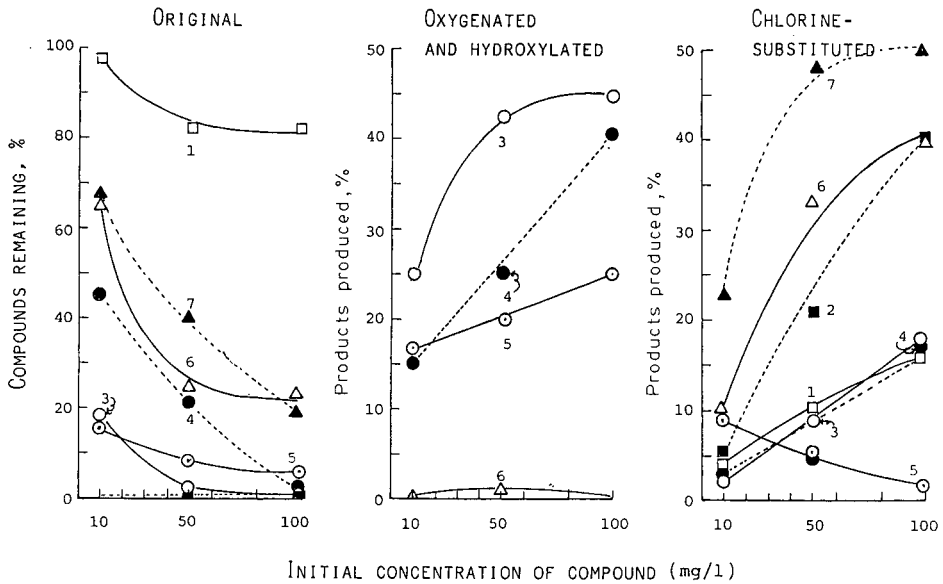


Fig. 8. Effect of the initial concentrations of compounds on the reactions of TCAHs with hypochlorite (20 equiv. of chlorine per mol of compound) in an acidic aqueous solution (pH 5) at 20°C for 24 h. Other details as in Fig. 6.

present during water treatment, different initial concentrations of TCAHs were treated with an excess of hypochlorite at pH 5 for 24 h. The results of GC determinations of the diethyl ether extracts from the chlorinated waters of TCAHs are shown in Fig. 8. It was observed that reactions of TCAHs with hypochlorite in water proceed more rapidly with increasing initial concentrations of both compounds. Oxygenated (quinones) and monochlorinated compounds were detected at comparatively high concentrations after treatment of even lower concentrations of TCAHs with an excess of hypochlorite under neutral and acidic conditions.

The higher concentrations of organic compounds and the corresponding high active chlorine levels described above have been found to represent extreme cases of contamination, such as encountered in some industrial water effluents[36,37] and in disinfection of the water mains and storage tanks after installation and maintenance[38].

It is worth stressing the relationship between the results reported here and the customary chemical treatment of waste-water. Industrial waste-waters are frequently treated with heavy doses of chlorine to destroy odours, to disinfect the waste-water and to improve sedimentation and filtration behaviour. As already mentioned, TCAHs are common components in such waste-water[29], unpurified water[27] and coal-tar coatings of water-supply systems[24,25] and this has led to the suggestion that oxygenated and chlorinated TCAHs may be generated during the chlorine treatment of such waters and in the water-supply system. This suggestion was clearly supported by an earlier report[30] in which several chlorinated and oxygenated TCAHs were found to be present in drinking water.

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PENTAFLUOROBENZOIC ANHYDRIDE AS A DERIVATIZING AGENT FOR ALCOHOLS AND HYDROXY FATTY ACID METHYL ESTERS DETECTED BY ELECTRON CAPTURE IN GAS CHROMATOGRAPHY

DONALD V. CRABTREE* and ALICE J. ADLER
Eye Research Institute of Retina Foundation, 20 Staniford Street, Boston, MA 02114 (U.S.A.)
and
GARRY J. HANDELMAN
Department of Biochemistry, Tufts University Medical School, 136 Harrison Avenue, Boston, MA 02111 (U.S.A.)

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SUMMARY

Pentafluorobenzoate derivatives of primary and secondary alcohols have been prepared using pentafluorobenzoic anhydride. The gas chromatographic properties of the homologous series of the methyl esters of 2-hydroxycarboxylic acids from C_{12} to C_{26} have been studied on the high-temperature stationary phase Poly-S 179. The suitability of determining trace amounts of these compounds using gas chromatography with an electron-capture detector and with electron-capture negative-ion chemical-ionization mass spectrometry is discussed.

INTRODUCTION

The development of this method for measuring hydroxy carboxylic acids was undertaken because of a need to quantify and identify hydroperoxides that may be formed in the retina[1]. The fatty acid side chains of these oxidation products of membrane phospholipids would be reduced to hydroxy fatty acids, transesterified to methyl esters and derivatized specifically at this hydroxyl to facilitate detection. Because of the small quantity of material available for analysis, it was imperative that the most sensitive detection method possible be used. Gas chromatography (GC) with electron-capture detection (ECD) provides good separation and detection of components in the picogram range[2]. For the development of this method a packed column was used. Those analysts interested in measuring only a few components will find the columns used here adequate. For those needing better separation, this method can be adapted to a capillary column. Useful work along these lines, preparing pentafluorobenzyl derivatives at the fatty acid ester linkage via transesterification of phospholipids with pentafluorobenzyl alcohol, has been carried out by Van Kuijk and co-workers[3,4].

After careful study, we decided that O-pentafluorobenzoylmethyl esters (as

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shown in Figs. 2 and 3) would provide the properties desired: good stability[5,6], optimal detector response[7,8] and possession of the highest probability of separation[9]. Furthermore, it was necessary that the reaction proceeded to completion for all components in a short period of time under gentle conditions. For this reason, pentafluorobenzoic anhydride (PFBA) was chosen as the derivatizing reagent. This choice avoids the formation of HCl that occurs with the acid chloride. In addition, the derivatives formed have been shown to be very useful in negative-ion chemical ionization (NICI) mass spectrometry (MS)[10].

The homologous series of 2-hydroxycarboxylic acids (C_{12}–C_{26}) was studied so that a relative retention index could be reported for subsequently observed unknowns. Included as examples of the use of this index are 16-hydroxyhexadecanoic acid and ricinoleic acid.

EXPERIMENTAL

Chemicals

Pentafluorobenzoyl chloride, benzoyl chloride, 1,1,1-trichloro-3,3,3-trifluoroacetone, 2-hydroxytetradecanoic acid and ethyl acetate were purchased from Fluka (Ronkonkoma, NY, U.S.A.). Potassium hydroxide, potassium bromide, 1-octanol, 3-octanol, decane, 1-decanol, 1,10-decanediol, 1-dodecanol, 1-pentadecanol, 1-octadecanol, 16-hydroxyhexadecanoic acid, cholesterol, cholesta-3,5-diene and trimethylcitrate were purchased from Aldrich (Milwaukee, WI, U.S.A.). 2-Hydroxydodecanoic acid and 2-hydroxyhexadecanoic acid were purchased from Lancaster Synthesis (Windham, NH, U.S.A.). Ricinoleic acid methyl ester (12-OH:18:1ω9), 2-hydroxystearic acid (2-OH:18:0), 2-hydroxybehenic acid (2-OH:22:0) and 2-hydroxycerotic acid (2-OH:26:0) were obtained from Serva (Westbury, NY, U.S.A.). Burdick & Jackson glass-distilled toluene was obtained from American Scientific Products (Boston, MA, U.S.A.). Silylation grade pyridine was obtained from Pierce (Rockford, IL, U.S.A.). N-Methyl-N-nitrosourea was obtained from ICN Pharmaceuticals. Diethyl ether was Fisher (Medford, MA, U.S.A.) HPLC grade. All chemicals were used without further purification.

Instrumentation

GC was performed on a Perkin-Elmer 3920 (purchased from Buck Scientific, Norwalk, CT, U.S.A.) with a flash vaporization injector interfaced to a Spectra-Physics (San Jose, CA, U.S.A.) SP 4100 integrator. When the baseline was extremely noisy, peak areas were determined by weighing photocopies of the peaks on a four-place Mettler balance. Emerson Electric (Hatfield, PA, U.S.A.) Model 8744A flow controllers were used for the carrier gas. The back pressure to the flow controllers was maintained at 78 p.s.i. The electron-capture detector was a Valco (Houston, TX, U.S.A.) Model 140 (180-μl volume) operating with a standing current of 0.6 nA. Periodically, it was cleaned by purging with hydrogen at a detector temperature of 370°C. The make-up gas was temperature equilibrated in the column oven prior to entering the detector. It was found that when the oven temperature was 230°C the optimal pressure for the make-up gas was 15 p.s.i. The injector temperature was maintained at its maximum temperature of 290°C and the column–detector interface at 330°C. The temperature of the column–detector interface was found not to influence

the response of the electron-capture detector. The oven temperature was calibrated with a Sensortek (Clifton, NJ, U.S.A.) Model BAT-12 thermocouple. It was found to be within 2°C of what it was set at. All oven temperatures are reported according to the setting and are ±2°C.

A Perkin-Elmer (Norwalk, CT, U.S.A.) 1310 IR spectrophotometer was used to measure infrared spectra. Potassium bromide pellets of the samples were made in a hand-held press.

The nuclear magnetic resonance (NMR) spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) XL-300 NMR spectrometer at ambient temperature under the conditions indicated in Table I. The reference and solvent for both spectra was $CFCl_3$. ^{19}F chemical shifts are reported relative to $CFCl_3$. ^{13}C chemical shifts are reported relative to tetramethylsilane. Chemical shifts are reported in parts per million (ppm) and coupling constants are reported in Hertz (Hz).

TABLE I

OPERATING CONDITIONS OF NMR

	^{19}F	^{13}C
Operating frequency (MHz)	282.2	75.4
Spectral width (Hz)	20 000	16 502
No. of repetitions	16	1024
Digital resolution (Hz)	3.36	0.55
Pulse repetition rate (s)	4.15	4.91

Mass spectra were recorded on a Finnigan (San Jose, CA, U.S.A.) 4000 quadrupole mass spectrometer interfaced to a Hewlett-Packard (North Hollywood, CA, U.S.A.) 5890 gas chromatograph. The gas chromatograph was equipped with a 25 m × 0.32 mm DB-1 capillary column having a film thickness of 0.25 μm (J&W Scientific, Rancho Cordova, CA, U.S.A.). The column was directly inserted into the ion source. The carrier gas was helium at a pressure of 13 p.s.i. The GC oven was initially set at 70°C and ramped linearly to 280°C at 30°/min. Sample concentrations were 3.5 ng/μl and injection volumes were one microliter. On-column injection technique and mass spectrometer operating parameters are described elsewhere[11] with the exception of scan time (for the *m/z* range 100–730) which was 1.0 s per scan for these measurements. The probe measurement was done on a VG Analytical SEQ-70 mass spectrometer.

Procedures

The 12 ft. × 2 mm column used to study GC properties was purchased from Supelco (Bellefonte, PA, U.S.A.). It was packed with 4.5 g of 3% Poly-S 179 (a polyphenyl ether sulfone) on 100–120 Gas Chrom Q II purchased from Alltech (Deerfield, IL, U.S.A.). Optimization of the derivatization reaction conditions was done with a 6 ft. × 2 mm Supelco column packed with 5% PPE-21 on 100–120 Chromosorb W AW (Supelco). Columns were installed with high-temperature Supeltex M-4 ferrules. The septum was an Alltech Ultrasep R and was changed every

50–75 injections (at room temperature). The carrier gas was grade 4.5 nitrogen and was passed through a molecular sieve/silica gel trap (Buck Scientific) and an R&D (N. Highlands, CA, U.S.A.) Model OT3 oxygen trap before entering the column. All bottled gases were purchased from Wesco (Billerica, MA, U.S.A.).

A Pasteur pipette connected to a Gilson Pipetteman was used to transfer organic liquids. Disposable micropipettes or Hamilton syringes were used to transfer quantities less than 50 μl. A 10-μl Hamilton syringe was used to inject samples into the gas chromatograph. A Bausch & Lomb 7× magnifier was used to read injection volumes. The volume (*ca.* 0.2 μl) remaining in the syringe after an injection was taken into consideration. All solutions were stored in either 20 ml or 6 ml glass scintillation vials. PTFE liners (Thomas Scientific, Swedesboro, NJ, U.S.A.) were inserted in all scintillation vial caps. Vials and liners were used without treatment and only once. A Multi-Blok heater from American Scientific Products was used to control the temperature of derivatization reactions. An Electrothermal melting point apparatus was used. Thermometers were calibrated with water. An Alltech 100-ml soap bubble rotameter in conjuction with a seven jewel Kaltron stopwatch accurate to 1/5 s was used to calibrate the carrier gas flow meters. Flow-rates were corrected for the vapor pressure of water and found to be a parabolic function of the meter reading. The same stopwatch was used for all kinetic measurements.

Synthesis of pentafluorobenzoic anhydride

Pentafluorobenzoic anhydride was synthesized following the general method of anhydride synthesis of Abdel-Baky and Giese[12]. This method is easy to use, high yields are obtained and the reactions all occur in one flask at room temperature. The product was recrystallized in anhydrous diethyl ether at −20.0°C, then dried under nitrogen and vacuum desiccated overnight. This process was repeated until a constant melting point was obtained (66–68°C)[12]. The product was further characterized with ^{19}F NMR, ^{13}C NMR and IR spectroscopy as will be discussed in the Results section.

Benzoic anhydride was also synthesized using the same method. Its melting point[12] and infrared spectrum[13] were in agreement with previous studies.

Methyl esterification with diazomethane

All free acids were first methyl-esterified with diazomethane[14] at 0°C prior to derivatization. That is to ensure that the carboxylic group does not interfere with the PFBA derivatization and to provide volatility on the GC column. This reaction was carried out in the fume hood. An explosion may result from the use of chipped glassware or ground-glass joints. We recommend to use protective gloves. Decant from one flask to another; do not use Pasteur pipettes to transfer solutions of diazomethane. For α,β-unsaturated fatty acids it has been reported that diazomethane can add to this double bond to yield pyrazolines[15].

In a 50-ml Erlenmeyer flask (first flask) approximately 25 ml of diethyl ether was added to 10.0 ml of cool 50% potassium hydroxide. The resulting two-phase system was allowed to chill in an ice bucket. The free acid to be methyl esterified was dissolved in a small amount of diethyl ether in a second 50-ml flask and also allowed to chill. After the solutions equilibrated to 0°C, approximately 5 mg of N-methyl-N-nitrosourea was added to the first (two-phase) flask. Diazomethane was generated in the aqueous phase and it bubbled into the ether layer. Gentle swirling may be required.

After a sufficient amount of diazomethane dissolved in the ether, as indicated by a yellow color, the ether layer was poured onto potassium hydroxide pellets in a third flask to dry. Then, the dried diazomethane solution was added to the second flask containing the free acid. This solution was maintained at 0°C for 20 min. If a yellow color still persisted the reaction was assumed to be complete. Then, the entire reaction mixture was dried down under nitrogen and the fatty acid methyl ester was redissolved in toluene or decane–toluene (1:1), except that ethyl acetate was used to dissolve methyl 16-hydroxyhexadecanoate.

PFBA-derivatization reaction

After the carboxyl groups were methyl esterified with diazomethane, the 2-hydroxy methyl esters were derivatized with PFBA to form 2-O-pentafluorobenzoyl-methyl esters. Methyl 2-hydroxyhexadecanoate was used to optimize the reaction. The objective of optimization was to obtain the highest yield in the shortest period of time using the mildest conditions possible. The optimal conditions were as follows.

A stock solution of 0.1 *M* PFBA in toluene was found to be stable at room temperature. This concentration was 25-fold in excess of the hydroxy methyl ester solutions. Hydroxy methyl esters were dissolved in toluene, decane–toluene (1:1) or ethyl acetate and stored at room temperature. Just prior to running the reaction, 20 μl of pyridine were added to 0.48 ml of 0.1 *M* PFBA in a 6.0-ml glass scintillation vial. The PFBA–pyridine solution and the hydroxy methyl ester solution were equilibrated to 50°C in a heating block. After temperature equilibration, 0.5 ml of the hydroxy methyl ester solution was added to the PFBA solution. Using these conditions, the reaction was found to be 98% complete after 1 h (see Fig. 1).

RESULTS

Characterization of pentafluorobenzoic anhydride

We wanted to be certain that we had synthesized the correct compound. There are very few references to this compound in the literature. Only the melting point has been reported[12]. For identification purposes we measured the ^{19}F NMR, the ^{13}C NMR, and IR spectrum of our product. First-order analysis was used for the NMR spectra.

The ^{19}F chemical shifts (ppm) were: *ortho*: 136.7, *meta*: 161.8, *para*: 145.3. Assignments were based on the assignments for pentafluorobenzoic acid[16] and pentafluorobenzoyl chloride[17]. The ^{13}C chemical shifts (ppm) and nearest neighbor coupling constants (Hz) were: C-1: 106.9, *ortho*: 147.5, J_{CF}: 263.3; *meta*: 139.1, J_{CF}: 252.7; *para*: 145.9, J_{CF}: 255.7; C=O: 153.2. These assignments were again based on assignments for pentafluorobenzoic acid[18] and pentafluorobenzoyl chloride[19].

The IR spectrum compared favorably with the vibrational spectrum of pentafluorobenzoyl chloride[20]. In anhydrides, the carbonyl stretch is split[21]. The splitting was 64 cm^{-1} and the bands occurred at 1741 cm^{-1} and 1805 cm^{-1}. The reported[21] values for benzoic anhydride (in *n*-hexane) are: 1740 cm^{-1} and 1801 cm^{-1}.

All measurements made on the product were consistent with its being pentafluorobenzoic anhydride. It was found to be stable (constant melting point) in a desiccator at room temperature.

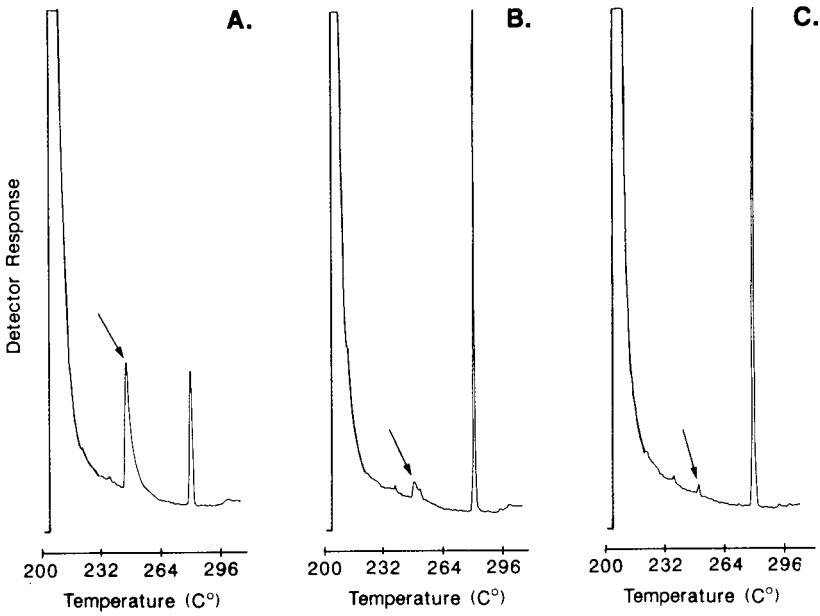

Fig. 1. Gas chromatograms of methyl 2-hydroxyhexadecanoate (arrow) and PFBA-derivatized methyl 2-hydroxyhexadecanoate during a derivatization reaction. The column was PPE-21. Chart speed is 0.5 cm/min. See Results and Experimental sections for further chromatographic conditions. Arrows indicate underivatized methyl 2-hydroxyhexadecanoate. (A) 1 min; (B) 36 min; (C) 62 min.

Optimization of the PFBA-derivatization

When optimizing the reaction parameters, we monitored the quantity of methyl 2-hydroxyhexadecanoate and derivatized methyl 2-hydroxyhexadecanoate using flame ionization detection (FID). The temperature program had an initial temperature of 200°C, a rate of 8°/min and a final temperature of 300°C (PPE-21 column). The reaction was 98% complete after 1 h using the optimal reaction conditions described in Experimental (Fig. 1). Based on unknown peaks in the chromatograms, which may be due to impurities, side reactions were limited to *ca.* 1.0% of derivatized methyl 2-hydroxyhexadecanoate. Upon standing for a few days the gas chromatogram of the reaction solution indicates that the reaction proceeded to 100% completion. From those observations, the FID response of derivatized methyl 2-hydroxyhexadecanoate is seen to be 1.36 times that of the underivatized compound. That relative response is consistent with the number of carbons found in each compound. Derivatized compounds in solution were found to be stable at room temperature for several months.

A plot of the disappearance of methyl 2-hydroxyhexadecanoate fits a first order curve with good correlation. The first order rate constant was found to vary linearly with PFBA concentration. The rate did not change appreciably with temperature. The pyridine concentration was found to be critical. Particularly at higher temperatures, the distillation of pyridine onto the sides of the vial and cap may slow the reaction and possibly the reaction may not proceed to completion, depending on how much

pyridine is lost from the solution. Consistent with previous results[10], when only pyridine was used as the solvent, the reaction solution turned yellow and then dark brown. Although it was not thoroughly investigated, derivatization with benzoic anhydride appears to proceed cleanly in pyridine.

The derivatization of 1-octanol, 3-octanol, 1-decanol, 1,10-decanediol, 1-dodecanol, 1-pentadecanol and 1-octadecanol all proceeded easily to completion, as in the case of the methyl 2-hydroxyhexadecanoate. The only tertiary alcohol attempted was trimethylcitrate. Even with a 50-fold excess of PFBA the reaction did not proceed to completion (overnight).

In this study, the reaction solution was injected directly into the gas chromatograph. Since these results indicate that this derivatizing agent may be used for measuring a large variety of primary and secondary alcohols, the mode of sample cleanup would vary according to the source of the alcohol. One way of removing excess reagents and contaminants is with an aqueous wash and subsequent use of a disposable silica column[10].

Mass spectra

To be certain of the structure of our PFBA-derivatized compounds, we measured the positive-ion chemical ionization (PICI) mass spectrum of derivatized methyl 2-hydroxyhexadecanoate (Fig. 2). Table II shows that the fragmentation pattern is consistent with the proposed structure. (See ref. 22 for a review of the CI-MS of lipids.) The similar relative abundances of $[M + H]^+$ and $[M - H]^+$ and the small relative abundances of $[M - CH_3OH]^+$ and $[M - H - CH_3OH]^+$, can be attributed to the relatively long alkyl chain [23]. Only the assignment of the ion at *m/z* 169 is questionable. It is probable that this ion is associated with the pentafluorobenzoyl moiety. Albeit remote, another possibility is $CH_3(CH_2)_{11}$. An ion at *m/z* 169 is often associated with perfluoroalkanes[24]. Possibly, this peak is due to a residual contaminant in the instrument[25].

The NICI mass spectra for derivatized methyl 2-hydroxyhexadecanoate and derivatized methyl 2-hydroxycerotate (C_{26}) at an ion-source temperature of 80°C are shown in Fig. 3. Contrary to 3-O-pentafluorobenzoyl-methyl myristate (3-OH:14:0) ref. 10, our compounds show significant fragmentation even at 80°C. Possibly, the greater fragmentation observed here is due to the closer proximity of the two ester groups to each other in our compounds, since the methane-PICI mass spectrum of methyl phthalate shows greater fragmentation than the methane-PICI mass spectrum

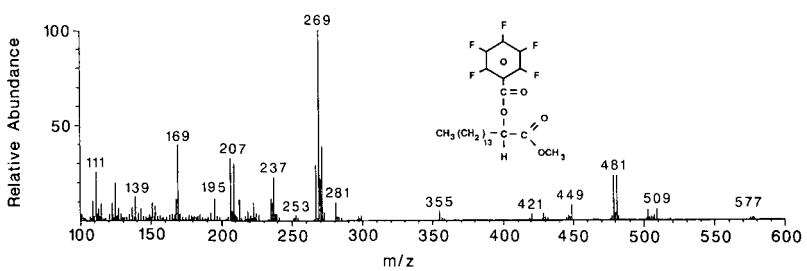

Fig. 2. Positive-ion chemical ionization mass spectrum of derivatized methyl 2-hydroxyhexadecanoate. Ion source temperature is 240°C.

TABLE II

PICI-MS FRAGMENTATION PATTERN OF DERIVATIZED METHYL 2-HYDROXYHEXADECANOATE

M is the molecular ion *m/z* 480. Ion-source temperature was 240°C.

m/z	*% Relative abundance*	*Positive ion assignment*
509	5.8	M + C_2H_5
481	23.2	M + H
479	23.0	M − H
449	6.8	[M + H] − (CH_3OH)
421	2.5	M − (CO_2CH_3)
355	4.9	Column bleed [$Si_5O_5(CH_3)_9$]
281	9.3	Column bleed [$Si_4O_4(CH_3)_7$]
269	100.0	$CH_3(CH_2)_{13}CHCO_2CH_3$
253	2.5	$CH_3(CH_2)_{13}CHCOCH_3$
237	22.7	$CH_3(CH_2)_{13}CCO$
207	31.3	Column bleed [$Si_3O_3(CH_3)_5$]
195	11.2	C_6F_5CO
169	39.3	C_6F_5H + H (?)
139	12.2	C_6F_2HCO
111	25.5	C_6F_2H

of methyl isophthalate[26]. Ion (*m/z*) assignments for fragments are: 130: C_6F_3H, 148: C_6F_4, 167: C_6F_5, 211: $C_6F_5CO_2$. Ion *m/z* 480 corresponds to the molecular ion for derivatized methyl 2-hydroxyhexadecanoate (Fig. 3A) and *m/z* 620 corresponds to the molecular ion for derivatized methyl 2-hydroxycerotate (Fig. 3B). The ion at *m/z* 193 has previously[8] been tentatively assigned as a tetrafluorohydroxybenzoyl system. Ion

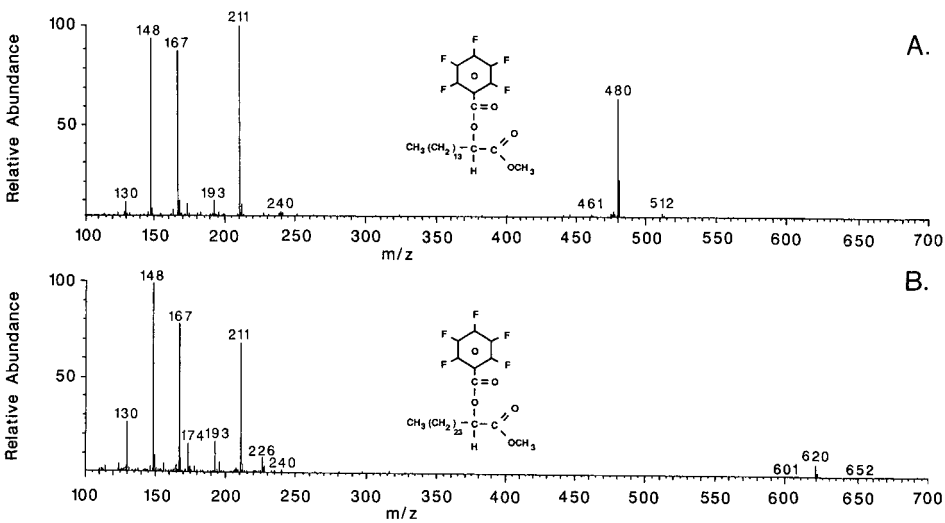

Fig. 3. Negative-ion chemical ionization mass spectrum of (A) derivatized methyl 2-hydroxyhexadecanoate; (B) derivatized methyl 2-hydroxycerotate. Ion source temperature is 80°C.

m/z 174 is consistent with this assignment (loss of fluorine). Another possibility is replacement of fluorine with hydrogen (*m/z* 211 − F + H). The ions at *m/z* 174 (*m/z* 211 − 2F + H) and *m/z* 130 (C_6F_3H) are consistent with this possibility.

Both compounds (C_{26} and C_{16}, see Fig. 3) show a small amount of $[M + 32]^-$. Possibly, this is due to the addition of CH_3OH. In a preliminary measurement using the direct-introduction probe technique (data not shown), the spectrum of *ca.* 1.0 μg of derivatized methyl 2-hydroxycerotate had $[M + 32]^-$ for its base peak (M^- had a relative abundance of 36%). The ions attributed to electron-capture processes approach a limiting value with large sample sizes[27]. If large sample concentrations are encountered, adduct ion formation may dominate the spectra[27] (leading to erroneous analysis of the spectrum). The presence of $[M + 32]^-$ in the spectra of Fig. 3 implies that even 3.5 ng of sample is slightly overloading the instrument. Taking into consideration that pentafluorobenzoyl derivatives can be observed in the femtogram region[8], this is not surprising.

Fig. 4 shows the ion-source temperature dependence of the NICI-MS frag-

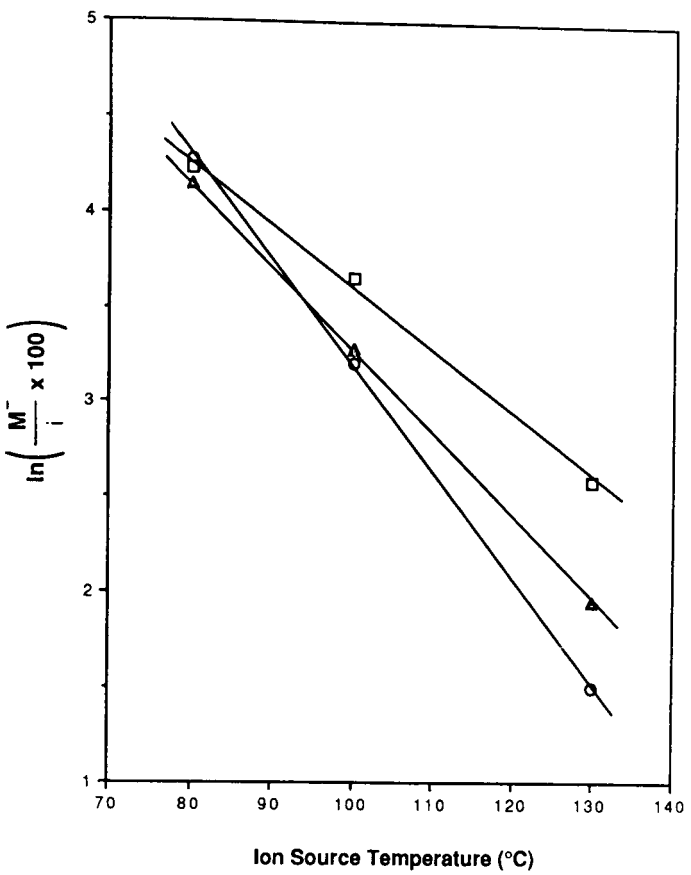

Fig. 4. This plot shows the temperature dependence of the natural logarithm of the ratio (× 100) of the relative abundances of the molecular anion (*m/z* 480) to the pentafluorobenzoyl anion *m/z* 211 (▲), to the pentafluorophenyl anion *m/z* 167 (○), and to the tetrafluorophenyl anion *m/z* 148 (□) for the NICI mass spectrum of derivatized methyl 2-hydroxyhexadecanoate.

mentation for derivatized methyl 2-hydroxyhexadecanoate. When the ion-source temperature was higher (240°C), the molecular ion (*m/z* 480) was not observable; only *m/z* 148 and *m/z* 167 were present in the spectrum. These data suggest a dissociative electron-capture mechanism[28,29]. This is consistent with our results using ECD for derivatized methyl 2-hydroxystearate (see below). From Fig. 3B one can see that the molecular ion (*m/z* 620) for derivatized methyl 2-hydroxycerotate (C_{26}) is barely observable even when the ion-source temperature is 80°C. This indicates, in agreement with previous measurements on comparable compounds[28–30], that for proper identification of these derivatives, within the confinements of the instrument, one must find the optimum ion-source temperature.

The total positive-ion chromatogram and the total negative-ion chromatogram of derivatized methyl 2-hydroxyhexadecanoate indicate that the reaction goes to 100% completion and there are not any observable side reactions. This agrees with our measurements using the PPE-21 column (Fig. 1) and the Poly-S 179 column (data not shown).

The sensitivity of the electron-capture detector

The response of the electron-capture detector is a function of the compound being detected and the temperature of the detector. Assuming that $A + e^- \rightleftharpoons A^-$ is an equilibrium process, it can be shown that[31]:

$$K_{eq} = CT^{-3/2} \exp(E_a/kT)$$

where A is the electron acceptor, K_{eq} is the equilibrium constant, C is a constant, T is the absolute temperature, E_a is the electron affinity, k is the Boltzman constant.

When low detection limits are required, it is important to find the optimum detector temperature for the compounds of interest[32]. The data for detector response with respect to detector temperature is usually plotted in the form $\ln(CK_{eq}T^{3/2})$ *vs.* $1/T$. CK_{eq} is the intensity of the response. This plot indicates the optimal temperature and the mechanism of electron capture[33]. Discontinuities in these plots indicate that the mechanism can change with temperature. Fig. 5 indicates that derivatized 1-dodecanol has a non-dissociative mechanism that favors use of low temperatures. The pentafluorobenzoate derivative of *n*-hexanol[34] exhibits the same mechanism. Contrary to that, but consistent with the NICI-MS data for derivatized methyl 2-hydroxyhexadecanoate (Fig. 4), derivatized methyl 2-hydroxystearate (Fig. 5) has a dissociative mechanism up to approximately 250°C. For this derivatized hydroxy fatty acid 250°C is the optimal temperature. The decline in response observed above 250°C is probably due to thermal electron detachment[30].

The linearity of ECD response with amount may be very limited. For example, butyl 3-hydroxydodecanoate derivatized with heptafluorobutyric anhydride has a linear response only between 5.0 pg and 50.0 pg[35]. Fig. 6 indicates that with our apparatus, the linear region for derivatized methyl 2-hydroxystearate occurs between 0.07 pmol (34 pg) and 7.0 pmol (3.4 ng). The upper limit of linearity is consistent with our NICI-MS data. Using an on-column injector and a capillary column, Mohamed *et al.*[8] were able to generate a linear calibration curve from 1 fg to 1 ng for N^4-pentafluorobenzoyl-1,3-dimethylcytosine.

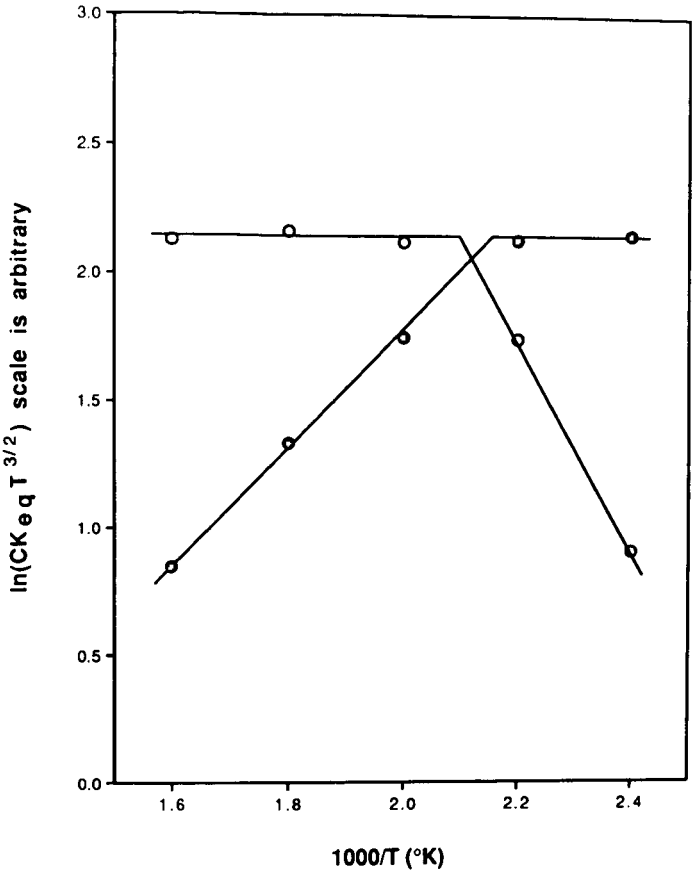

Fig. 5. Plot showing temperature dependence of ECD response. $\ln(CK_{eq}T^{3/2})$ *vs.* $1/T$ for derivatized dodecyl alcohol (●) and derivatized methyl 2-hydroxystearate (○). Scale has been normalized to same maximum response for both compounds.

Calculation of specific retention volumes

The goal here was to measure and report the retention volumes of the homologous series of derivatized 2-hydroxy fatty acid methyl esters so that they could be used as a standard for the identification of unknowns in subsequent measurements. It is customary to simply report retention times or retention temperatures (when using temperature programming); however, these were not measured directly because the carrier gas flow-rate was found to be a function of column temperature as will be shown. Therefore, measured retention times were converted to specific retention volumes taking into consideration that the carrier gas flow-rate was not constant.

Because of the large dead volume between the column and the electron-capture detector, the high temperatures employed in these measurements, the length of the column (12 ft.), and the use of 100–120 mesh support, maximum flow of the carrier gas was required to obtain reasonable peak shape. Under these conditions, the carrier gas flow-rate (as it entered the instrument) plotted against $1/T^{0.7}$ yielded a straight line

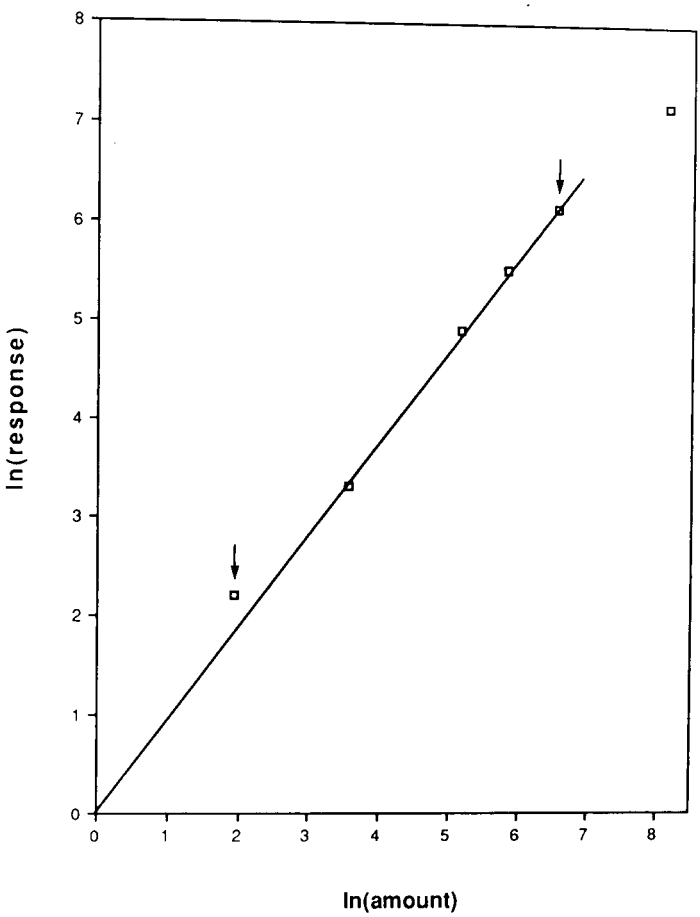

Fig. 6. Standard curve of ECD response *vs.* amount of derivatized methyl 2-hydroxystearate. Arrows indicate 0.07 and 7.0 pmol.

(Fig. 7). Since the reciprocal of the viscosity of a gas as a function of temperature approximately fits the same curve[36], this indicated that the flow controllers were inoperative and the measurements were being made at constant pressure rather than constant flow.

For use in constructing temperature programs[37], isothermal retention time *vs.* temperature was measured. It is generally accepted[38] that:

$$V_r - V_{ds} = A \exp(\Delta H/RT)$$

where V_r is the isothermal retention volume, V_{ds} is the dead space volume, A is assumed to be independent of temperature and it has been shown[39] that $R\ln A$ is equal to the entropy of solution of the component in the stationary liquid phase, ΔH is the heat of evaporation of the component from the stationary liquid phase, R is the gas constant, and T is the absolute temperature. When operating under constant pressure, because of the required corrections, it is much more convenient to use retention volumes rather

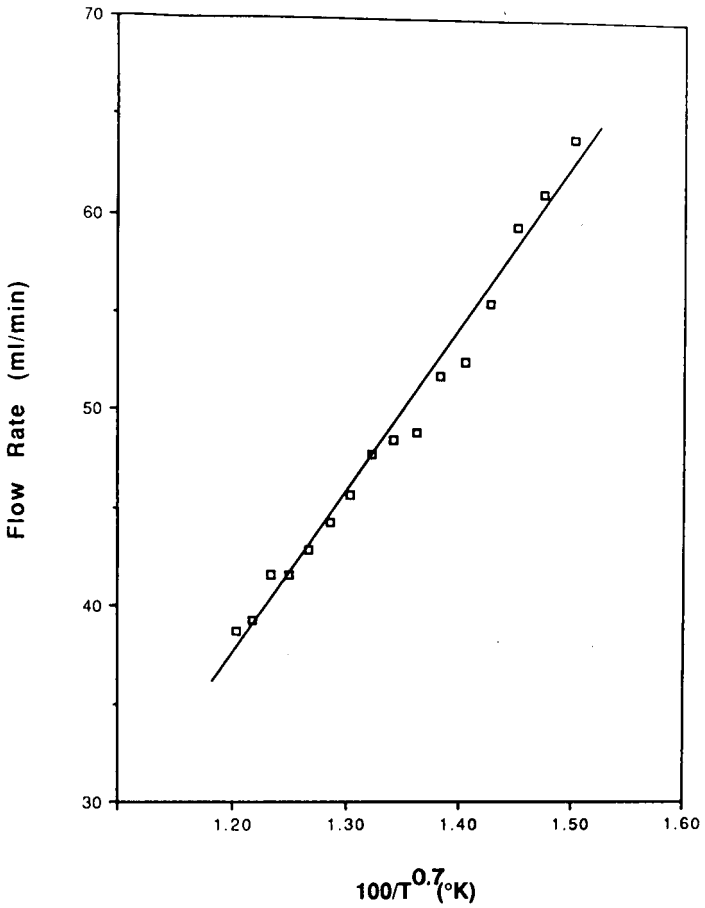

Fig. 7. Plot showing temperature dependence of flow-rate at column inlet. Flow-rate at column inlet *vs.* $1/T^{0.7}$ is plotted.

than retention times. Taking into consideration that the flow-rate was measured at the column inlet, the measured retention volume (V_{meas}) was corrected for the pressure differential in the column according to the method of James and Martin[40].

$$V_R = j' V_{meas}$$

$$j' = 1.5\{[(P_0^2/P_i^2) - 1]/[(P_0^3/P_i^3) - 1]\}$$

where V_R is the corrected retention volume, P_i is the inlet pressure (78.0 p.s.i.), and P_0 is the outlet pressure (14.7 p.s.i.). Then, according to the method of Littlewood *et al.*[41] V_R was converted to the specific retention volume, V_g.

All of the above corrections yield:

$$F(T) = (79\,326.0/T^{0.7}) - 592.2$$

$$V_g = (t_R - t_{ds})\, F(T)$$

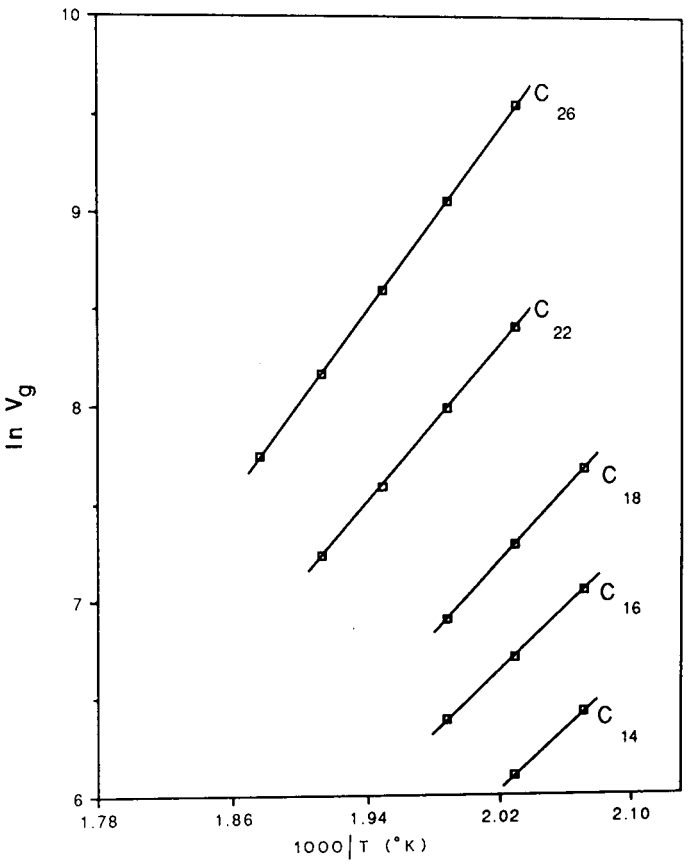

Fig. 8. Temperature dependence of the specific retention volume (V_g). ln V_g of non-derivatized 2-hydroxy fatty acids *vs.* $1/T$ is plotted.

where $F(T)$ is the corrected flow-rate at 0°C per gram of stationary phase (ml/min-g), t_R is the retention time and t_{ds} is the t_R for dead space. According to Littlewood *et al.*[41]:

$$V_g = A \exp(\Delta H/RT)$$

where ΔH is the heat of evaporation of the component from the stationary phase at 0°C. From plots of ln V_g *vs.* $1/T$ for the homologous series of 2-hydroxy fatty acid methyl esters (Fig. 8 non-derivatized compounds and Fig. 9 derivatized compounds), Table III was generated. The difference in $\Delta H/R$ between non-derivatized compounds and derivatized compounds appears to be a constant independent of carbon number and has a mean of 2310. This implies that the derivatized products form a homologous series which is analogous to the homologous series of the commercially-obtained non-derivatized 2-hydroxy fatty acids.

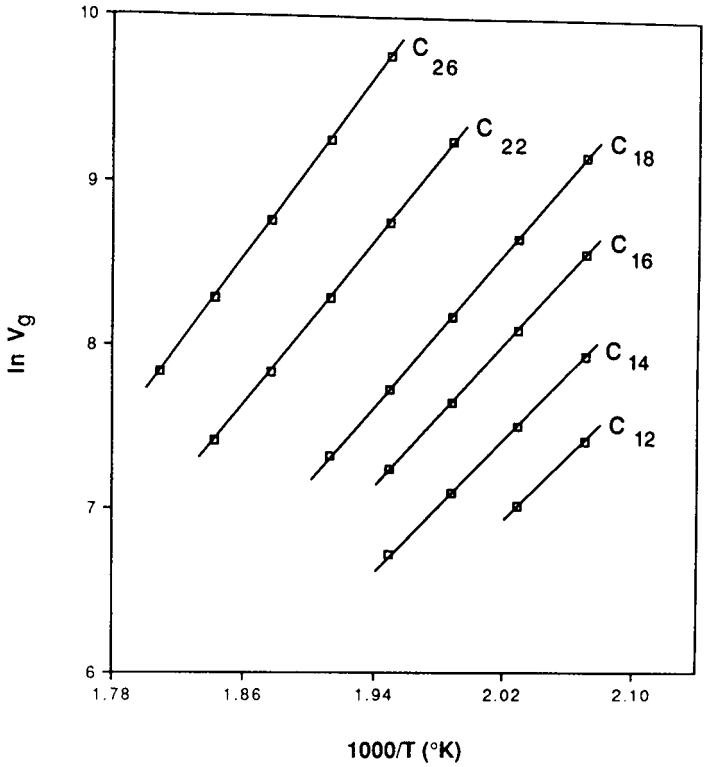

Fig. 9. Temperature dependence of the specific retention volume (V_g). $\ln(V_g)$ of derivatized 2-hydroxy fatty acids *vs.* $1/T$ is plotted.

TABLE III

THERMODYNAMIC PROPERTIES OF THE HOMOLOGOUS SERIES

The column packing was Poly-S 179. The carrier gas was nitrogen.

Carbon number	*Non-derivatized*		*Derivatized*		*Difference*[a]
	$\Delta H/R$	*ln A*	$\Delta H/R$	*ln A*	
12	–	–	9760	−12.78	–
14	7860	−9.84	10 160	−13.09	2300
16	8140	−9.80	10 930	−14.06	2790
18	9360	−11.69	11 570	−14.81	2210
22	10 160	−12.20	12 490	−15.58	2330
26	11 800	−14.40	13 710	−16.96	1910
				Mean:	2310
				Standard deviation:	283

[a] This is the difference between $\Delta H/R$ for derivatized compounds and $\Delta H/R$ for non-derivatized compounds.

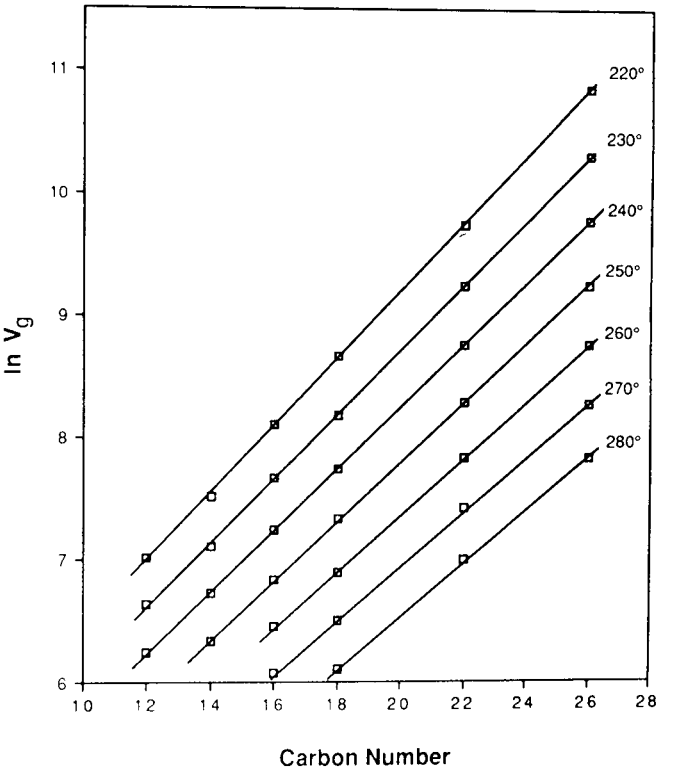

Fig. 10. Plot showing relation between ln V_g and the number of carbons for derivatized hydroxy fatty acid methyl esters.

TABLE IV

COMPARISON OF OBSERVED AND PREDICTED RETENTION TEMPERATURES FOR DERIVATIZED COMPOUNDS FOR THE TEMPERATURE PROGRAM T_i = 210°C, rate = 2°/min

A 12 ft. × 2 mm column was packed with 3% Poly-S 179. Carrier gas was nitrogen. Inlet pressure was 78 p.s.i.

Carbon number	T_R (°C)	
	Observed	*Predicted*
12	216.8	216.9
14	221.5	220.6
16	227.5	227.1
18	235.1	234.6
22	253.5	253.4
26	272.7	273.9

Temperature programming

To test the usefulness of the data in Table III, they were used to predict retention temperatures for the following temperature program: $T_i = 210°C$, rate = 2° per min, $T_f = 310°C$. Since V_{ds} was found not to vary with temperature (over the range studied), this term was neglected. From Harris and Habgood[38], to calculate the retention temperature, one must evaluate the following integral:

$$r/F_{T_i} = (1/A) \int_{T_i}^{T_R} F'(T)\exp(-\Delta H/RT)\mathrm{d}T$$

where r is the heating rate (deg/min), F_{T_i} is the corrected flow-rate per gram of stationary phase at the initial temperature, $F'(T)$ is the corrected flow-rate at the column outlet, T_R is the retention temperature and $\Delta H/R$ is assumed to be independent of temperature.

Since $PV = nRT$, where n is the number of moles,

$$F'(T) = \frac{F(T)}{T}$$

Consequently (for this system):

$$4.38 \cdot 10^{-3} = (1/A) \int_{T_i}^{T_R} [F(T)/T]\exp(-\Delta H/RT)\mathrm{d}T$$

Since this integral can not be integrated in closed form[42], it must be evaluated numerically. This calculation was done on a personal computer (Tandy 1000 HD with an 8088 microprocessor and an 8087 math coprocessor) using Turbo Pascal Numerical Methods Toolbox (Version 4.0)[43] and the method of Akporhonor *et al.*[44]. Table IV indicates excellent agreement between observed and predicted values.

A retention index for ECD-sensitive derivatized hydroxy fatty acids

Fig. 10 shows that for the homologous series of derivatized 2-hydroxy fatty acid methyl esters there is a linear variation of $\ln V_g$ with number of carbons. Therefore, it is possible to define a retention index[45] of an unknown, I_x, for the derivatized compounds such that:

$$I_x = 100\left[z + \left(\frac{\ln V_{g_x} - \ln V_{g_z}}{\ln V_{g_{(z+1)}} - \ln V_{g_z}}\right)\right]$$

where z is the number of carbon atoms. Using this index at a column temperature of 220°C with interpolated values, yields $I = 1887$ for derivatized methyl ricinoleate (12-OH:18:1ω9) and $I = 1989$ for methyl 16-hydroxyhexadecanoate (16-OH:16:0). The anomolously high retention index of this derivatized 16-hydroxy compound is consistent with previous work on acetoxy fatty acid methyl esters[46]. In subsequent

work, when mass spectral data are unavailable, unknown peaks will be reported according to this retention index, although it may be subject to modification if a capillary column is used.

Derivatization of cholesterol

Since our intent is to use this method to study lipid peroxidation in the retina, we were concerned that the large quantity of cholesterol[47] present might overwhelm the electron-capture detector. When the kinetic course of the derivatization of cholesterol was monitored using GC–FID, the cholesterol peak disappeared as a function of reaction time. The product peak appeared at a much shorter retention time than cholesterol, indicating that it probably was not cholesteryl pentafluorobenzoate. It has been shown that cholesteryl heptafluorobutyrate thermally decomposes to cholesta-3,5-diene[48]. The retention time of our product peak matched that of commercially obtained cholesta-3,5-diene. Since cholesta-3,5-diene has a very weak ECD response, it is anticipated that cholesterol will not interfere with future measurements.

DISCUSSION

Although the pentafluorobenzyl esters of carboxylic acids[49] and pentafluorobenzoate esters of phenols[11,50] have been measured in the past, use of pentafluorobenzoic anhydride to derivatize secondary alcohols has not been reported. Our results indicate that this is an excellent derivatizing reagent for primary and secondary alcohols.

From the chromatographic data it is clear that the retention temperature of hydroxy fatty acid methyl esters is significantly increased with the addition of the pentafluorobenzoate group (Figs. 8 and 9). With the use of Poly-S 179 packing the high temperatures required for elution were not a significant problem. This packing has been shown to be useful in the separation of a variety of compounds at temperatures as high as 395°C[51,52]. Although, we do not recommend using temperatures that high for these derivatives since they may undergo pyrolytic elimination of the pentafluorobenzoyl group[53,54]. The polarity of Poly-S 179 has been shown to be comparable to Carbowax 20M[55].

The efficiency of the column for derivatized methyl 2-hydroxycerotate (C_{26}) and derivatized 2-hydroxybehenate (C_{22}) is 630 and 530 theoretical plates, respectively. This yields a resolution[56] of 6.3 between those two components, indicating that an additional five peaks may be resolved between these two homologues[57]. This resolution may be improved by reducing the dead volume between the column outlet and the detector. Comparable resolution occurs between derivatized methyl 2-hydroxystearate (C_{18}) and derivatized methyl 2-hydroxybehenate. A capillary column coated with Poly-S 176 (similar to Poly-S 179) has been shown to provide good separations of fatty acid methyl esters[58] and would provide much better resolution of the derivatized compounds. Recently, a Poly-S 179 capillary column was prepared and used to separate polycyclic aromatic hydrocarbons and triglycerides at temperatures as high as 380°C using GC–FID[59]. The favorable results reported indicate that this column may be very useful in studying the oxidation products of lipids in the retina using GC–ECD.

Previous methods for detecting trace levels of hydroxy fatty acids derivatize the

hydroxyl group with a trimethylsilyl group and esterify the carboxyl group with pentafluorobenzyl bromide[49] or transesterify the carboxyl group with pentafluorobenzyl alcohol[3]. A disadvantage of trimethylsilyl derivatives is that they are vulnerable to hydrolysis and subsequent side reactions[3]. The result of using pentafluorobenzyl bromide or pentafluorobenzyl alcohol to esterify or transesterify the carboxyl group is that all fatty acids in the mixture to be analyzed have a large electron-capture response. Single-ion monitoring (SIM) MS alleviates that problem to a large extent[3], but coelution can still occur in complex biological mixtures. When PFBA is used to esterify the hydroxyl group, then the specificity of the method is increased substantially because only the fatty acids of interest [*i.e.* those that have been peroxidized (and subsequently reduced to alcohols)] are derivatized.

SIM-NICI-MS with the pentafluorobenzyl group on the carboxyl yields a sensitivity of *ca.* 10 pg[3]. This is extremely good considering that the observed ions are carboxylate anions sans the pentafluorobenzyl group. Without SIM, sensitivity only in the nanogram region has been reported for pentafluorobenzyl esters when using NICI-MS[49]. Our results indicate that when pentafluorobenzoate is on the hydroxyl group (O-pentafluorobenzoyl-methyl esters), a large amount of the sample introduced into the mass spectrometer does not undergo any fragmentation. Consequently, the observed anion still maintains its (extremely good) electron-capture sensitivity. When the electron-capturing group is on the hydroxyl group (where it remains), then a 20-fold increase in sensitivity (*ca.* 0.3 pg) is observed in the SIM-NICI-MS of 3-O-pentafluorobenzoyl methyl myristate[10]. Also, pentafluorobenzoyl derivatives of cytosine have a sensitivity of one femtogram using NICI-MS[8]. For the specific analysis of lipid hydroperoxides our method should be a useful complement to the method of Van Kuijk and co-workers[3,4].

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CHROM. 21 190

DETERMINATION OF BENZENES AND NAPHTHALENES IN WATER BY PURGE AND TRAP ISOLATION AND CAPILLARY COLUMN CHROMATOGRAPHY

TRYGG N. BARNUNG[a] and OTTO GRAHL-NIELSEN*
Department of Chemistry, University of Bergen, Allégt 41, N-5007 Bergen (Norway)
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SUMMARY

A method for determination of volatile, aromatic hydrocarbons in the range from benzene to C_2-naphthalenes has been adapted for monitoring the concentration of these components in water. The hydrocarbons are purged from the water by helium, trapped on an adsorbent, Tenax-GC, desorbed from this by rapid heating, transferred directly to a fused-silica gas chromatography column, which is cooled to $-80°C$ for initial cryotrapping, and finally chromatographed by programmed raising of the temperature of the column. The whole process is automated and takes 40 min, plus 10 min for temperature equilibration for the next experiment. With 5 ml water samples, concentrations of 0.1 μg/l (ppb) of the individual components were determined. The method was applied to monitoring of a biotest system, and compared to the traditional liquid extraction method.

INTRODUCTION

Conventional solvent extraction techniques are not well suited for determination of volatile petroleum hydrocarbons, in the range up to approximately C_{12},in water, due to the ease of evaporative loss of the analytes[1]. This will occur during all stages in the procedure, and in particular when the extract is concentrated before the final analysis. The alternative to solvent extraction is to isolate the volatiles from the vapour phase above the water. This can be done in two principally different ways: either by head space sampling, static[2] or dynamic[3], or by extracting the components from the water with an inert gas and trapping them on an adsorbent. This can either be done with a given amount of gas which is pumped continuously through a closed loop with the trap included[4], or by an open-ended system with delivery of gas from a reservoir[5]. This latter method is usually termed purge and trap (P&T), and several devices are commercially available, *e.g.*, from Hewlett-Packard, Chrompack, Chemical Data Systems.

[a] Present address: Institute of Marine Research, Division of Aquaculture, Matre Aquaculture Station, N-5198 Matredal, Norway. This study was submitted in partial fulfilment of the requirements for the degree of Cand. Scient. by T.N.B.

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The possibility of complete automation and short analysis times makes the P&T method attractive. The method was originally developed for use with packed gas chromatography (GC) columns[6]. It is, however, desirable to utilize the high resolution power of the present-day capillary columns in connection with P&T sampling. Interfacing of a P&T sampler with a capillary column has been attempted in several cases[7–9]. In all cases the flow of inert gas through the P&T sampler during the desorption of analytes from the trap was higher than the flow through the capillary column. It was therefore necessary to split the gas flow, allowing only a minor part, *e.g.*, one tenth[9], to enter the column. This results in loss in sensitivity.

Pankow and Rosen[10] have installed a direct interface, without splitting, between a P&T sampler and a fused-silica capillary column. The column terminated at the ion source in the high vacuum of a mass spectrometer. With this low pressure at the end of the column, and a fairly high head pressure of 80 p.s.i., the flow through the trap and chromatography column in the desorption step was approximately 20 ml/min. The head pressure was reduced to 10 p.s.i. during the subsequent chromatography.

In another investigation, Pankow and Kristensen[11] have studied the effect of the flow-rate through a Tenax[12] trap on the desorbability of polycyclic aromatic hydrocarbons. They found that naphthalene was completely desorbed by a 2.8 ml/min flow of helium for 20 min at a trap temperature of 250°C. Lower flow-rates or shorter times were not tested.

When the duration of the desorption is several minutes a trapping of the analytes at the beginning of the column is necessary to obtain proper chromatographic results with sharp peaks. This was accomplished by Trussell *et al.*[7] and Dreisch and Munson[8] by immersing the first loop of the column in liquid nitrogen. According to Pankow and Rosen[10] it is better to cool the whole column, *i.e.*, by letting liquid nitrogen into the GC oven, thereby denoting the method as P&T/WCC, *i.e.*, Purge and Trap with Whole Column Cryotrapping. They used a temperature of −80°C, Pankow and Kristensen[11] used −30°C and Adlard and Davenport[9] cooled the GC oven to −50°C.

A crucial point when the P&T sampler is coupled to a capillary column is obviously the flow-rate during the desorption step relative to the flow-rate during the chromatography step. The first purpose of the present investigation was to see if the flow-rate applied for capillary columns, *i.e.*, of the order of 1 ml/min, could be used during thermal desorption of benzene and naphthalene and their alkylated homologues from Tenax-GC. This was desirable because a commercial P&T sampler, Hewlett-Packard 7675 A, should be directly interfaced, without splitting, to a narrow-bore fused-silica column, and since extra manipulations with head pressures would make automation more difficult.

A further purpose of this investigation was to study the potential of the P&T method for detection of very low levels, *i.e.*, 1 μg/l (ppb) and below, of petroleum hydrocarbons in water. Pankow and Rosen[10] have worked with concentrations of the individual compounds of 10 μg/l. When samples of polluted water are collected close to the pollution source, in time and space, concentrations higher than 10 μg/l are usually detected. However, in studies of the dilution and evaporation of the volatile aromatics a detection limit below 1 μg/l is desirable.

Finally, the goal was to monitor the concentration of the water-soluble light aromatics in a system used for biotesting of the effects of these compounds on eggs and larvae of cod, *Gadus morhua*[13–15]. In this system the supply of the polluted water was

limited, and a P&T method, which, in addition to other advantages, also demands small sample volumes, *i.e.*, 5–10 ml, would be ideal for monitoring of the concentrations. The results from this method should be compared with results from a determination based on liquid extraction.

EXPERIMENTAL

Materials

Stock solutions in methanol were prepared of the following aromatic hydrocarbons: benzene, toluene, *m*-, *p*- and *o*-xylene, ethylbenzene, *n*-propylbenzene, naphthalene, 1-methylnaphthalene and 1,4-dimethylnaphthalene. Mixtures of all standards were prepared from aliquots of the individual solutions, and test solutions containing 0.1, 1.0 and 10.0 μg/l, respectively, of each component, were prepared by diluting aliquots of the mixtures to 1000 ml in clean sea-water, pumped from a depth of 120 m in Byfjorden, Bergen, Norway.

Immediately after preparation, seven samples were transferred from each solution to purge vessels. Culture tubes, with a total volume of 16 ml, were used as purge vessels. They were completely filled, tightly sealed with screw caps and stored in a refrigerator until analysis. Seven samples of water from a biotest system[15] were collected in similar tubes and treated in the same manner.

Immediately before analysis, approximately 2/3 of the water in the tubes were decanted off, leaving about 5 ml, which were purged as described below. The exact amount of water was determined by weighing after the purging was completed.

Instrumentation

An Hewlett-Packard 7675 A P&T sampler was used. It was supplied with a 100 mm × 4.8 mm I.D. stainless-steel trap containing 1.3 ml Tenax-GC (60–80 mesh). The transfer line supplied with the sampler was replaced by a 30-cm glass-lined metal tubing, GLT, from Scientific Glass Engineering, of 1/16 in. O.D. and 0.4 mm I.D., which was connected directly with a nut and ferrule to the six-port valve of the sampler. With the sampler placed on top of an Hewlett-Packard 5790 gas chromatograph, the tubing ended inside the GC oven. The tubing was passed through a 1/16-in. male fitting, with 1 mm protruding, and silver-soldered to it. An Hewlett-Packard 25 m × 0.2 mm I.D. fused-silica column was led 2 mm into the tubing and connected by way of a graphite ferrule, the flat end being towards the end of the transfer line, and an extended nut. The liner was maintained at 150°C to avoid condensation of components.

The stationary phase of the column was 5% phenyl 95% methyl silicone of thickness 0.33 μm. Helium was used as the carrier gas at a flow-rate of 1 ml/min, led through the P&T sampler.

The purge vessel, containing 5 ml water to be analysed, was securely fastened to the purge unit and purged with helium at a flow-rate of 40 ml/min. Various purge times were tested. The trap was kept at ambient temperature. The GC oven was cooled to −80°C by liquid nitrogen.

For the desorption step, the trap was ballistically heated to 250°C within approximately 1 min, a flow of 1 ml/min helium was led through the trap and further directly through the transfer line to the capillary column in the cooled oven. Various

desorption times were tested. After desorption, the trap was vented for 5 min at 300°C, and thereafter cooled to ambient temperature by compressed air.

The chromatographic step commenced immediately after the desorption step. The oven was heated from −80 to 0°C in 30 s, kept at 0°C for 5 min, then raised at 10°C/min. The eluates from the column were detected by a flame ionization detector at 350°C and with nitrogen, 30 ml/min, as a make-up gas. The peak areas were determined by an Hewlett-Packard 3390 recording integrator.

RESULTS AND DISCUSSION

To find the optimum volume of purge gas, seven replicates, each 5 ml, of sea-water containing 1 μg/l of, respectively, benzene, *o*-xylene, naphtalene and 1,4-dimethylnaphthalene, were purged for 5, 12.5 and 15 min, with a purge flow of 40 ml/min. The resulting areas of the peaks of the four components are given in Fig. 1.

For benzene the recovery is smaller at 500 ml than at 200 ml, showing that the breakthrough volume for benzene lies between these numbers, while the optimum volume for the other three components apparently is 500 ml. This purge volume, *i.e.*, a purge time of 12.5 min at a flow-rate of 40 ml/min, was chosen for the rest of the investigation, even when the recovery of benzene is only 85% of that obtained with 200 ml.

With a desorption temperature of 250°C, which is 50°C below the maximum recommended operating temperature of Tenax-GC, and a flow-rate of 1 ml/min of helium back-flushing the trap, a desorption time of 5 min was tested first. After

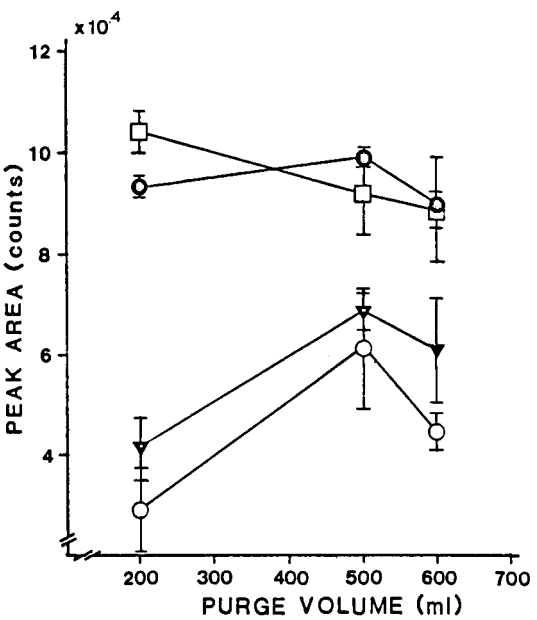

Fig. 1. Effect of different purge volumes on recovery of benzene (□), *o*-xylene (●), naphthalene (▼) and 1,4-dimethylnaphthalene (○) from 5 ml water containing 1 μg/l of each component. The data are given as mean ± standard deviation (n = 5–8).

chromatography of the desorbed material, the trap was re-desorbed for another 5 min. Chromatography showed that the desorption was not complete after the first 5 min. With an initial desorption time of 10 min the desorption was found to be complete for all components from benzene to 1,4-dimethylnaphthalene. In a systematic investigation of the desorbability of different components from Tenax-GC, Pankow and Kristensen[11] obtained complete recovery of naphthalene at a flow-rate of 2.8 ml/min for 20 min at 250°C. Since their investigation covered components with lower desorbability than naphthalene, *i.e.*, larger aromatic hydrocarbons and pesticides, they did not test lower desorption volumes. On the contrary, they needed much higher volumes and higher temperature for the least desorbable components.

Typical chromatograms from analyses of samples of the three different concentrations of test solutions and of a sample of water from the biotest system are

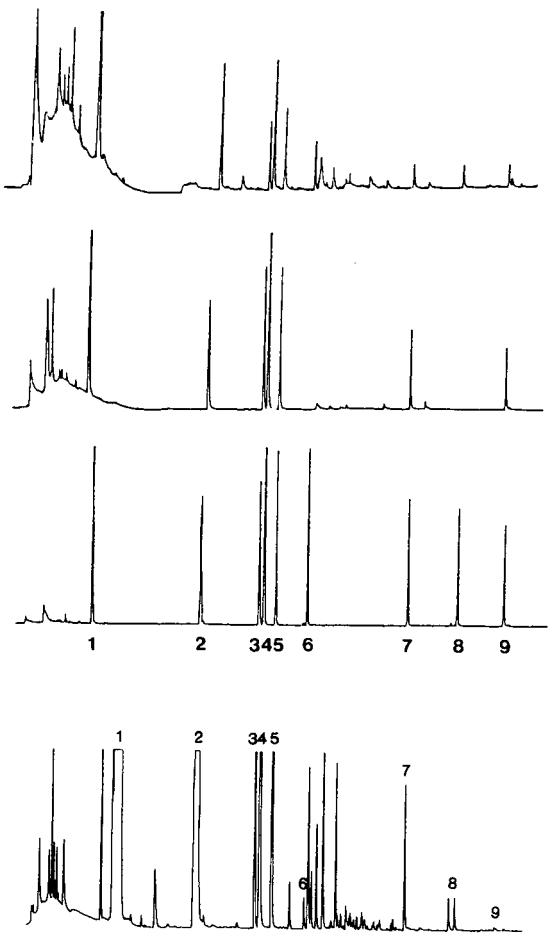

Fig. 2. Chromatograms of standard solutions of aromatic hydrocarbons in water at the following concentrations of each component; from top, 0.1, 1.0 and 10.0 μg/l; and, at the bottom, of water from a biotest system. Peaks: 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = *m*- plus *p*-xylene; 5 = *o*-xylene; 6 = *n*-propylbenzene; 7 = naphthalene; 8 = 1-methylnaphthalene; 9 = 1,4-dimethylnaphthalene.

shown in Fig. 2. The elevated baseline at the beginning of the chromatograms, particularly evident at the lowest concentration of the standards, is due to changes in the flow-rate of the carrier gas during the rapid temperature rise from −80 to 0°C. It was necessary to install extra charcoal filters on the helium supply line to obtain stable baselines of the chromatograms.

Even at the lowest concentration, all components give well defined peaks. At this level, peaks representing components naturally present in the water are seen between the aromatics. The signal-to-noise ratio is high, implying that the attenuation might have been decreased. In addition, the volume of water might be increased well above the 5 ml used here. This means that the method has potential for determination of concentrations even lower than 0.1 μg/l of the individual components.

The chromatographic retention time of benzene varies somewhat between the different experiments. A small variation is also seen for toluene, but from ethylbenzene and upwards the retention times are reproducible. The variation for the two most volatile components must be due to the lack of reproducibility of the temperature in the GC oven during the first part of the chromatography from −80°C. This does not, however, pose any problems in the identification of the various peaks, in that the pattern and relative sizes of the peaks are used as guidelines.

The methylnaphthalenes, with the 2-methyl derivative eluted first, are present in equal concentrations in the water. The water had been accommodated by gentle stirring below an oil layer without the formation of a dispersion. The flow-through in the 50-l accommodation tank was 40–60 ml water and 3–4 ml Statfjord crude oil per min[15].

The mean peak areas of the replicate determinations of the ten standards at three different concentrations are given in Table I. The precision in the case of benzene and alkylated benzenes at 1 and 10 μg/l is very acceptable, with a relative standard deviation of between 1 and 6%. Determination of concentrations lower than 1 μg/l obviously has lower precision, especially for the most volatile components.

TABLE I

PEAK AREAS FROM P&T DETERMINATION OF STANDARD SOLUTIONS OF 0.1, 1.0 AND 10.0 μg/l OF EACH OF TEN HYDROCARBONS IN SEA-WATER

The numbers are means and relative standard deviations of seven replicate determinations.

Compound	*0.1 μg/l*		*1.0 μg/l*		*10.0 μg/l*	
	Area	*% S.D.*	*Area*	*% S.D.*	*Area*	*% S.D.*
Benzene	144	45	1007	5	9057	3
Toluene	247	28	1066	3	10058	2
Ethylbenzene	124	8	998	1	9911	3
m- and *p*-xylene	264	13	1894	1	18698	3
o-Xylene	153	5	964	2	9279	4
n-Propylbenzene	81	5	[a]		8938	6
Naphthalene	43	12	518	11	5670	10
1-Methylnaphthalene	44	4	[a]		5751	11
1,4-Dimethylnaphthalene	36	23	371	25	5156	17

[a] Not present in the 1.0 μg/l solution.

The lowest concentration of benzene and the alkylated benzenes gives relatively larger peak areas than the two higher concentrations. This indicates contamination of the samples. However, the source of possible contaminations has not been found. Procedural blanks of the sea-water did not show any benzene or alkylated benzenes. Both the lower precision and the unexplained higher peak areas imply that determination of benzenes becomes increasingly ambiguous as the concentrations decrease below 1 μg/l.

Naphthalene and alkyl-substituted naphthalenes behave differently to benzene and alkylated benzenes in two ways: the precision of the determination and the peak areas are both lower. The flame ionization detector on the gas chromatograph is a gram-carbon detector, and theoretically the response of naphthalene should be 1.016 times that of benzene. It is apparent that the recovery of the naphthalenes by this method is much lower than that of the benzenes. The low recovery also gives a lower precision of the method. As mentioned above, no naphthalenes remain in the water sample after 500-ml purging, and no loss occurs in the desorption step. Apart from this, no further tests have been carried out to detect where the naphthalenes are lost. However, quantitation of the naphthalenes is still possible, although with lower precision than for the benzenes, by taking the actual response factors into consideration.

A chromatogram from the P&T determination of a 5-ml sample of water from the exposure tank of a system for biotesting the effects of "water-soluble" petroleum hydrocarbons on marine organisms[15] is shown in Fig. 2. The peaks corresponding to the 9 (10) standards were easily identified. The unidentified peaks between *o*-xylene and naphthalene very likely represent C_3- and C_4-alkylated benzenes, as is the case in a similar chromatogram of groundwater contaminated with gasoline presented by Pankow and Rosen[10].

The identified components were quantitated in six replicate samples of the biotest water, collected immediately after each other, by the use of response factors obtained from Table I. The results are shown in Table II. The precision of the analyses is good.

A sample of 3 l of the same water, collected immediately after the samples for the P&T analysis, was analysed by Westrheim and Palmork[16], employing liquid extraction with dichloromethane followed by GC. The results, given in Table II, correspond well with the results of the P&T determination. The somewhat lower values for the liquid extraction indicate that an evaporation might have taken place. However, no replicates were obtained here, so that the precision of that method is not known.

The present P&T method has been used successfully in the testing of a new biotest system for studying the long term effect of oil on fish eggs and larvae[17].

In conclusion, the attractive features of the the P&T method for determination of petroleum hydrocarbons in water are as follows: the method allows determination of benzenes and naphthalenes in concentrations down to below 0.1 μg/l. At concentrations of 1 μg/l and above, the precision is high, especially for benzenes. Small sample volumes are needed. This is important in cases where the availability of water is low, *i.e.*, in biotest systems supplying small volumes of water, or in cases when the samples have to be transported from the sampling site to the laboratory. The method is automatic in that the only manipulation of the samples during the determination is limited to attaching the sample tube to the sampler and pressing the start button.

TABLE II

CONCENTRATIONS, IN μg/l OR ppb, OF VARIOUS AROMATIC HYDROCARBONS IN SEAWATER IN THE EXPOSURE AQUARIUM OF A BIOTEST SYSTEM WITH STATFJORD CRUDE OIL AS THE SOURCE OF THE HYDROCARBONS

The numbers are means and standard deviations of six P&T determinations. The result of one determination based on liquid extraction[a] is also given.

Compound	*P&T*	*Liquid extraction*
Benzene	81.0 ± 4.9	78
Toluene	65.9 ± 1.3	56
Ethylbenzene	2.2 ± 0.1	[b]
m- and *p*-xylene	12.6 ± 0.3	16
o-Xylene	8.0 ± 0.2	
n-Propylbenzene	1.1 ± 0.1	
Naphthalene	1.4 ± 0.2	
Methylnaphthalenes	0.8 ± 0.2	

[a] See ref. 16.
[b] Less than 2 μg/l.

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AUTHENTICATION OF COCOA IN MAYA VESSELS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES

W. JEFFREY HURST*, ROBERT A. MARTIN, Jr. and STANLEY M. TARKA, Jr.
Hershey Foods Technical Center, P.O. Box 805, Hershey, PA 17033-0805 (U.S.A.)
and
GRANT D. HALL
Department of Anthropology, Harvard University, Cambridge, MA 02138 (U.S.A.)
(Received January 16th, 1989)

SUMMARY

Samples of a dry residue collected from the interiors of ceramic vessels at the Maya site of Rio Azul in northeastern Guatemala were analyzed by a variety of high-performance liquid chromatographic techniques. Archeologists at the site had strong indications that the vessels contained cocoa. Since the literature indicated that cocoa would tend to be the only Mesoamerican commodity that would contain both theobromine and caffeine, initial studies concentrated on the determination of these compounds. Reversed-phase chromatography coupled with photodiode array and mass spectrometry detection confirmed the existence of these cocoa alkoloids in several of the vessels. Amino acid and fatty acid analysis were also conducted on the residues.

INTRODUCTION

Literature indicates that cocoa cultivation had been well established by the Aztecs in Mexico and the Maya in Central America for hundreds of years when Columbus first saw the commodity in 1502 during his fourth voyage. During that voyage, he saw a cargo of cocoa beans in a ship that was sighted off the coast of the Gulf of Honduras. In 1519, Cortes and the conquistadores were introduced to cocoa when the palaces of Montezuma were captured and large amounts of cocoa beans were found[1–3]. Cocoa was introduced to the European continent shortly after this time where its consumption grew steadily.

In 1984 at the site of Rio Azul in Guatemala, location shown in Fig. 1, excavations uncovered a burial site that was later designated as Tomb 19. The tomb contained the remains of a middle-aged man and many other artifacts including fourteen pottery vessels. One of the vessels was highly decorated with a unique lid, as can be seen in Fig. 2. Further information indicated that it contained some type of liquid substance. Hieroglyphics from the vessel can be seen in Fig. 3 and were deciphered to indicate ka-ka-w(a) or cocoa[4,5]. It is not the purpose of this paper to cover the topic of cocoa

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Fig. 1. Map indicating location of Rio Azul.

history or the status of the Rio Azul expedition since both areas have been very well covered in other publications[6–8].

A series of samples from several of the vessels were provided by archeologists at the site and analyzed by a variety of techniques. After initial analytical studies further investigations centered on the vessel seen in Fig. 2, which was given the designation as

Fig. 2. Photographs of vessels that contained samples for analysis.

Vessel 15. Archeological evidence indicated that the vessels in Tomb 19 should be assigned to the period 460–480 AD during the Early Classic Period of Mayan culture[5]. The studies that were conducted centered on the determination of theobromine and caffeine since those compounds tended to be unique to cocoa. Additional data were also obtained in the areas of fatty acid analysis, general lipid analysis and amino acid analysis.

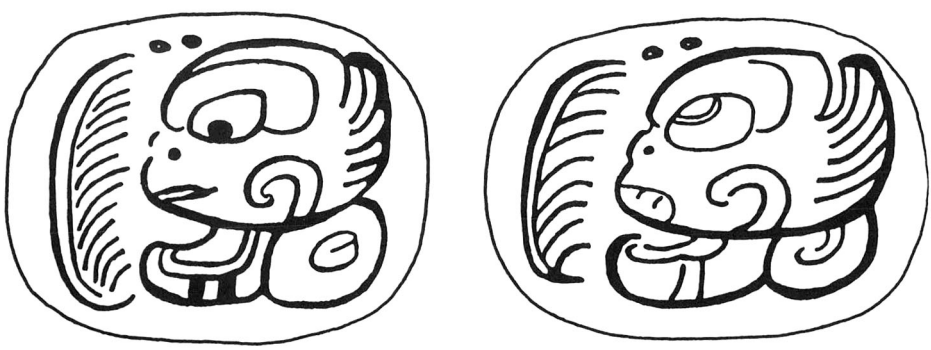

Fig. 3. Hieroglyphic from vessel.

EXPERIMENTAL

Instrumentation

The high-performance liquid chromatography (HPLC) apparatus was assembled from commercially available components. The pumps utilized were a Model 510 solvent delivery system (Waters) and a Model 6A liquid chromatograph (Shimadzu). A number of detectors were used in various stages of the study. Initial investigations were conducted with a Model 990 photodiode array (PDA) detector (Waters) for the determination of theobromine and caffeine while confirmatory data were obtained using a Model 201 liquid chromatography–mass spectrometry (LC–MS) apparatus (Vestec). Fatty acid analysis used a Model SPD-2A UV detector at 254 nm (Shimadzu) while amino acid analysis used a Model 440 UV detector at 254 nm (Waters). Data acquisition was accomplished by the use of an NEC APC III computer and associated hardware for the PDA detector and the IBM-based Teknivent Vector One mass spectrometry workstation. Differential scanning calorimetry (DSC) was accomplished through the use of a Model 910 DSC apparatus coupled to a Model 1090 thermal analyzer (DuPont Instruments).

Due to the diversity of assays accomplished a number of columns and mobile phases were used. The particulars are outlined in Tables I and II.

Standards

Standard compounds were obtained from a variety of sources. The theobromine and caffeine were repurified by sublimation. These were made up to approximate concentrations of 10 μg/ml in water and stored refrigerated at −4°C.

TABLE I
HPLC COLUMNS UTILIZED

Column type	*Size*	*Manufacturer*	*Assay*
5 μm Spherisorb ODS	30 cm × 3.9 mm	HPLC Technol.	Theobromine and caffeine
Resolve C_{18}	10 cm × 8 mm	Waters	Theobromine and caffeine
5 μm Spherisorb ODS	30 cm × 3.9 mm	HPLC Technol.	Fatty acid
Pico-Tag	15 cm × 3.9 mm	Waters	Amino acid

TABLE II
MOBILE PHASES USED

Composition (v/v)	*Assay*	*Detector*	*Reference*
Water–methanol–acetic acid (74:25:1)	Theobromine–caffeine	PDA	12
0.1 *M* amonium acetate–methanol	Theobromine–caffeine	MS	–
Gradient from 80:20 acetonitrile–water to 100 acetonitrile	Fatty acid	UV	8
Gradient from Pico-Tag buffers A to B	Amino acid	UV	11

The initial standard was used for the entire course of the study. Fatty acid standards were made up to varying concentrations in chloroform and stored at −20°C.

Other reagents

Fatty acid analysis was accomplished using the panacyl bromide derivative procedure that was reported earlier[9].

Amino acid analyses were accomplished using the Pico-Tag method[12].

Sample preparation

Samples were provided by the University of Texas at San Antonio and used as received. The samples in question not only contained some of the vessel contents but also seemed to contain some of the interior of the vessel. No attempt was made to differentiate the various components due to the small sample size available. In Table III the sample preparation techniques used for each assay are outlined. After sample preparation, all samples were analyzed using the technique of interest.

RESULTS AND DISCUSSION

Initial studies with UV detection indicated peaks in the chromatogram of the sample extract at the same point as theobromine and caffeine. Since the occurrence of peaks at the same retention times as pure compounds is inconclusive, further studies were conducted more fully utilizing the capabilities of the PDA detector. The PDA detector will provide a three-dimensional plot of time *versus* wavelength *versus* absorbance for each injection. It additionally will allow one to obtain a UV spectrum at any portion of a peak of interest whether it be leading edge, apex or trailing edge. Fig. 4 and 5 provide a chromatogram of standard and sample extract indicating the peaks of interest at 254 and 280 nm. The PDA detector additionally allowed the determination of derivative spectra of theobromine and caffeine. First and second derivative spectra were obtained for the peaks of interest and found to agree.

A series of LC–MS studies was conducted to provide further confirmation of theobromine and caffeine. Fig. 6 illustrates the LC–MS data on the series of standard compounds. The mobile phase and column used are given in Tables I and II. The mobile phase flow-rate was 1.3 ml/min. The MS interface operating temperatures were as follows: Vaporizer, 155°C; block heater, 220°C; tip heater, 233°C; lens heater, 85°C.

TABLE III
SAMPLE PREPARATION TECHNIQUES USED

Sample preparation	*Assay*	*Reference*
Dissolve in water and filter	Theobromine and caffeine	12
Extract with chloroform, make potassium salt and derivative	Fatty acid	10
Extract with 0.1*M* HCl and make derivative	Amino acid	11

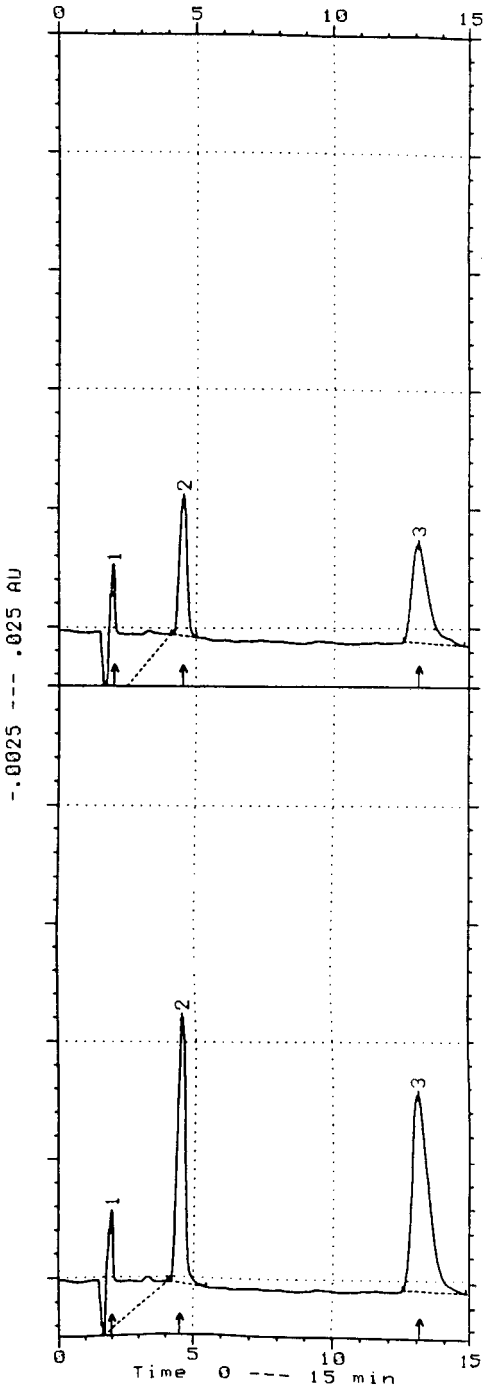

Fig. 4. Chromatograms of standard compounds. Top: 244 nm; bottom: 280 nm.

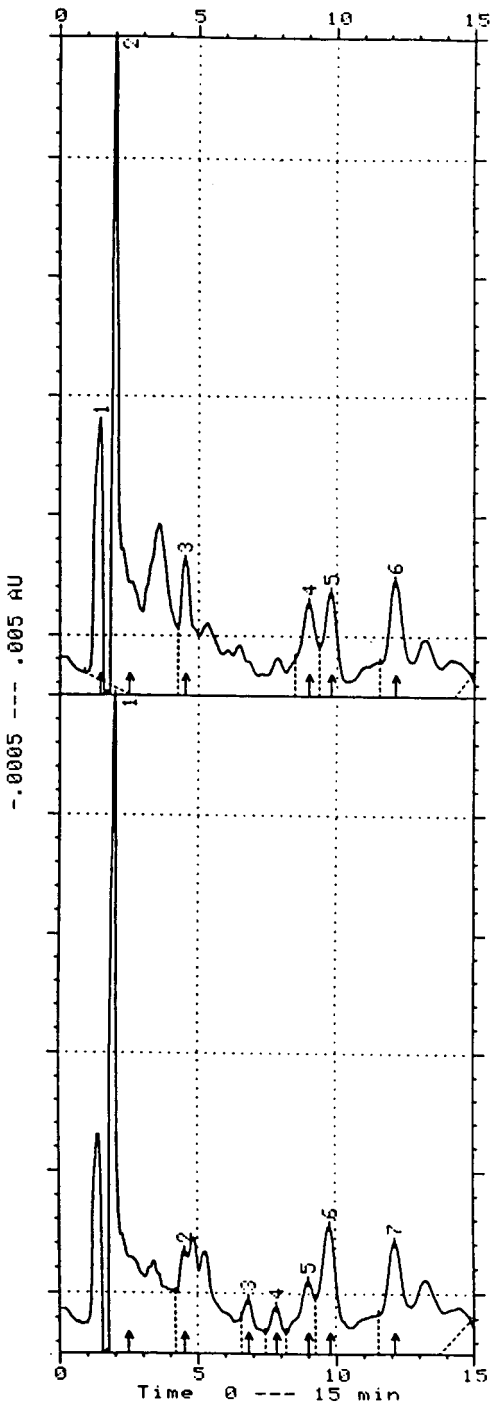

Fig. 5. Chromatograms of extract from Vessel 15. Top: 254 nm; bottom: 280 nm.

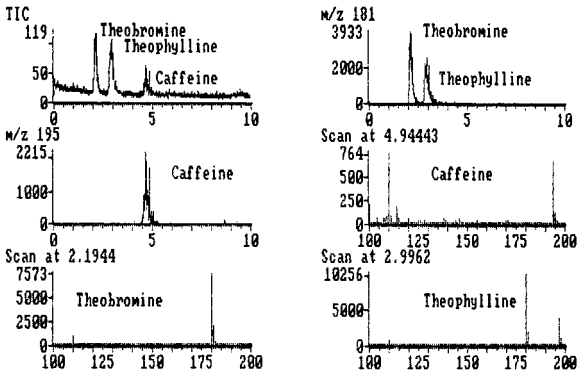

Fig. 6. LC–MS data on standard compounds.

The peaks of interest were collected and injected into the LC–MS and the extract was also injected in the "column-out" mode of operation. The total ion current (TIC) and a sample spectrum are shown in Fig. 7. Since LC–MS in the thermospray mode can be classified as a "soft-ionization" technique[13] the major ion seen is the MH + for the compound of interest. These ions are *m/z* 181 for theobromine and *m/z* 195 for caffeine. A related compound, theophylline, also exhibits an MH^+ ion at *m/z* 181 but does not have the same retention time as theobromine. This data provides further evidence to the existence of these compounds in this sample.

Other chemical assays were accomplished to provide further data on the contents of Vessel 15. The DSC results indicated that the vessel contained no detectable amount of lipid material. This data was further reinforced by the negative results from the fatty acid analysis. The lack of lipid is not disconcerting since even under ideal conditions one sees lipid degradation due to oxidation or other mechanisms.

An amino acid profile was also accomplished with the standards chromatogram shown in Fig. 8 and the sample chromatogram shown in Fig. 9. The results provide no information as to the existence of cocoa in the vessel but are intriguing since they do indicate the existence of some amino acids in this sample irrespective of the source.

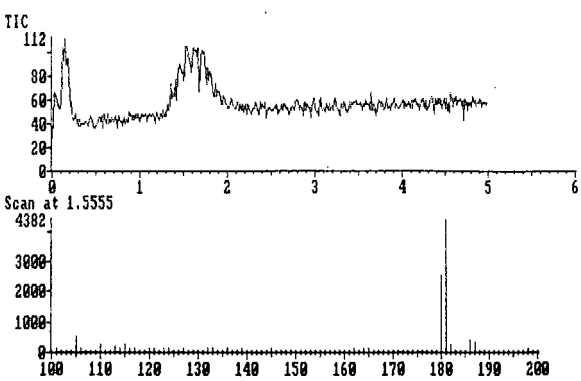

Fig. 7. Positive ion spectrum of theobromine peak from residue of Rio Azul cocoa vessel.

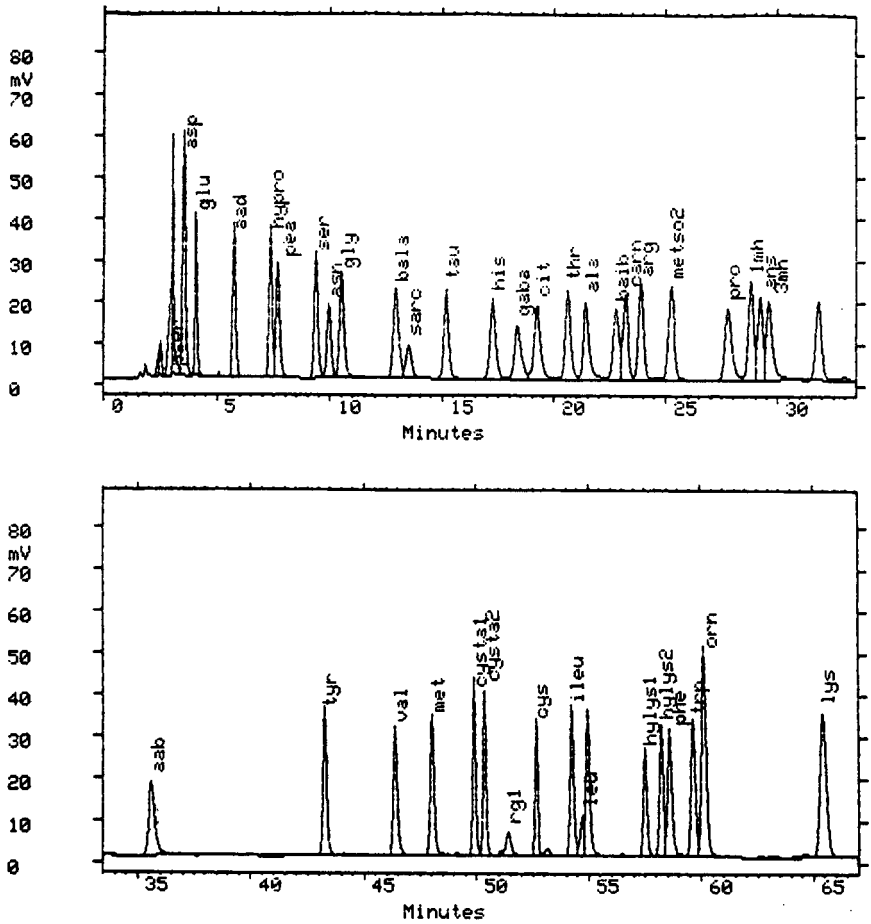

Fig. 8. Standard Pico-Tag amino acid profile.

It also is noteworthy since it provides information on the use of this assay on an archeological sample.

CONCLUSIONS

These results provide valuable information to epigraphists on the contents of this vessel and show that it did contain cocoa in some form. For reasons of length all aspects of each assay were not outlined in detail. This study serves to illustrate the results that can be obtained from a multidisciplinary team in solving this type of problem.

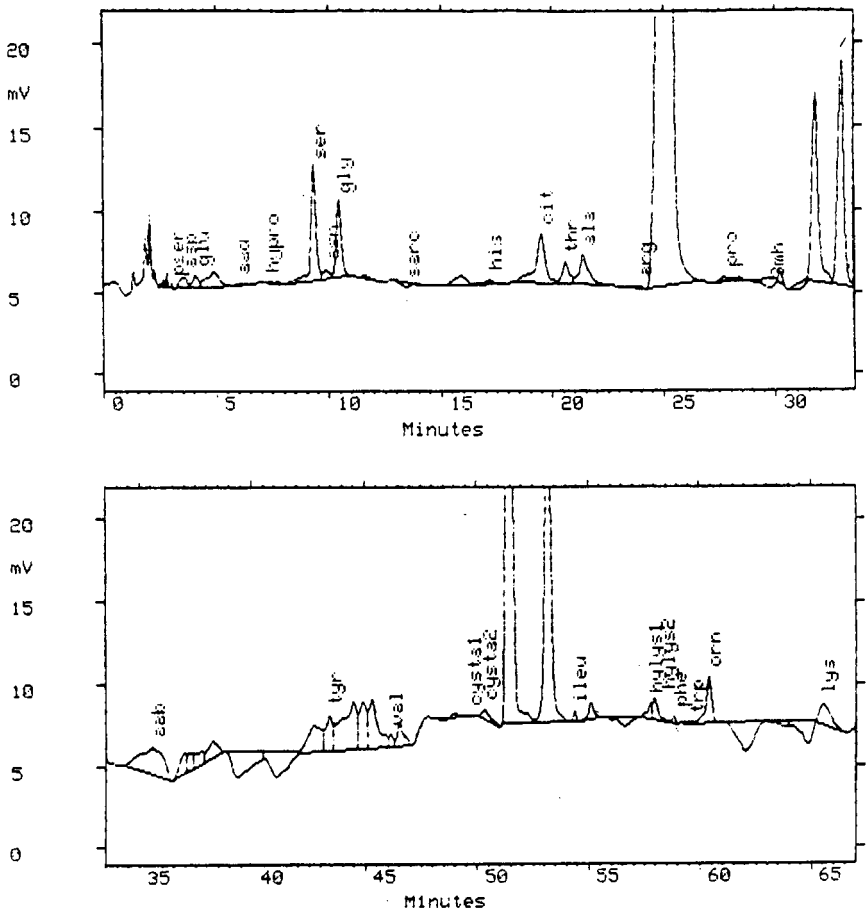

Fig. 9. Pico-tag amino acid profile of vessel extract.

ACKNOWLEDGEMENTS

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ANION–CATION SEPARATIONS ON A MIXED BED ALUMINA–SILICA COLUMN

DAVID M. BROWN and DONALD J. PIETRZYK*
Department of Chemistry, University of Iowa, Iowa City, IA 52242 (U.S.A.)
(First received August 30th, 1988; revised manuscript received December 16th, 1988)

SUMMARY

Mixed bed ion-exchange (MBIE) columns containing alumina and silica were evaluated for the simultaneous separation of anion and cation analytes. At the mobile phase pH used alumina provides anion exchange sites while silica provides cation exchange sites. Since alumina and silica exhibit weak acid and base properties, their anion and/or cation exchange properties are pH dependent. Ion exchange capacities, rates of exchange and analyte ion exchange selectivities are also pH dependent. The major mobile phase parameters affecting analyte anion and cation resolution and elution order are pH and type and concentration of counter anion and counter cation, respectively. The weight ratio of the two exchangers and/or the exchange capacities of the two in the column can also be used to alter resolution and elution order. Several examples of the simultaneous separation of inorganic mono- and divalent anions and cations using a single sample injection, a single column and a single detector (conductivity) illustrate the parameters and scope of the alumina–silica MBIE column.

INTRODUCTION

A mixed bed column of ion exchangers is the incorporation of both cation and anion exchanger particles interspersed throughout the same column. Historically, these kinds of columns have been used as an alternative to a two stage or multistage approach where anion and cation columns are connected in series for the removal of electrolytes from water in water treatment processes.

Applications of mixed beds of anion and cation exchangers (hereafter referred to as MBIE columns) to analytical separations has been limited. MBIE columns were used to separate alkaline earths, transition metals and rare earth[1–5] from mobile phases containing ligands that converted metal ions into anionic complexes. Subsequent work suggested that the separations were often not improved over the sole use of either an anion or cation exchanger[6]. MBIE silica based columns[7,8] containing phenyl modified silica were used for the separation of acidic and basic proteins while polymer based MBIE columns were used for the simultaneous separation of inorganic anions and cations[9]. The latter study also demonstrated that the column and mobile phase parameters affecting retention, selectivity and resolution are typical of conventional anion and cation exchange.

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Other chromatographic strategies other than MBIE columns have been used for the simultaneous separation of anions and cations. Dual anion and cation columns with a single conductivity detection with synchronal sample injection[10] and anion and cation columns connected in series with indirect photometric detection[11,12] have been used. In another approach a single sample, anion and cation column in series, and two detectors were used[13–15]. It is also possible to use a ligand in the mobile phase which converts metal ions into anionic complexes. These are separated on an anion exchanger along with other anions present in the sample[16,17]. Column switching techniques have also been described[18,19].

The ion exchange properties of inorganic oxides have been recognized for a long time[20]. Recent studies with modern, spherical alumina[21,22] and silica[23,24] microparticles have shown that these oxides can function as efficient stationary phase ion exchangers for liquid chromatographic anion and cation exchange separations, respectively. Exchange rates, exchange capacities and ion selectivities for these microparticles[22,24] were found to be vastly improved over the characteristics reported for the classical, irregular shaped alumina and silica macroparticles[20]. Unlike polymeric derived strong acid or base ion exchangers, oxides are generally weak acid or base ion exchangers and pH plays a major role in determining the oxide's exchange capacity, ion selectivity and its ability to act as an anion or cation exchanger since many oxides exhibit amphoteric behavior.

This report focuses on using a MBIE column composed of alumina and silica microparticles. Because of the mobile phase pH, alumina provides anion exchange sites while silica provides cation exchange sites. Thus, it is possible to efficiently separate anions and cations simultaneously by using one alumina–silica MBIE column with a single sample injection, one eluent and one detector while maintaining the unique analyte ion selectivities provided by alumina and silica.

EXPERIMENTAL

Reagents

Inorganic salts used as electrolytes and buffers in the mobile phase and as analytes were analytical reagent grade when possible. LC water was obtained by passing distilled water through a Sybron/Barnstead water purification unit. Bulk form, spherical alumina (5 and 10 μm, Spherisorb A5Y and A10Y from Phase Separations) and silica (6 μm, Zorbax silica from DuPont) were used in stainless-steel 150 mm $\times$ 4.1 mm I.D. columns.

Instrumentation

A Varian M2010 pump, Waters U6K injector and Waters M430 conductivity detector were used. Column temperature was maintained at 35°C by circulated water from a constant temperature bath. Peak area was determined with a Spectra Physics M4100 Integrator.

Procedures

Weighed quantities of alumina and silica were added to 20 ml of a solution that contained 100 g sodium chloride per liter of LC water. The slurry mixture was carefully stirred in a column packing reservoir for 10 min. Columns were packed upward using

a procedure outlined elsewhere[22]. To ensure hydration of the alumina and silica, newly packed columns were conditioned overnight with an aqueous 2.5 m*M* lithium acetate mobile phase. In general, columns were conditioned for several hours at 1 ml/min when switching from one mobile phase to another. Typical column efficiencies were 8000 plates/meter for Na^+ and 25 000 plates/meter for Cl^- using an aqueous pH 5.03, 2.50 m*M* lithium acetate mobile phase. All newly prepared columns and periodic testing of columns used during the studies were compared to these efficiency standards. Inlet column pressure at 1 ml/min was typically 500 to 900 p.s.i. Analyte solutions were prepared by dissolving weighed amounts of salt in water at about 1 mg/ml in glass vials. Sample aliquots delivered by 10-μl syringe were 1–10 μl. Aqueous mobile phases were prepared by titrating standard acetic acid solution with a standard lithium hydroxide solution to the desired pH followed by dilution to volume.

RESULTS AND DISCUSSION

As mobile phase pH is changed from acidic to basic the surface charge on silica and alumina changes from positive to negative, respectively. This transition point or isoelectric point pH is dependent on the chemical environment and the conditions used for the oxide preparation[20–26]. Depending on the buffer components the isoelectric point pH for silica is about 2 while for alumina the value varies between 3.5 to 9.2. Silica does not exhibit a useful amphoteric behavior because of its reactivity in acidic solutions. Thus, in column experiments only its cation exchange behavior above approximately pH 2 is useful. Alumina in contrast is capable of exhibiting both anion (low pH) and cation (high pH) exchange because of its chemical stability in both environments. Since the two oxides exhibit weak acid and base characteristics, the available and useful exchange capacities of the two oxides are also pH dependent. Under favorable pH conditions and in the presence of suitable buffer components cation exchange capacity on silica and anion exchange capacity on alumina can approach about 3.5 and 4 mequiv./g, respectively[25].

When the two oxides are intimately mixed in an aqueous solution and the pH is adjusted between 2 and 8, silica is a cation exchanger and alumina is an anion exchanger. As the pH is increased cation exchange capacity for silica increases and anion exchange capacity for alumina decreases while at the lower pH the reverse occurs. Adjustment of pH within this approximate pH range alters the two exchange capacities and as either of the two extremes are approached a sufficient number of exchange sites for the lesser one is still present for modern ion-exchange chromatography. Since the isoelectric point pH is also dependent on the buffer components[20–26], exchange capacity at a given pH is also dependnt on the type of buffer components and their concentration.

A second important ion exchange characteristic of oxides is that the exchange selectivities often differ not only from oxide to oxide but also when compared to polymeric based strong acid and base ion exchangers. For example, alkali metal and alkaline earth retention order on silica at cation exchange conditions is identical to that on a sulfonated styrene based cation exchanger. In contrast anion retention order on alumina under anion exchange conditions is significantly different than with quaternary ammonium styrene based anion exchangers. On alumina anion elution order is $F^- > SO_4^{2-} > Cl^- > Br^- > I^- > ClO_4^-$ while for the styrene based anion exchanger it is SO_4^{2-}, $ClO_4^- > I^- > Br^- > Cl^- > F^-$ (ref. 22).

Mixed bed column performance

High pressure packing of a well stirred slurry mixture of the two oxides provided a uniformly interspersed packed column. Reproducible chromatographic data were obtained from a series of identically packed columns and visual examination of the packing when it was purposely extruded from a packed column supported this conclusion. Initial column experiments using several different salts as sources of cation and anion analytes indicated a favorable column performance and that anions and cations could be efficiently separated and detected by conductivity.

A 2.5 m*M* acetic acid, 1.85 m*M* lithium hydroxide, pH 5.03 aqueous mobile phase, a 150 mm × 4.1 mm column packed with alumina and silica at a 1:1 ratio and KI as an analyte were used to evaluate column performance. When the flow-rate was increased by 0.5-ml/min increments up to 2.0 ml/min, capacity factor, k', values for K^+ retention decreased from 3.02 (0.5 ml/min) to 2.53 (2.0 ml/min) while for I^-, k' values were constant at 7.25. Plates/m for K^+ changed from 2800 to 2760 while for I^- the change was from 24300 to 18700 plates/m. Change in peak symmetry was insignificant. At 1.0 ml/min column inlet pressure was about 600 p.s.i. Using a constant injected sample volume of 1 μl the k' value for K^+ and I^- remained constant as the amount of KI injected increased from 0.1 to 8 μg. Similarly, when using a sample that was 0.6 μg of KI/μl, no change in k' was observed as the sample volume was increased from 2 to 30 μl. Thus, neither a mass or volume overload was indicated over the range of conditions studied suggesting that linear anion and cation ion exchange isotherms are present. Since anion and cation exchange capacity for alumina and silica, respectively, are pH dependent the mass and volume overload condition will also depend on the pH. At pH 5.03, which was used in most of these experiments, anion and cation exchange capacities for alumina and silica are about 0.5 and 0.1 mequiv./g, respectively; no attempt was made to accurately determine the exchange capacities at the experimental conditions used. When the ratio of alumina to silica was changed retention times of analyte anions and cations changed accordingly. If alumina was in excess anion retention increased and cation retention decreased while if silica was in excess the reverse effect was observed. In experiments reported here 1:1 alumina:silica was used.

Conductivity detection was used in all experiments reported here. Thus, selection of mobile phase counter cations and counter anions affects not only the ion exchange equilibria as a result of ion exchange selectivity, but also detection because of differences in equivalent ionic conductances between counter ions and analyte ions. Lithium acetate provides suitable counter anion and cation ion exchange selectivities and equivalent conductances and was used as the mobile phase electrolyte in most of the experiments described here.

Peak identification was achieved by using combinations of different salts as analytes. For example, alkali metal and halide peaks were identified by recording and comparing the chromatograms obtained for each alkali metal halide salt as an analyte. When combined with chromatograms obtained with other salts as analytes peak assignment is further supported.

pH

Mobile phase pH determines the ion exchange capacity of alumina and silica. As pH increases, cation exchange capacity of silica increases while the anion exchange

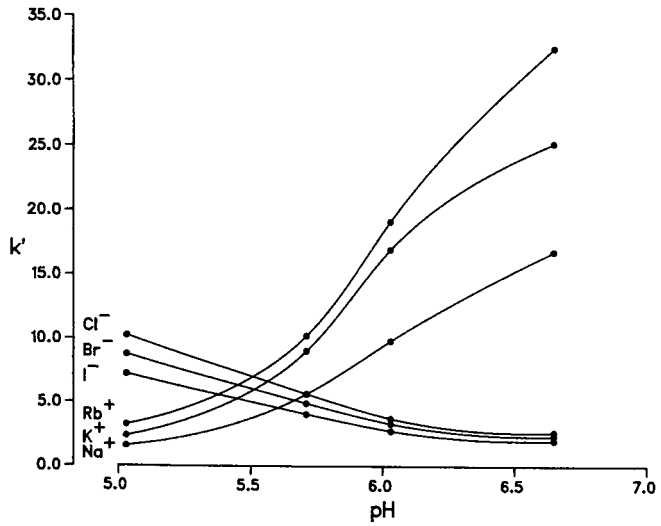

Fig. 1. Effect of mobile phase pH on analyte ion retention. The aqueous mobile phase is 2.50 m*M* acetic acid solution containing 1.85, 2.31, 2.39 or 4.90 m*M* LiOH at 1.0 ml/min and 35.0°C.

capacity of alumina decreases. The reverse occurs as pH is lowered. Since analyte ion retention time is influenced by exchange capacity, it must therefore also be influenced by pH. A high mobile phase pH should cause analyte cation retention to increase and analyte anion retention to decrease. The reverse should occur as pH is lowered. Fig. 1 illustrates these trends, where retention expressed as k' is plotted as a function of mobile phase pH using alkali metal halide salts as the analytes. The effect of pH on retention is significant and over a 2 pH unit change from pH 5 to 7 the alkali metal cation and halide anion retention is reversed. As shown in Fig. 2 as little as a 1.5 pH shift in the mobile phase is sufficient enough to reverse the elution order of cations and

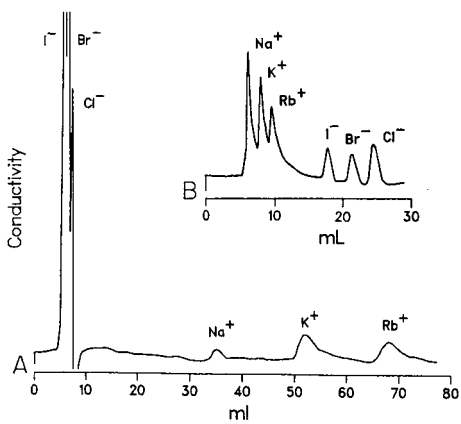

Fig. 2. Simultaneous separation of monovalent cations and anions on a 1:1 alumina:silica column as a function of pH. The aqueous mobile phases are (A) 2.50 m*M* acetic acid, 4.90 m*M* LiOH at pH 6.65 and (B) 2.50 m*M* acetic acid, 1.90 m*M* LiOH, pH 5.03 at 1.0 ml/min and 35.0°C.

anions. Figs. 1 and 2 also demonstrate that the anion elution order for the alumina–silica MBIE column is characteristic of alumina functioning as an anion exchanger[22] and the cation order is characteristic of silica functioning as a cation exchanger[24].

Mobile phase ionic strength

Increasing the mobile phase counter cation and anion concentration will decrease analyte cation and anion retention, respectively, which is consistent with an ion exchange process. This effect is illustrated in Fig. 3 where analyte anion and cation retention is plotted as a function of mobile phase ionic strength using lithium acetate as the ionic strength salt. If a counter cation of higher ion exchange selectivity is used analyte cation retention is lowered. Similarly using a counter anion of higher ion exchange selectivity lowers analyte anion retention. When lithium acetate was used (see Fig. 3) the effect of increasing acetate concentration on decreasing analyte anion retention is larger than the effect of increasing Li^+ concentration on decreasing analyte cation retention. Several reasons contribute to this observation. (1) Acetáte exchange selectivity towards anions is more favorable than Li^+ selectivity toward cations. (2) Increasing acetate concentration reduces the isoelectric point pH which lowers the anion exchange capacity of alumina[21,22,23] and thus analyte anion retention is reduced.

Fig. 4 illustrates how an increase in mobile phase counter ion concentraion can alter elution order. Two major effects can be realized. (1) Increasing electrolyte concentration automatically decreases both analyte anion and cation retention since both counter cation and anion concentration must increase. (2) Because ion exchange selectivities are unique it is possible, for example, to select a mobile phase salt that provides a counter cation that causes retention of analyte cations to be reduced to

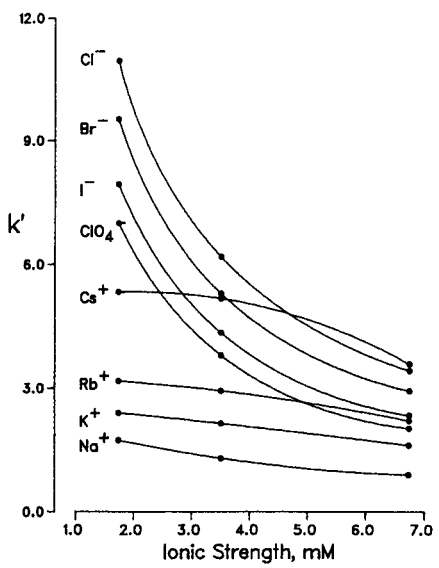

Fig. 3. Effect of mobile phase ionic strength on analyte ion retention. The aqueous mobile phases are lithium acetate pH 5.0, at 1.0 ml/min and 35.0°C.

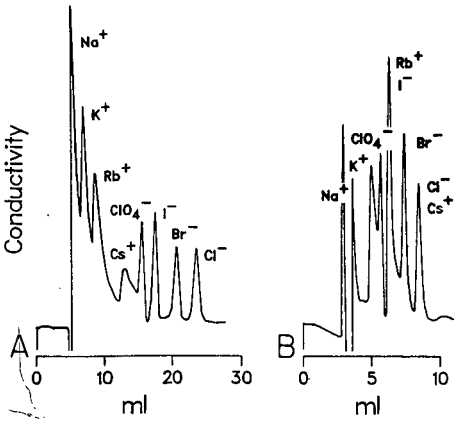

Fig. 4. Effect of mobile phase ionic strength on the separation of monovalent cations and anions on a 1:1 alumina:silica column. The aqueous mobile phases are (A) 2.50 m*M* acetic acid, 1.85 m*M* LiOH, pH 5.01 and (B) 10.0 m*M* acetic acid, 7.00 m*M* LiOH, pH 5.00 at 1.0 ml/min and 35.0°C.

a greater extent than the affect of the counter anion on analyte anion retention. Alternatively, a salt can be chosen, as in Fig. 4, that provides a counter anion that has a more significant affect on anion retention than the counter cations effect on cation retention.

Calibration graph

Calibration graph reproducibility for the simultaneous separation and determination of anions and cations on the alumina–silica MBIE column was evaluated using NaCl and $CaCl_2$ as analytes. A Micrometrics M725 autoinjector (sample injection 20 μl) was used in these experiments. For NaCl the aqueous mobile phase was 2.50 m*M* acetic acid, 1.85 m*M* LiOH, pH 5.03, while for $CaCl_2$ it was 10.0 m*M* acetic acid, 7.13 m*M* KOH, pH 5.02. The column was 150 mm × 4.1 mm, alumina–silica. A stronger eluent (increased counter ion concentration and K^+ which has a higher cation exchange selectivity compared to Li^+) was used for the $CaCl_2$ analyte because of the higher retention of Ca^{2+}.

For NaCl the calibration graph was linear from 0.50 to 254 μg NaCl per 20-μl injection; no attempt was made to determine the upper limit of linearity. For Na^+ the calibration graph corresponded to: peak area (10^7) = 3.679 (ng Na^+) (10^4) + 0.02254 with a correlation coefficient of 0.9979, while for Cl^- it was peak area (10^7) = 0.9844 (ng Cl^-) (10^4) − 0.006334 with a correlation coefficient of 0.9999. The detection limits at the mobile phase–detector conditions used were 0.5 and 0.03 μg per 20 μl injection for Na^+ and Cl^-, respectively. For $CaCl_2$, which was studied over the range of 0.45 to 90.7 μg of $CaCl_2$ per 20 μl injection, linear calibration graphs for Ca^{2+} and Cl^- were: peak area (10^6) = 0.7790 (ng Ca^{2+}) (10^3) + 0.2459 with a correlation coefficient of 0.9994 and peak area (10^6) = 0.5323 (ng Cl^-) (10^4) − 0.005676 with a correlation coefficient of 0.9998, respectively. Detection limits for the experimental conditions used were 0.100 μg Ca^{2+} and 0.071 μg Cl^- per 20-μl injection, respectively. For both NaCl and $CaCl_2$ as analytes retention times for Na^+ and Cl^- and Ca^{2+} and Cl^- were constant over the range of NaCl and $CaCl_2$ injected.

Separations

The alumina–silica MBIE column performance is favorable, reliable and the column is applicable to the separation of a variety of mono- and divalent anion and cation analyte ions. Under favorable conditions peak shapes are well defined and column efficiencies, which are typical of polymeric exchangers particularly anion exchangers, approach 8000 and 25 000 plates per meter for cations and anions, respectively. Manipulation of the alumina to silica ratio, pH, eluent counter ions and their concentration are the major factors used to improve resolution and/or to shift retention order of anion analytes relative to cation analytes and *vice versa*. Analyte retention times were reproducible providing the mobile phase pH was maintained within the pH stability range of alumina and silica. Certain eluent counter ions should be avoided when using alumina[22] and silica[24] because they can cause column plugging due to particle breakdown and/or change the exchange sites due to chemical reaction. This is particularly true of alumina where F^- and multivalent anions cause a permanent change in alumina's performance as an anion exchanger. The effect of F^- is rapid with a permanent loss in anion exchange capacity while, in general, multivalent counter anion effects are more gradual and often only reduce exchange capacity. Counter ions that exhibit ligand characteristics toward Al and/or Si should also be avoided.

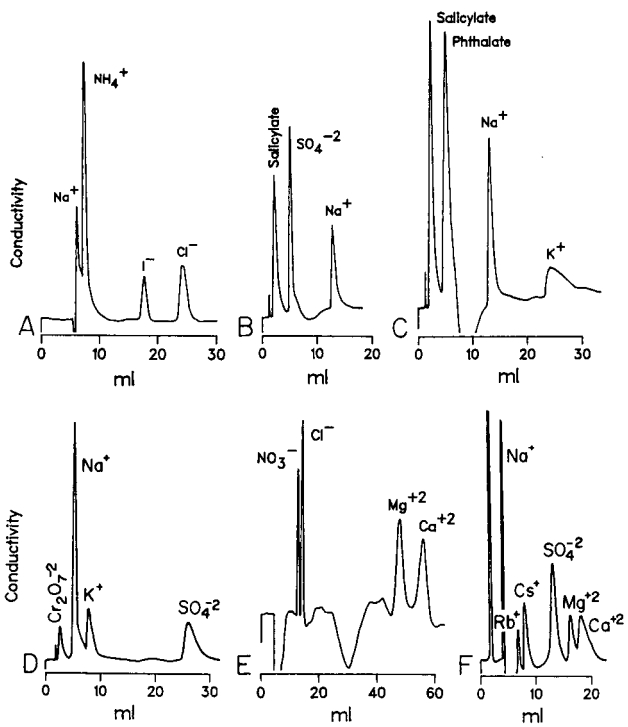

Fig. 5. Simultaneous separation of cations and anions on a 1:1 alumina:silica column. The aqueous mobile phases are (A) 2.50 m*M* acetic acid, 1.85 m*M* LiOH, pH 5.03, (B) and (C) 2.00 m*M* H_3PO_4, 2.00 m*M* LiOH, pH 4.75, (D) 2.00 m*M* H_3PO_4, 1.90 m*M* LiOH, pH 3.97, (E) 5.00 m*M* actic acid,3.60 m*M* KOH, pH 5.03, (F) 4.00 m*M* H_3PO_4, 3.90 m*M* KOH, 2.00 m*M* KCl, pH 3.93 at 1.00 ml/min and 35.0°C.

Several examples of mono- and divalent anion and cation separations are illustrated in Fig. 5. In all cases a 150 mm × 4.1 mm, 1:1 alumina–silica MBIE column and conductivity detection was used. In general, salts used as analytes were first injected individually at about 1 μg or less in 1 to 10 μl volumes to establish cation–anion retention. Figs. 2, 4 and 5A demonstrate that monovalent anions and cations are easily separated using an aqueous lithium acetate mobile phase at about pH 5. If the mobile phase pH is increased anion retention decreases and cation retention increases; a pH decrease reverses the effect. These trends are due to the effect of pH on exchange capacity and the isoelectric point pH. Mobile phase pH will also affect analyte retention through its influence on analyte ionization. Thus, in Fig. 5A at pH 5.0 ammonia is retained and separated from Na^+ as the cation. In Fig. 5A the injected sample contained 1.0 μg of NaI and 0.74 μg of NH_4Cl.

Divalent analyte ions are more retained than monovalent analyte ions. Thus, to maintain reasonable elution times, eluent strength must be increased. Fig. 5B, C and D illustrate separations of monovalent cations and highly retained anions. Eluent strength was increased by using a lithium phosphate mobile phase. Since pH is 4.75 (Fig. 5B, C) and 3.97 (Fig. 5D) the eluent counter anion is primarily an equilibrium mixture of $H_2PO_4^-/HPO_4^{2-}$ and this provides a much stronger eluent counter anion mixture than obtained with an acetate mobile phase. The change in pH between Fig. 5B, C and D is also a factor. At the higher pH (Fig. 5B, C) cation exchange capacity of silica is enhanced and anion exchange capacity of alumina is reduced while the lower pH (Fig. 5D) causes the reverse effect. This accounts for the significant difference in retention times for Na^+, K^+ and SO_4^{2-} between Fig. 5B, C and D. Fig. 5B and C also show that organic analyte anions can be effectively separated in an alumina–silica MBIE column. Since these are derived from weak acids and their ionization is pH dependent, their elution time is also affected by pH.

The selection of multivalent anions as mobile phase counter ions must be done carefully. Because of strong interactions between alumina or silica and multivalent anions and the resulting change in the oxide surface[22,24–26] not all multivalent counter anions are useful as eluents. For example, when a 2.5 m*M* lithium acetate mobile phase is used Na^+ retention is significantly lower (see Fig. 2B) than when the lithium phosphate mobile phase (see Fig. 5B and C) is used even though Li^+ is the counter cation in the two mobile phases. A similar effect on anion elution was also observed. The slight difference in Li^+ concentration does not account for this observation. The increase in retention is apparently the result of an irreversible change in the alumina–silica surface due to a phosphate interaction. Even after reconditioning the column with excessive quantities of 0.10 *M* NaCl mobile phase the original Na^+ retention from a lithium acetate mobile phase could not be obtained. Other multivalent eluent counter anions or anions that complex with alumina and/or silica will also cause irreversible oxide surface change[22,24]. Thus, while a phosphate mobile phase can be used to effectively and reproducibly elute more highly retained anions it does so by permanently changing the column. The change, however, does not eliminate preparation of reproducible peak shapes and calibration graphs (see calibration graph section).

Fig. 5E illustrates the separation of divalent cations from monovalent anions. In this case a potassium acetate mobile phase was used. The counter cation K^+, which has a higher cation exchange selectivity relative to Li^+, was used in the mobile phase; its

concentration relative to Li^+ was also increased in order to increase eluent strength. Fig. 5F illustrates a separation where both a strong eluent cation (K^+) and anion (phosphate buffer) are used effectively to separate highly retained cations and anions. Counter ion concentration was also increased to improve eluent strength and pH was lowered to decrease cation exchange capacity of silica and thus to reduce analyte cation retention.

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CHROM. 21 193

CHARACTERIZATION OF OAKMOSS PRODUCTS USED IN PERFUMERY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. SCHULZ* and G. ALBROSCHEIT
DRAGOCO, Research Department, Analytical Laboratories, D-3450 Holzminden (F.R.G.)
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SUMMARY

An high-performance liquid chromatographic (HPLC) method was developed to identify and to quantify characteristic substances in commercially available oakmoss products. The procedure offers a rapid and reliable method for routine process and/or quality control. The identity of the registered peaks was confirmed using HPLC coupled on-line with ultraviolet–visible spectroscopy as well as by spectral analysis (^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry) of the isolated substances. Whereas a freshly prepared laboratory extract of *Evernia prunastri* contains mainly evernic acid, heating at 118°C results in decomposition products such as evernyl, orsellic acid, everninic acid and other phenol derivatives. The results indicate that the often used gas chromatographic method is not readily applicable to the study of lichen compounds, because these are not sufficiently volatile or too unstable at elevated temperatures.

INTRODUCTION

Oakmoss [*Evernia prunastri* (L.) *Ach.*] is a lichen belonging to the family *Usneaceae* which grows primarily on oak trees. It is collected particularly in Yugoslavia, France, Morocco and Algeria. Solvent extracts of oakmoss are extensively used in perfumery. Extracts and absolues are also prepared from the so-called "tree-moss" furnished by the two related lichens *Evernia furfuracea* and *Usnea barbata* which grow predominantly on conifer bark.

Whereas the volatile part of the moss products is well analyzed by gas chromatography–mass spectrometric (GC–MS) methods[1–4], little information exists concerning the amount of high boiling constituents which cannot be vaporized without decomposition.

While odour quality in most cases is placed above other considerations in the processing of natural raw materials for perfumes, there should be other "objective" criteria in order to guarantee the reproducibility of the industrial processing, the use of the right botanical species and the absence of solvents or other natural or synthetic perfume materials. Existing GC methods to characterize oakmoss products have limited applicability for quality control purposes. Therefore, a new high-performance

0021-9673/89/$03.50

liquid chromatography (HPLC) method is proposed for the rapid quantitative and non-destructive characterization of the most relevant oakmoss constituents.

EXPERIMENTAL

Materials

Usnic acid and atranorin were obtained from C. Roth (Karlsruhe, F.R.G.), evernic acid and orcinol from Sigma (Deisenhofen, F.R.G.) and evernyl from Roure Bertrand Dupont (France). Methyl and ethyl evernate were prepared by esterification of evernic acid. 3-Methoxy-5-hydroxy- and 3,5-dimethoxytoluene were synthesized by reaction of orcinol with dimethyl sulphate.

The identity of the synthesized reference substances was confirmed by the individual GC–MS as well as the ^{1}H and ^{13}C NMR data.

Acetonitrile and water used for the mobile phase were of HPLC grade (Merck, Darmstadt, F.R.G.).

Extracts from *Evernia prunastri* were obtained by refluxing the commercially available plant material (Dalmacijabilje, Yugoslavia) for 2 h in a soxhlet apparatus with methanol. After evaporation to dryness, the residue was reconstituted in a few millilitres of the solvent, filtered through a 0.8-μm Millipore filter and applied to HPLC without further treatment. HPLC determination of the industrial extraction batches could be performed directly after dilution and removal of insoluble matter by filtration.

Chromatographic conditions

The HPLC system consisted of an Hewlett-Packard HP 1090 liquid chromatograph with a DR 5 solvent delivery system, variable-volume auto-injector, auto-sampler, thermostatically controlled column compartment and an HP 1040 A diode-array detector which measures absorbance at all wavelengths in the range from 190 to 600 nm simultaneously. The mobile phase was phosphoric acid, adjusted to pH 2.8 with distilled water (A) and acetonitrile (B). The gradient of these two solutions was controlled by the following time programme: 0.0 min, 80.0% A; 0.1–5.0 min, 80.0–70.0% A (linear gradient); 5.1–8.0 min, 70.0% A; 8.1–16.0 min, 70.0–5.0% A (linear gradient). The mobile phase was sparged with helium prior and throughout the analysis to prevent bubble formation. The flow-rate was 0.5 ml/min. For analytical separations an Hypersil ODS 5-μm microbore column, 100 mm × 2.1 mm I.D., obtained from Hewlett-Packard, was used.

RESULTS AND DISCUSSION

A typical chromatogram obtained from commercially available extracts of oakmoss is illustrated in Fig. 1. Using a microbore reversed-phase column in combination with a binary gradient solvent system of dipotassium hydrogenphosphate–phosphoric acid solution and acetonitrile, a very good resolution of most relevant peaks can be achieved within 15 min.

Table I lists the retention times and absorption maxima of all compounds recognized in the lichen extracts. The identity of the registered peaks was confirmed by comparing both the observed retention times and the UV–VIS spectra of the individual peaks with the individual data of the standard compounds.

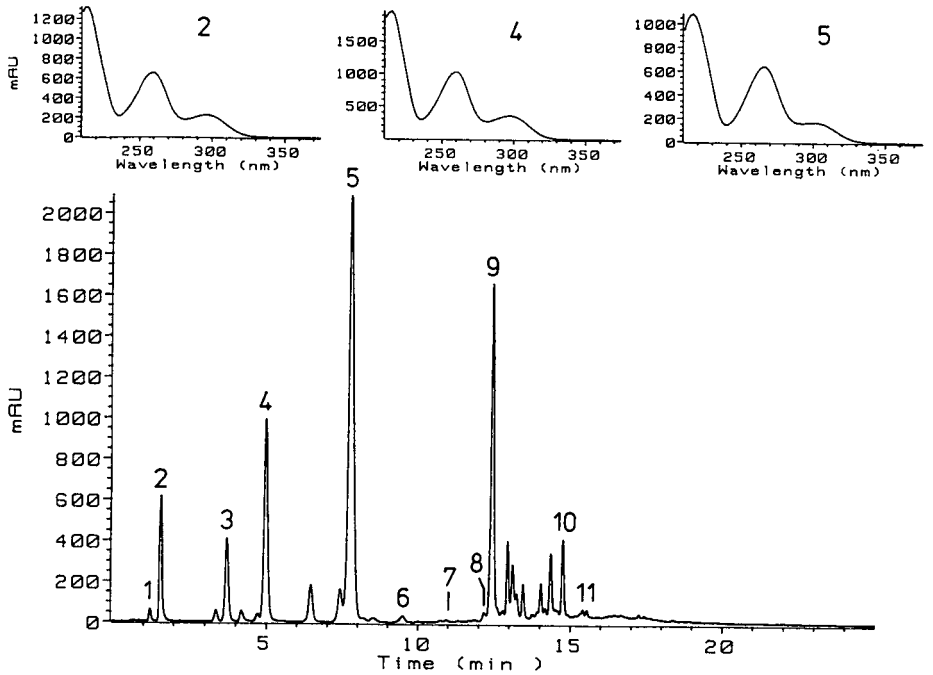

Fig. 1. Reversed-phase HPLC of a commercially available oakmoss extract, detected at λ = 260 nm. UV–VIS spectra presented for peak numbers 2, 4 and 5. Peak identification according to Table I.

TABLE I

ABSORPTION MAXIMA AND RETENTION TIMES OF THE COMPOUNDS DETECTED

Peak No.	*Compound*	*Absorption maxima (nm)*	*Retention time (min)*
1	Orcinol (3-Hydroxy-5-methylphenol)	272	1.1
2	Orsellic acid (2,4-Dihydroxy-6-methylbenzoic acid)	266, 300	1.6
3	Orcinol monomethyl ether (3-Methoxy-5-hydroxytoluene)	272	3.4
4	Everninic acid (2-Hydroxy-4-methoxy-6-methylbenzoic acid)	266, 300	5.0
5	Evernyl (Methyl 2,4-dihydroxy-3,6-dimethylbenzoate)	266, 300	7.7
6	3,5-Dimethoxytoluene	272	9.6
7	Methyl evernate	258, 300	11.0
8	Ethyl evernate	260, 298	12.1
9	Evernic acid	266, 302	12.5
10	Usnic acid	232, 280	14.3
11	Atranorin	248	15.5

In order to identify the compounds corresponding to peaks 2 and 4, it was necessary to isolate the two substances from the extract, because no standards existed. By freezing out the oakmoss extract from chloroform, a crude mixture of both substances was obtained. This fraction was thorougly washed with cold chloroform, dried and subsequently separated by preparative HPLC (C_{18} column, gradient elution with methanol–water). Based on spectral analysis (^1H and ^{13}C NMR, MS) the two pure substances obtained were identified as orsellic acid and everninic acid, respectively[5,6].

Evernyl, which is regarded as having the most important contribution to the typical odour of oakmoss extract, was found to be the main ingredient beside evernic acid in commercially available products (content almost >15%). Due to the similarities in chemical structure, the UV–VIS spectra of orsellic acid, everninic acid and evernyl (Fig. 1) are very similar. Ethyl and methyl evernate were detected only in very small amounts. According to earlier GC studies[4], the presence of orcinol, orcinol monoethyl ether and orcinol dimethyl ether was confirmed. Usnic acid, which to our knowledge has not been found in oakmoss extracts before, is eluted as a well resolved, narrow peak at a retention time of about 15 min. Although it was not possible to get

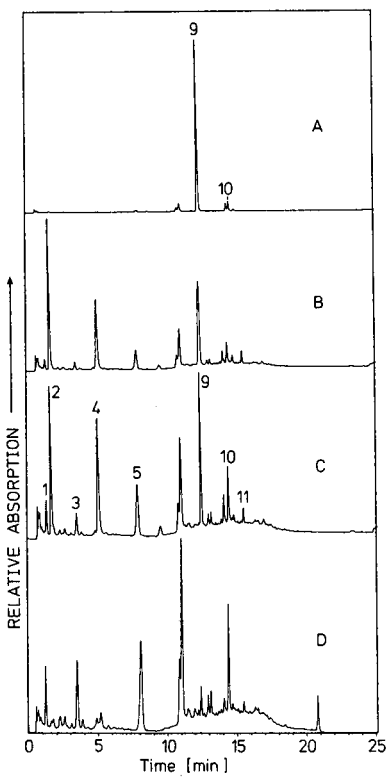

Fig. 2. Chromatograms of a laboratory prepared methanolic extract of *Evernia prunastri* (A) and of the decomposition compounds obtained during a tempering process at 118°C after 1 (B), 1.5 (C) and 3 h (D). Peak identification according to Table I.

a UV–VIS spectrum of the two very small peaks at about 15.5 min, one of them was characterized as the depsid atranorin by its retention time. Furthermore, the assignment is confirmed by the results of earlier studies[7].

Strack *et al.*[7] first performed HPLC separations of lichen substances and found that the main part of a methanolic extract from *Evernia prunastri* consists of evernic acid. Atranorin and chloratranorin were detected only in very small amounts. The existing discrepancy when compared to the corresponding GC results described in the literature[1,3,4] is not discussed by the authors.

In order to clarify the cause of the different results, a laboratory extract from *E. prunastri* was analysed both by an HPLC and a GC method. The HPLC separation was found to be in good agreement to the corresponding results of Strack *et al.* (Fig. 2A), whereas the gas chromatogram shows a composition comparable to that of commercially available products. This fact leads to the conclusion that the characteristic ingredients of *E. prunastri* cannot be vaporized without decomposition.

It is of interest in this context that the laboratory lichen extract does not show any odour characteristics of the corresponding industrially produced materials.

In order to clarify the mechanism of formation of the substances which are responsible for the typical "oakmoss note", the residue of a freshly prepared methanolic extract was heated at 118°C for some hours. The composition of the residue at various stages of this tempering process was monitored by HPLC as illustrated in Fig. 2.

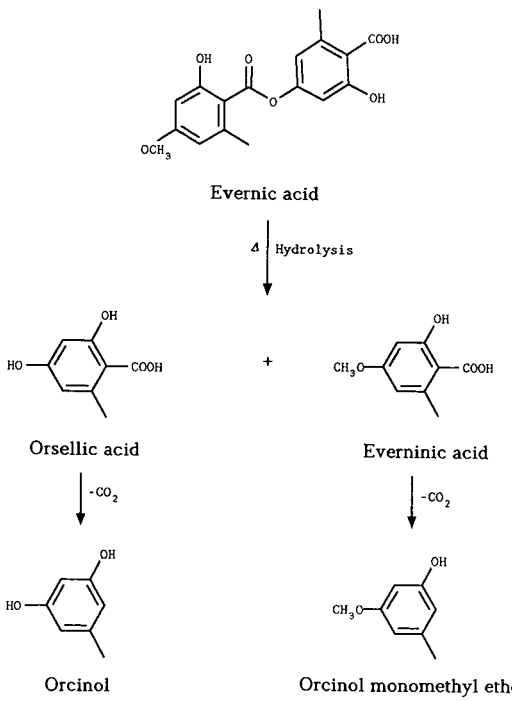

Fig. 3. Degradation reactions of thermally processed evernic acid.

After 1 h, the content of evernic acid decreases and numerous decomposition products result. The degradation of the depsid in a first step mainly yields orsellic acid and everninic acid (Fig. 2B), and after decarboxylation orcinol and orcinol monomethyl ether (Fig. 2D) are formed. The supposed degradation principle is outlined in Fig. 3. A similar scheme was postulated by Pfau[8] in 1937. The same results can be obtained by performing the thermal process with pure evernic acid. On heating the oakmoss extract for 1.5 h (Fig. 2C), the distribution pattern of the detected compounds is very similar to that of a commercially available extract, presented in Fig. 1. On tempering for 3 h, only very small amounts of evernyl and evernic acid are present in the product.

The results obtained lead to the conclusion that GC is of little use in lichen studies, owing to the thermal lability and low volatility of most well known ingredients. As reported[9], especially evernic acid was found to be very unstable to GC analysis even when it was first converted into its trimethylsilyl derivative.

The advantages of the HPLC method presented are:

(i) rapid characterization both of the typical lichen compounds and of the artefacts resulting from the extraction procedure,

(ii) the possibility of quantifying characteristic oakmoss substances in commercially available products for quality control purposes,

(iii) The possibilities of establishing a process control correlating the individual concentrations of the detected ingredients with a desired odour quality.

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CHROM. 21 182

GAS CHROMATOGRAPHIC DETERMINATION OF NICOTINAMIDE IN MEATS AND MEAT PRODUCTS AS 3-CYANOPYRIDINE

AKIO TANAKA*, MASAO IIJIMA, YOSHINORI KIKUCHI, YOJI HOSHINO and NORIHIDE NOSE

Saitama Institute of Public Health, Kamiokubo-Higashi, 639-1, Urawa, Saitama (Japan)

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SUMMARY

The determination of nicotinamide as 3-cyanopyridine after dehydration by heptafluorobutyric anhydride (HFB) was performed by gas–liquid chromatography (GLC) with flame ionization detection (GLC–FID) and a column of 5% OV-17 on Chromosorb W AW DMCS at 130°C. Determination was possible with 3–100 μg of the dehydrated reaction mixture. The procedure for determining nicotinamide in various meats and meat products involves direct analysis by GLC–FID without a clean-up stage; the detection limit is 5 ppm and the recovery ranged from 93.4 to 104.6% (average 98.0%). Various possible interferents in the samples did not interfere in the production or determination of 3-cyanopyridine. The procedure is suitable for routine use. The dehydrated derivative of nicotinamide was confirmed as 3-cyanopyridine by combined gas chromatography–mass spectrometry and infrared spectrometry.

INTRODUCTION

Nicotinamide is one of the B vitamins and serves as a precursor in the biosynthesis of the coenzymes NAD and NADP, which are involved in many enzymatic reactions and metabolic pathways. Nicotinamide deficiency manifests itself as the disease pellagra, which, although common in corn-eating areas two centuries ago, is now rarely seen except in occasional cases of alcoholism. On the other hand, nicotinamide is used as a vitamine-enriching agent and in several countries an official tolerance has been established. Several investigators have reported the use of nicotinamide as a colour-producing agent or for maintaining the colour quality of meat, leading to temporary poisoning with symptoms of facial or cutaneous flushing and itching[1–4]. The development of a simple, rapid and accurate method for the determination of nicotinamide in meat samples is therefore of interest.

The various methods used for determining nicotinamide in foods include colour reactions and absorption measurement[5], microbiological assay[6], high-performance liquid chromatography (HPLC)[2,4,7,8] and gas chromatography (GC)[1,3]. However, several of these methods are not suitable for the determination of nicotinamide

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because they involve the determination of total nicotinic acid after the decomposition of nicotinamide and require complex pretreatments[5–8]. Further, the simultaneous determination of nicotinamide and nicotinic acid using HPLC reported by Yoshida *et al.*[2] is influenced by ascorbic acid.

Determinations of nicotinamide in foods by gas chromatography have been described. Aoyama *et al.*[3] studied the direct determination of nicotinamide by gas–liquid chromatography (GLC) with flame ionization detection (FID). This method is simple but has poor sensitivity. Miyama and Imaida[1] studied the determination of nicotinamide as methyl nicotinate after separation by ion exchange and decomposition of nicotinamide, followed by methylation in the presence of methanol and hydrochloric acid and then GLC–FID. This method lacks specificity, which is crucially important for the determination of nicotinamide because of the determination of nicotinic acid after decomposition of nicotinamide, and also requires a long time for the methylation stage. However, we found that 3-cyanopyridine is over six times more sensitive than nicotinamide in GLC; it can be prepared quantitatively by the instantaneous dehydration of nicotinamide with heptafluorobutyric anhydride (HFB).

Nicotinamide in foods was extracted with acetonitrile without the need for a clean-up stage. The proposed method is simple and selective and offers a practical means of determining nicotinamide in various meats and meat products. The recovery of nicotinamide added to various samples was satisfactory.

EXPERIMENTAL

Reagents and apparatus

Nicotinamide (Tokyo Kasei Kogyo, Tokyo, Japan) was dried at 100°C for 5 h under vacuum immediately before use. A stock solution was prepared by dissolving nicotinamide in acetonitrile to give a concentration of 100 μg/ml. HFB (Wako, Osaka, Japan) stage was of specially pure grade. The internal standard solution for GC was prepared by dissolving 100 μg of 1,2,4-trichlorobenzene in 1 ml of diethyl ether. 3-Cyanopyridine (Tokyo Kasei Kogyo) was of specially pure grade and was used without further purification.

The column packing materials for GLC, *viz.*, Chromosorb W AW DMCS, Advance, DEGS, OV-17, OV-330, PEG-20M, SE-30 and XE-60, were of high purity and were obtained from Nihon Chromato (Tokyo, Japan). All other reagents and solvents were of high purity from Wako.

For identification of the dehydration product of nicotinamide, a Shimadzu GC-MS QP-1000A combined gas chromatograph–mass spectrometer and an infrared spectrometer were used. For GC, a glass tube (2 m × 3 mm I.D.) packed with OV-17 on Chromosorb W AW DMCS was used; the carrier gas (helium) flow-rate was 30 ml/min and the column temperature was 130°C. The conditions for mass spectrometry (MS) were as follows: separator temperature, 180°C; ion source temperature, 210°C; trap current, 60 μA; electron energy, 70 eV; and accelerating potential, 3.5 keV.

The 3-cyanopyridine for IR analysis was prepared as follows. A 0.3-g portion of nicotinamide was placed in a 10-ml test-tube with a ground-glass stopper, then 2 ml of diethyl ether and 0.1 g of HFB were added. The mixture was allowed to react for 10 min at room temperature with occasional shaking. After reaction, 0.5 ml of water (*ca.* pH

8.0) was added and the mixture was shaken gently for 5 min and centrifuged at 1400 *g* for 2 min. The supernatant solution was applied to the column (10 cm × 1.0 cm I.D., prepared with 2 g of activated alumina topped by 0.5 g of anhydrous sodium sulphate) and eluted with diethyl ether. A 10-ml volume of effluent was collected and then evaporated at room temperature. The residue was kept in a desiccator for 1 day, then mixed with a suitable amount of dried potassium bromide. The IR spectra of both nicotinamide and 3-cyanopyridine were measured with a Shimadzu IR-435 recording spectrophotometer.

Preparation of 3-cyanopyridine

A suitable amount of nicotinamide (1–100 μg) or extract dissolved in 2 ml of acetonitrile was placed in a Pyrex test-tube (11.5 cm × 15 mm I.D.), and the solvent was evaporated to dryness under reduced pressure at 40°C for 10 min in a water-bath. To the dried residue were directly added 0.5 ml of internal standard solution followed by 20 μl of HFB, and the reaction was allowed to proceed in a test-tube fitted with a ground-glass stopper at room temperature for 5 min with occasional shaking. A 3-μl volume of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-7A gas chromatograph with a flame ionization detector was used for all analyses. The column consisted of a glass tube (2 m × 3 mm I.D.) packed with 5% of OV-17 on Chromosorb W AW DMCS (80–100 mesh) and was conditioned and operated at 130°C; the detector and injector temperatures were 190°C and the flow-rate of the carrier gas (nitrogen), hydrogen and air were 50, 50 and 800 ml/min, respectively.

Calibration graph

A series of working standard nicotinamide solutions were prepared by dilution of the stock solution with acetonitrile. Aliquots were placed into a Pyrex test-tube to give 3, 10, 30, 50, 70 and 100 μg of nicotinamide, and the solvent was removed by evaporation at 40°C for 10 min in a water-bath. After dehydration by addition of internal standard solution and HFB according to the procedure described above, 3-μl aliquots of the resulting solutions were injected into the GC column at 130°C. As shown in Fig. 1, the retention time (3.6 min) of the 3-cyanopyridine relative to that of 1,2,4-trichlorobenzene was 0.53. The minimum detectable amount of 3-cyanopyridine in this method was *ca.* 2.0 μg. The peak-height ratio of 3-cyanopyridine to 1,2,4-trichlorobenzene was plotted against the amount of nicotinamide analysed; a typical calibration graph is shown in Fig. 2.

Preparation and analysis of various meats and meat products

An accurately weighed sample (generally about 10 g) of finely ground sample was placed in the 300-ml stainless-steel container of a homogenizer, 80 ml of acetonitrile were added and the mixture was homogenized at high speed for 10 min. The extracted solution was filtered and diluted accurately to 100 ml with acetonitrile. A 2-ml volume of the filtrate was placed in a 5-ml test-tube fitted with a ground-glass stopper and the solvent was evaporated to dryness under reduced pressure at 40°C for 10 min in a water-bath. To the dried residue were directly added 0.5 ml of the internal

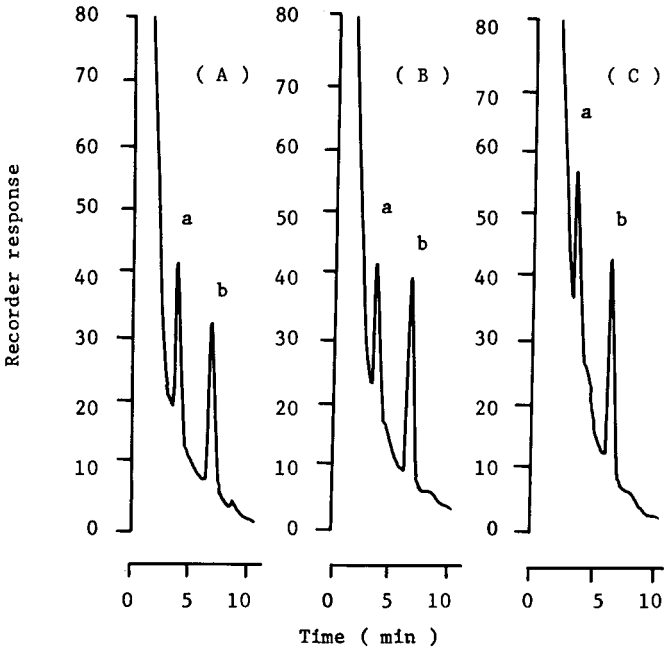

Fig. 1. Gas chromatograms of the dehydration product of (A) a standard reaction mixture to which nicotinamide was added at level of 50 ppm, (B) chicken meat and (C) tuna meat. The sample size was 3 μl. Shimadzu GC-7A gas chromatograph with FID. Column, 5% OV-17 on Chromosorb W HP (80–100 mesh) (2 m × 3 mm I.D.); column temperature, 130°C; injector and detector temperatures, 190°C; flow-rates of nitrogen carrier gas, hydrogen and air, 50, 50 and 800 ml/min, respectively. Peaks: a, 3-cyanopyridine; b, 1,2,4-trichlorobenzene.

standard solution followed by 20 μl of HFB. The mixture was allowed to react as described above and analysed by GC under the described conditions.

The contents of nicotinamide in foods were determined by GLC as described above using the internal standard method and comparison with the calibration graph.

RESULTS AND DISCUSSION

Standard assay

For the GLC assay using the described procedure, there was a linear relationship between peak height and amount of nicotinamide. As shown in Fig. 2, the calibration graph was linear from 3 to 100 μg of nicotinamide and the average relative standard deviations of five determinations were 4.5% for 3 μg, 3.8% for 10 μg, 2.1% for 30 and 50 μg and 2.4% for 70 and 100 μg; the reproducibility was considered to be satisfactory.

Dehydration of nicotinamide

A chromatogram of the dehydrated derivative of nicotinamide is shown in Fig. 1A; the retention time was 3.6 min. The optimum amount of reagent and the optimum time were investigated by using HFB, and the results are shown in Figs. 3 and 4. For 100 μg of nicotinamide, at least 29.8 μg of HFB in 0.5 ml of diethyl ether were required.

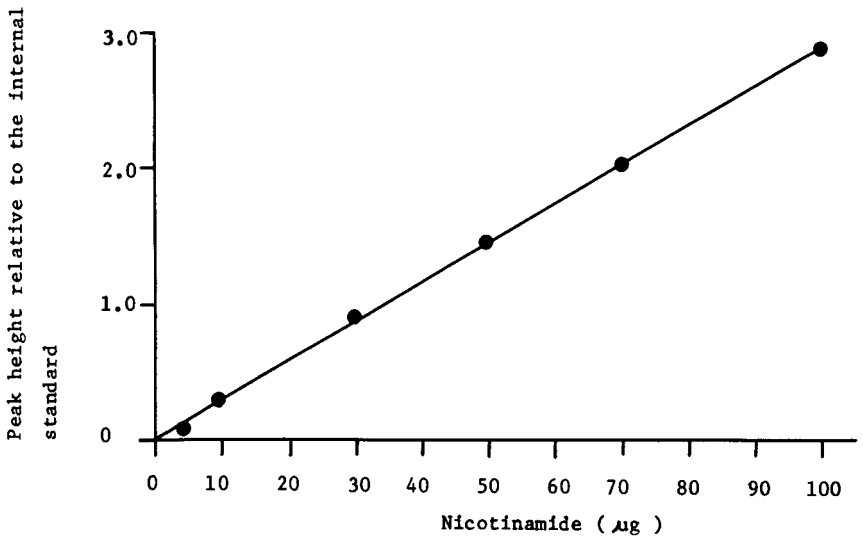

Fig. 2. Calibration graph for nicotinamide. Dehydration of nicotinamide was carried out at room temperature for 5 min. The sample size for GLC was 3 μl; the column temperature was 130°C and the nitrogen flow-rate was 50 ml/min. The abscissa shows the nicotinamide content of the reaction mixture and the ordinate the detector response measured as the peak height relative to that of the internal standard (1,2,4-trichlorobenzene; 100 ng per μl of reaction mixture).

The reaction proceeded fairly rapidly and when HFB solution in diethyl ether (40 μl/ml) was added to the solid residue of nicotinamide, the yield of the dehydrated product reached 100% within 3 min, and in practice 20 μl of reagent and a reaction time of 5 min were used.

To obtain good dehydration reactivities towards nicotinamide, aliquots of 20 μl of various acid anhydride reagents were added to 100 μg of nicotinamide dissolved in

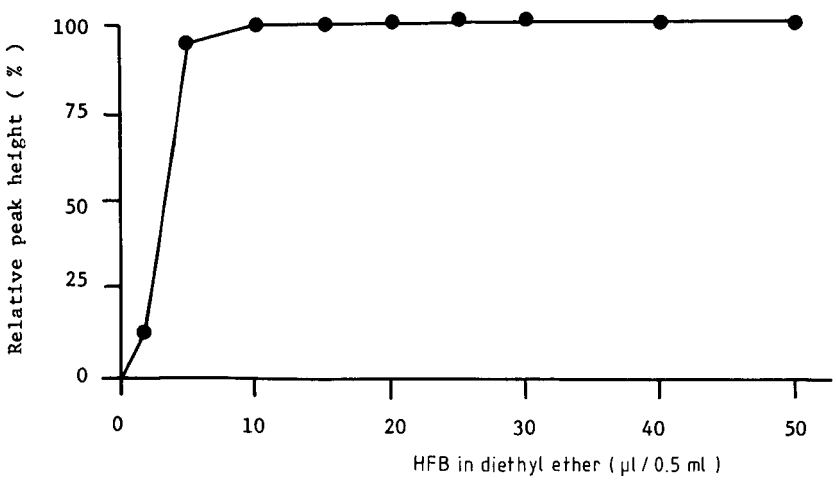

Fig. 3. Effect of amount of HFB on the dehydration of nicotinamide. To 100 μg of nicotinamide was added HFB in 0.5 ml of diethyl ether at room temperature and the product was analysed by GLC after 5 min.

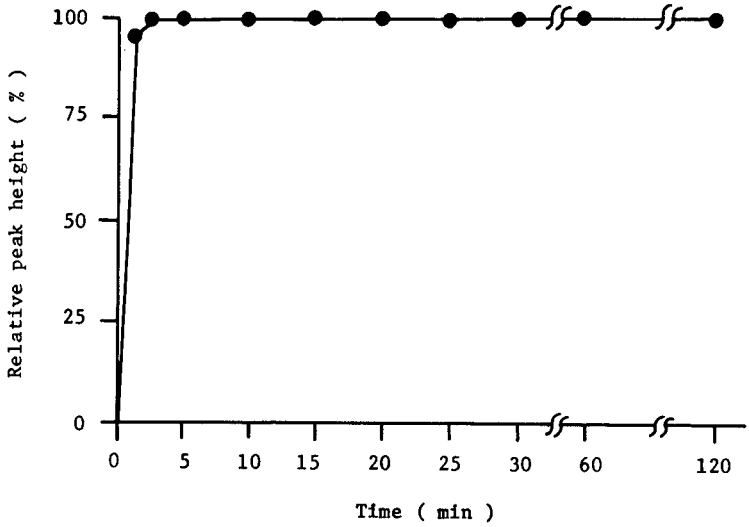

Fig. 4. Time course of formation of the dehydration product after addition of HFB to nicotinamide. To 100 μg of nicotinamide was added HFB in 0.5 ml of diethyl ether at room temperature and the product was analysed by GLC.

TABLE I

EFFECT OF VARIOUS ACID ANHYDRIDE REAGENTS ON DEHYDRATION OF NICOTINAMIDE

20 μl of acid anhydride reagent were added to 100 μg of nicotinamide dissolved in 0.5 ml of diethyl ether. Reaction and GLC conditions as in the described procedure.

Acid anhydride	*Relative peak height (%)*
Acetic anhydride	5.2
Trifluoroacetic anhydride	75.1
Propionic anhydride	29.9
Pentafluoropropionic anhydride	94.8
Butyric anhydride	63.7
Heptafluorobutyric anhydride	100.0

TABLE II

EFFECT OF SOLVENT ON DEHYDRATION OF NICOTINAMIDE

Reaction and GLC conditions as in the described procedure. Each reaction mixture (0.5 ml) contained nicotinamide (100 μg) and HFB (20 μl).

Solvent	*Relative peak height (%)*
Diethyl ether	100.0
Acetonitrile	74.8
Acetone	41.8
Ethyl acetate	27.4
Benzene	2.7
Hexane	2.7
Methanol	0.5

0.5 ml of diethyl ether. The relative yields obtained from the gas chromatograms are shown in Table I. It is assumed that the reaction is influenced by the presence or absence and number of fluorine atoms in the acid anhydride structure. The most suitable reagent for the dehydration of nicotinamide was HFB, and it gave good chromatogram (Fig.1A). Ethyl acetate, acetone, diethyl ether, acetonitrile, benzene, hexane and methanol were tried as reaction solvents in the dehydration of nicotinamide. The most suitable was diethyl ether and the least suitable was methanol, as shown in Table II. We chose diethyl ether because of its good solvent properties for 3-cyanopyridine and 1,2,4-trichlorobenzene.

Gas chromatographic sensitivity

Columns containing Advance (2%, w/w), DEGS (5%, w/w), OV-17 (5%, w/w), OV-330 (5%, w/w), PEG-20M (5%, w/w), SE-30 (5%, w/w) and XE-60 (3%, w/w) on Chromosorb W AW DMCS were tested. All of the columns showed the peak of 3-cyanopyridine; particularly good peak characteristics and sensitivity were achieved with OV-17 under the conditions described above. A low temperature and a long column were preferable for the GLC of the 3-cyanopyridine. At 130°C, a 2.0-m column containing OV-17 on Chromosorb W AW DMCS gave a good gas chromatogram; the retention times of 3-cyanopyridine relative to that of the internal standard was 0.53. This GC determination of nicotinamide after dehydration with HFB was six times more sensitive than the direct determination reported by Aoyama *et al.*[3]. After dehydration, the reaction mixture should be injected into the gas chromatograph as soon as possible; at room temperature the sample was stable for at least 5 h, but after 24 h the content of 3-cyanopyridine had decreased to 96.3%.

Influence of evaporation of the solvent on the recovery of nicotinamide and 3-cyanopyridine

Prior to dehydration of nicotinamide it was necessary to evaporate the acetonitrile in order to change to diethyl ether as the preferred reaction solvent. A 2-ml volume of acetonitrile containing 100 μg of nicotinamide was evaporated under reduced pressure at 40°C for 10 min. No loss of nicotinamide during or after the evaporation was observed. On the other hand, when the evaporation was performed at room temperature in order to eliminate excess of HFB after dehydration, a significant decrease in the amount of 3-cyanopyridine was observed. The losses of 3-cyanopyridine as a function of evaporation time at room temperature were 9.3% after 2 min, 21.2% after 5 min, 35.7% after 10 min and 68.3% after 20 min. Therefore, the dehydrated sample was injected directly into the GC column without evaporation.

Interferences

Nicotinamide can be extracted from foods with acetonitrile[1–4] and this simple and rapid extraction permits the determination of nicotinamide in foods by GLC without effects from interfering substances. To investigate the effects of various possible interferents, 100-μg portions of nicotinamide were added to various amounts of substances, and each mixture was analysed by direct dehydration without clean-up. As shown in Table III, none of them had much effect on the determination. Another possible interferent is the water derived from the samples, but the addition of 1–10 g of water to 100 μg of nicotinamide did not affect the determination. It could be presumed

TABLE III

INFLUENCE OF VARIOUS SUBSTANCES ON RECOVERY OF NICOTINAMIDE

Each amount of substance was added to 100 μg of nicotinamide dissolved in 0.5 ml of diethyl ether. Reaction and GLC conditions as in the described procedure.

Substance	*Amount added (μg)*	*Recovery of nicotinamide (%)*
Uracil	50	98.5
	100	97.1
Cytosine	50	94.2
	100	92.7
Adenine	50	95.3
	100	94.6
Guanine	50	94.7
	100	95.3
Xanthine	50	99.7
	100	107.1
5′-Inosinic acid	50	101.2
	100	105.0
Adenosine 5′-(tetrahydrogentriphosphate) (ATP)	50	99.2
	100	98.0
Nicotinic acid	50	97.0
	100	100.3
Sorbic acid	50	99.1
	100	101.0
Butylhydroxyanisole	50	101.0
	100	99.7
Butylhydroxytoluene	50	97.5
	100	95.5
Benzoic acid	50	99.1
	100	100.0
p-Hydroxybenzoic acid	50	98.9
	100	99.7

TABLE IV

RECOVERY (%) OF NICOTINAMIDE ADDED TO VARIOUS MEATS AND MEAT PRODUCTS

Each result is the average of five determinations.

Sample	*Amount of nicotinamide added (μg/g)*			
	10	*20*	*50*	*100*
Chicken	98.4	97.6	98.0	99.1
Pork	97.3	98.7	104.6	98.0
Beef	93.4	95.7	94.7	96.4
Tuna	100.3	101.6	98.1	99.6
Ham	94.7	95.3	98.0	101.1

TABLE V

COMPARISON OF GC AND HPLC METHODS WITH THE PROPOSED METHOD FOR THE DETERMINATION OF NICOTINAMIDE IN VARIOUS MEATS AND MEAT PRODUCTS

Each sample was purchased commercially and each result is the average of four determinations.

Sample	*Nicotinamide (ppm)*		
	GC method[1]	*HPLC method*[2]	*Proposed method*
Chicken	98.6	88.6	96.6
Pork	45.2	44.6	41.8
Beef	20.5	17.4	25.4
Tuna	131.8	121.3	119.0
Ham	32.3	28.4	31.0

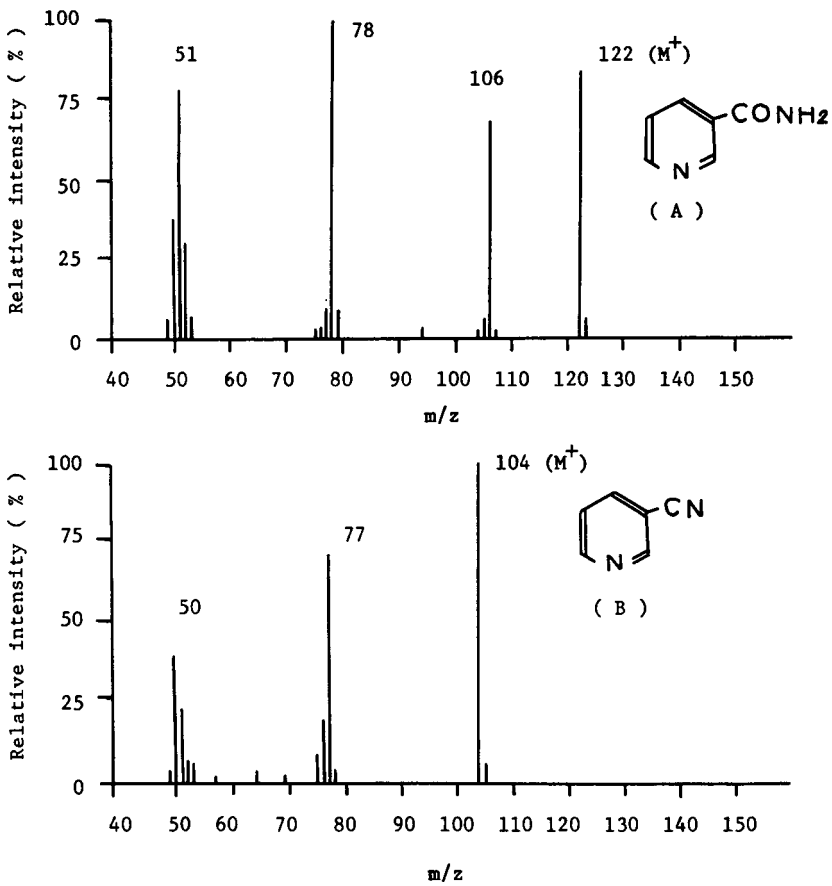

Fig. 5. Mass spectra of (A) nicotinamide and (B) its dehydration product.

that the water was eliminated during or after the evaporation of the acetonitrile extract of nicotinamide. Therefore, further dehydration of the acetonitrile extract was not necessary.

The method described was tested on the analysis of chicken and tuna meat and the results of the analysis of the sample digest by GLC after the formation of 3-cyanopyridine are shown in Fig. 1B and C. The dehydrated extract obtained from both meats gave gas chromatograms with good peak characteristics.

Recoveries and application

Nicotinamide (10, 20, 50 and 100 μg) added to 10-g samples of chicken, pork, beef, tuna and ham chopped and then ground in a porcelain pestle and mortar was determined by the proposed method. Five determinations were carried out at each concentration. As shown in Table IV, the average recovery was 98.0% and ranged from 93.4% at the lower concentrations to 104.6% at the higher concentrations. The detection limit was 5 ppm.

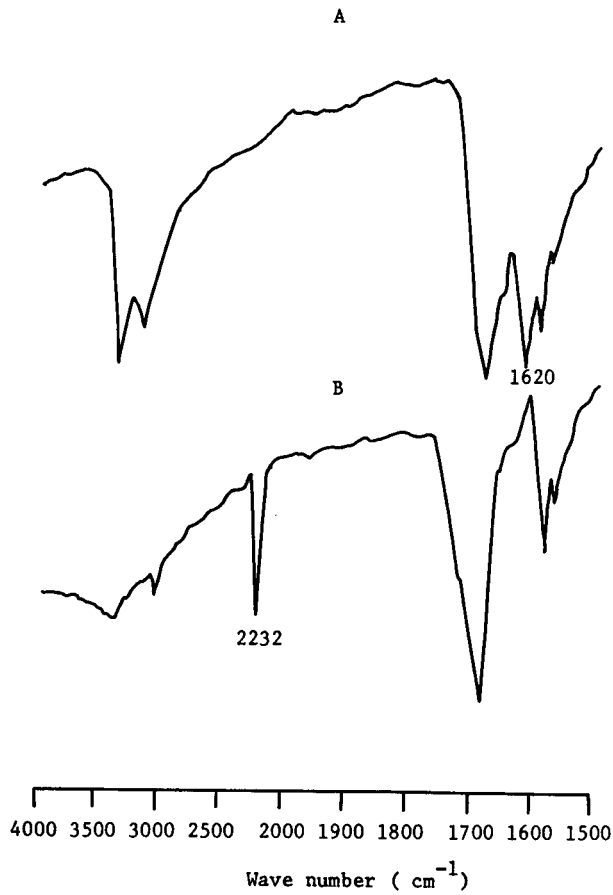

Fig. 6. IR spectra of (A) nicotinamide and (B) its dehydration product in potassium bromide.

Further, the proposed method was compared with another GC method[1] and an HPLC method[2]. The results from the different procedures correlated well (Table V) and it is concluded that the proposed method is suitable for routine use.

Identification of the dehydration product of nicotinamide

The mass spectrum of the product derived from the dehydration of nicotinamide by HFB was identical with the standard spectrum of nicotinamide, with ion peaks at m/z 122 (M^+), 106 ($M^+ - NH_2$), 78 (−CO) and 51 (−HCN), as shown in Fig. 5A. The mass spectrum corresponding to the peak obtained by dehydration of nicotinamide is shown in Fig. 5B, with m/z 104 (M^+), 77 ($M^+ - HCN$) and 50 (−HCN). The parent peak (m/z 122) for nicotinamide and that at m/z 104 for the dehydration product correspond to the molecular weight of each compound. The shift of the peaks from m/z 104 to 77 for the dehydration product could be ascribed to removal of a cyano group. Further, the elution time (3.6 min) and the mass fragmentation pattern of the hydrated product agreed with those of the 3-cyanopyridine standard. Partial characteristics of the IR spectra of nicotinamide and its dehydration product are shown in Fig. 6. In the spectrum of the dehydration product, the absorption at 1620 cm^{-1} resulting from the carbonyl group in the nicotinamide spectrum has disappeared, and a medium absorption at 2232 cm^{-1} resulting from a cyanide group is observed.

These experiments confirmed that the dehydration product of nicotinamide was 3-cyanopyridine.

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AFFINITY CHROMATOGRAPHY OF BOVINE HEART LACTATE DEHYDROGENASE USING DYE LIGANDS LINKED DIRECTLY OR SPACER-MEDIATED TO BEAD CELLULOSE

M. NAUMANN, R. REUTER, P. METZ and G. KOPPERSCHLÄGER*
Institute of Biochemistry, Karl-Marx-University Leipzig, Liebigstrasse 16, Leipzig, 7010 (G.D.R.)
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SUMMARY

A number of reactive dyes coupled to bead cellulose directly or spacer-mediated has been investigated in respect of their interaction with lactate dehydrogenase (LDH; E.C. 1.1.1.28) from heart muscle. The Procion dyes Red HE-7B and Navy H-ER, as well as the Remazol dyes Brilliant Blue R and Brilliant Red 5-BN directly bound to bead cellulose provide high binding of LDH and the adsorbed enzyme is eluted specifically from these affinity adsorbents in high yield. In contrast, under the same conditions no binding of LDH has been found to the Procion dyes Green H-4G, Yellow HE-3G, Scarlet MX-G and Orange MX-G, although an opposite behaviour was expected from the results of affinity partitioning in aqueous two-phase systems. However, immobilizing these dyes via a spacer generated strong binding of the enzyme to the affinity adsorbent. The influence of the length of the spacer was studied in respect of the binding capacity and the yield of the enzyme specifically eluted. The applicability of Procion Scarlet MX-G-(diaminohexyl)-bead cellulose for the purification of LDH from muscle extract in one chromatographic step was demonstrated.

INTRODUCTION

The use of triazine dyes for the purification of proteins especially of enzymes by dye-ligand affinity chromatography is well established on the laboratory scale (for reviews see refs. 1 and 2). In the last decade, besides the reactive dyes Cibacron Blue F3G-A and Procion Red HE-3B, a series of other textile dyes were tested for their application as affinity ligands[3,4]. Supports of dyes frequently used are polysaccharides like agarose and diverse Sephadex types. Cellulose may also be a good support because reactive dyes were developed predominantly for the textile dyeing and printing industry. However, previous results showed that Cibacron Blue F3G-A bound to powered cellulose binds phosphofructokinase from yeast, but the enzyme was not eluted under different conditions[5]. The advantages of macroporous bead cellulose for dye-ligand affinity chromatography owing to its high porosity, sufficient rigidity and its low cost were first demonstrated by Mislovičová *et al.*[6].

In this study a commercially available macroporous bead cellulose has been

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tested for its usefulness as a matrix in dye-ligand affinity chromatography in respect of the adsorption and desorption behaviour of lactate dehydrogenase (LDH) from heart muscle. Particularly, the influence of the mode of coupling of the dyes on the properties of the resulting affinity adsorbent has been analyzed in more detail. Finally, a simple procedure for the purification of LDH to homogeneity was elaborated.

EXPERIMENTAL

Materials

Substrates and substances for buffers were obtained from Boehringer (Mannheim, F.R.G.), VEB Laborchemie (Apolda, G.D.R.) and VEB Arzneimittelwerk (Dresden, G.D.R.). All were of analytical reagent grade. The dyes of the Procion type (ICI, Manchester, U.K.), Cibacron Blue F3G-A (Ciba Geigy, Basle, Switzerland) and the Remazol dyes (Hoechst, Frankfurt/Main, F.R.G.) were gifts from VEB Chemisches Kombinat Bitterfeld (G.D.R.) and were used without further purification. Bead cellulose (sizes 80–200 and 200–400 μm) was a product from VEB Arzneimittelwerk (Leipzig, G.D.R.). Poly(ethylene glycol) (PEG 6000) was obtained from Serva (Heidelberg, F.R.G.), Dextran M 70 from VEB Serumwerke (Bernburg, G.D.R.). Cadoxen [tris(ethylenediamine) cadmium(II) hydroxide] was a gift from the Institute of Organic Chemistry of the Slovakian Academy of Sciences, Bratislava.

Methods

Preparation of lactate dehydrogenase. LDH was partially purified from bovine heart muscle as follows.

Extraction of minced muscle at room temperature with a ten-fold volume of distilled water containing 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol. After centrifugation (9000 *g*, 30 min) the pH of the supernatant was lowered to 5.5 with 1 *M* H_3PO_4. The specific activity of LDH was 4–6 units/mg protein, where 1 unit is defined as the amount of enzyme which reduces 1.0 μmol pyruvate to lactate per minute.

Binding of LDH to DEAE-Sephadex A-50 equilibrated with 30 m*M* potassium phosphate buffer, pH 5.5, 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol (buffer A). Ion-exchange chromatography was carried out in a batch procedure by stirring the gel with the supernatant (60 units of LDH per g of the exchanger) for about 30 min. In order to remove the unbound protein, the DEAE-cellulose was washed with buffer A (10-fold volume of the exchanger).

Elution of LDH from the ion exchanger by adding 0.1 *M* KCl to buffer A.

Precipitation of LDH by ammonium sulphate to 65% saturation at pH 7.4. The precipitate was suspended in buffer A, pH 7.4, saturated with ammonium sulphate and was designated as prepurified LDH (specific activity 30–70 units/mg protein).

Preparation of dye derivatives of bead cellulose. Commercial bead cellulose was prepared in a mixture of water and chlorobenzene. Before use the matrix was washed exhaustively with distilled water to remove traces of the organic solvent. The chlorotriazine dyes (listed in Table I) were covalently attached to the support according to the method of Lowe and Pearson[1]; 10 g of the moist gel cake were suspended with 45 ml of distilled water and 5 ml of the dye solution (20 mg per ml of distilled water). The suspension was gently stirred at 40–60°C for 30 min. Then NaCl

was added to a final concentration of 2% and the gel suspension was stirred 30 min prior adding solid Na_2CO_3 to a final concentration of 1%. The gel suspension was stirred again for 2 h. After keeping overnight at room temperature, the gel was exhaustively washed on a sintered glass funnel with distilled water, 0.1 *M* Na_2CO_3 and again distilled water until the final washing was colourless.

The Remazol dye derivatives of bead cellulose were prepared according to Mislovičová *et al.*[6]. A 10-g amount of cellulose (wet weight) was suspended in 10 ml of 0.25 *M* sodium hydroxide solution and after the addition of 100 mg of the dye the suspension was stirred at room temperature for 4 h. The product was washed thoroughly with distilled water and 0.1 *M* $NaHCO_3$ to remove the unbound dye. The affinity adsorbents were stored as suspensions in water at 4°C in the presence of 0.02% sodium azide. Before use they were exhaustively washed and equilibrated with the respective buffer.

Preparation of dye-(spacer)-bead cellulose. The preparation of the dye–spacer bead cellulose was carried out in three steps as summarized in Fig. 1.

Activation of the cellulose by N-chlorocarbonyloxy-5-norbornene-2,3-dicarboximide (Cl-CO-ONB)[7]: the cellulose (I) was transferred from water to acetone by stepwise washing with water–acetone mixtures (9:1, 7:3, 3:7, 1:9, v/v) and finally with dry acetone. A 10-g amount of this material was suspended in a solution of 8.3 m*M* Cl-CO-ONB in 10 ml of dry acetone. The suspension was gently tumbled for 16 h at room temperature. Then the acetone was sucked off and the resin was washed twice with dry acetone. The activated support (II) was stored in dry acetone.

Synthesis of dye-(spacer)-conjugate: 60 mmol of α,ω-diaminoalkane (III) were dissolved in 30 ml water and the solution was adjusted to pH 10 with 2 *M* HCl. A 3-mmol amount of monochloro- or dichlorotriazine dye (IV), dissolved in 30 ml water, was added dropwise and the reactants were stirred for 1 h at 50°C. After cooling to room temperature, the precipitate formed (V) was collected by filtration and dried. The structure of the dye–spacer conjugates was confirmed by ^1H NMR spectroscopy.

Coupling of dye-(spacer)-conjugate to activated bead cellulose: 500 mg of the dye–spacer conjugate (V) were dissolved in 30 ml of dimethyl sulphoxide (DMSO)–0.1 *M* $NaHCO_3$ (1:1, v/v). A 5-g amount of the activated bead cellulose swollen in water by stepwise treatment with acetone–water mixtures (9:1, 7:3, 3:7, 1:9, v/v) was suspended in 30 ml of ligand solution (V) and allowed to tumble for 16 h at 20°C. The reaction product (VI) was separated by filtration on a büchner funnel and washed with DMSO until the filtrate became colourless. Finally, the dye-(spacer)-bead cellulose was treated with 100 ml 0.1 *M* $NaHCO_3$, 100 ml water and stored at 4°C. For the preparation of dye-(spacer)-celluloses the following dyes were used: Cibacron Blue F3G-A and the Procion dyes Red HE-3B, Orange MX-G, Scarlet MX-G, Green H-4G and Yellow HE-3G (see also Fig. 1).

Determination of the degree of dye substitution. As already described for agarose matrices[1], the determination of the dye concentration bound to bead cellulose by acid hydrolysis was not successful. Therefore the affinity adsorbents (50–300 mg) with dyes directly linked to cellulose were dissolved in 2 ml of Cadoxen, which is a good solvent for bead cellulose as described by Gemeiner and Pasteka[8]. For a standard, 2–10 mg of the free dyes were also dissolved in 2 ml of Cadoxen in order to compensate changes in spectral properties of the dyes caused by the solvent. After dissolving the dye–cellulose, the absorbances of the solutions were measured immediately at λ_{max} using a Specord

M 40 spectrophotometer (VEB Carl-Zeiss, Jena, G.D.R.) and compared with the respective calibration graph for the standards. This procedure is common practice when either the structure of a dye is unknown or the molar absorbance of the dye is influenced by the solvent. The method cannot be applied for the determination of spacer-mediated dye substituents because these affinity matrices are insoluble in Cadoxen, probably due to some cross-links within the matrix generated by the activation procedure.

Affinity chromatography of LDH. All experiments were carried out in 20 m*M* potassium phosphate buffer, pH 7.0, 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol (buffer B). The prepurified LDH was exhaustively dialyzed against buffer B. After centrifugation (20 000 *g*, 15 min) the enzyme was loaded onto a column (10 cm × 1 cm) packed with the affinity adsorbent. The resin was washed using a 12-fold column volume of buffer B to remove unbound protein. Then the adsorbed enzyme was eluted with buffer B containing either 1 *M* KCl or 0.05 m*M* oxidized nicotinamide–adenine dinucleotide (NAD^+) plus 1 m*M* sodium sulphite. Fractions of 2–5 ml were collected for assaying the LDH activity. The pooled enzyme was concentrated by ultrafiltration using collodium bags (Sartorius, Göttingen, F.R.G.). The affinity columns were regenerated by treatment with 0.1 *M* NaOH, water and buffer B.

Partition of LDH in two-phase systems. The partition of LDH in aqueous two-phase systems was performed as described previously[9] with minor modifications. The two-phase systems were prepared from aqueous stock solutions of PEG 6000 (40%, w/w), Dextran M 70 (20%, w/w) and 0.1 *M* triethanolamine–HCl buffer, pH 7.0, 50 m*M* 2-mercaptoethanol and 10 m*M* EDTA by weighing the respective amounts so that samples of 4 g contain finally 5.5% PEG 6000, 8.25% Dextran M 70 in 25 m*M* triethanolamine–HCl buffer, pH 7.0, and the respective amount of the enzyme. In the case of affinity partitioning, 2% of the total PEG was replaced by dye-liganded PEG 6000. The enzyme (0.01–0.05 ml) was dialyzed for 2 h against 200 ml of 25 m*M* triethanolamine–HCl buffer, pH 7.0, to remove the ammonium sulphate. A 1–2 μl volume of the dialyzed LDH containing 3–6 units of enzyme activity was added to each tube cooled to 0°C. The mixture was kept for 30 min at 0°C, mixed again for 15 s and centrifuged at 2000 *g* for 2 min at 0°C. For the enzyme assay, appropriate volumes of each of the phases were carefully removed. The partition coefficient, *K*, is defined as the ratio of the enzyme concentration in the upper and in the lower phases determined by the activity in both phases. The term $\Delta \log K$ is the difference between the log *K* value of a dye–PEG-containing system and that of a system without dye–PEG.

Assay conditions. The activities of LDH and malate dehydrogenase (MDH, E.C. 1.1.1.37) were measured spectrophotometrically at 340 nm using the following conditions: potassium phosphate buffer, pH 7.0, 0.8 m*M* pyruvate and 0.8 m*M* oxaloacetate, respectively, and 0.2 m*M* $NADH_2$.

Protein was assayed according to Bradford[10] with dried human albumin as a standard.

RESULTS AND DISCUSSION

A number of triazine dyes directly bound to bead cellulose have been tested for their affinity to LDH from bovine heart muscle. If the original muscle extract was applied to a limited amount of the affinity adsorbent, MDH was predominantly bound

due to its higher quantity in the extract in comparison to LDH. For example, Procion Navy H-ER-bead cellulose binds about 150 units of MDH and only 25 units of LDH per gram (wet weight). The competition of the two enzymes for the dye ligands is one of the reasons why various dye–bead celluloses exhibiting high binding ability for purified LDH do not efffectively adsorb the enzyme from the crude extract. In order to avoid this competition, LDH was prepurified to 30 to 70 units/mg of protein (see Methods) containing only 1.5% of MDH activity relative to the activity of LDH.

In Table I the binding capacities of various dye–bead celluloses for LDH, the eluted activity and the recovery of the enzyme are summarized. The variation of the amount of LDH bound to one type of dye–cellulose depends on the specific activity of the LDH used. The higher the specific activity the higher is the amount of LDH adsorbed and *vice versa*. The results show that Cibacron Blue F3G-A, Procion Red HE-7B, Procion Yellow HE-4R and Procion Navy H-ER are suitable affinity ligands for LDH. The various adsorbents show only small differences in respect to the recovery and the purification of the enzyme. The specific activity of the LDH was about 200–300 units/mg.

TABLE I

BINDING CAPACITIES OF DYE DERIVATIVES OF BEAD CELLULOSE AND RECOVERY OF ELUTED LACTATE DEHYDROGENASE

The binding capacity of dye–bead cellulose was determined using LDH prepurified by DEAE-Sephadex chromatography (specific activity 30–70 units/mg). Columns (10 cm × 1 cm) containing 5 g of the respective dye–cellulose were loaded with an excess of LDH (about 400 units/g cellulose) at 10°C. The amount of activity adsorbed (defined as 100%) was calculated from the difference between the total activity of LDH loaded onto the column and the unbound one determined in the breakthrough fraction and the wash pool. The purification factor was calculated from the increase in the specific activity of the enzyme. The degree of dye substitution of dye cellulose was determined after dissolving the adsorbents in cadoxen (see *Methods*).

Dye	*Degree of dye substitution [mg dye/g cellulose (wet weight)]*	*Binding capacity of the dye–bead cellulose for LDH [units/g cellulose (wet weight)]*	*LDH activity eluted in the main fractions (%)*	*Purification factor*
Cibacron Blue F3G-A	2.0	200–245	55–65	3–4
Procion dyes				
Red HE-3B	2.0	10–30	70	
Red HE-7B	5.7	190–270	75–90	n.d.
Navy H-ER	3.7	140–200	80–90	4–5
Brown HE-G	n.d.[a]	70–150	50–75	5–6
Yellow HE-4R	3.2	100–250	70	3
Scarlet MX-G	3.8	No binding	–	3–4
Orange MX-G	1.2	No binding	–	–
Green H-4G	2.0	No binding	–	–
Yellow HE-3G	1.3	No binding	–	–
Remazol dyes				
Brilliant Blue R	20.0	100–250	80–90	3–5
Brilliant Red 5 BN	n.d.	100–300	70–90	3–5

[a] n.d. = Not determined.

Immobilized Procion Red HE-3B known as a preferential adsorbent of $NADP^+$-linked dehydrogenases[12] had the lowest binding capacity for the LDH.

The two Remazol dye–bead celluloses successfully used in the purification of rat liver LDH[6] provided high binding also for the enzyme from heart muscle. Furthermore, these affinity adsorbents showed a recovery of 90–100% of the adsorbed enzyme and the LDH appeared in a small volume of the eluate.

Table I also shows that the Procion dyes Scarlet MX-G, Orange MX-G, Green H-4G and Yellow HE-3G, when directly bound to bead cellulose, are incapable of interacting with LDH. This result was not expected because affinity partitioning of pure LDH in aqueous two-phase systems using triazine dye-substituted poly(ethylene glycol) showed that the enzyme binds also to Procion Green H-4G and Procion Orange MX-G[9]. Moreover, Procion Scarlet MX-G and Procion Orange MX-G were found to interact specifically with pig heart LDH[11].

In order to rationalize these contradictory results, the affinity of prepurified LDH from heart muscle to diverse triazine dyes was screened by means of affinity partitioning in aqueous two-phase systems (Table II). Here, the $\Delta \log K$ values, as a measure of the strength of interaction of the enzyme with dyes[9], are shown. Values higher than one found under the experimental conditions indicate sufficient interaction of the dye molecule with the enzyme. This holds for most of the dye stuffs except Procion Scarlet MX-G and Procion Orange MX-G which exhibit weaker interactions.

Dyes listed in group 1 of Table II are able to interact with LDH if they are coupled directly to the matrix. In group 2 those dyes are listed which did not exert affinity to LDH when coupled directly to cellulose (see Table I). This was not expected, particularly for Procion Green H-4G and Procion Yellow HE-3G where no significant difference in the Δ log K values was observed compared with the dyes in group 1.

One possibility for the lack in affinity of the immobilized dyes of group 2 for LDH may be a steric hindrance to the dye–enzyme interaction caused by the matrix. Therefore the dyes in group 2 of Table II were coupled to bead cellulose via a spacer. The length of the spacer was varied between two and six carbon atoms using diamino-ethane, -butane and -hexane (see Fig. 1).

TABLE II

INTERACTION OF HEART MUSCLE LDH WITH DIFFERENT TRIAZINE DYES STUDIED BY MEANS OF AFFINITY PARTITIONING

The two-phase systems contained 8.25% Dextran M 70 and 5.5% PEG 6000. In the case of affinity partitioning, 2% of the total PEG was replaced by dye-liganded PEG. For further experimental details see *Methods*. The term $\Delta \log K$ is the difference between the log K values of a two-phase system with and without dye–PEG. In group 1 triazine dyes are listed which show binding of LDH when they are directly coupled to bead cellulose. In group 2 dyes are listed which do not interact with LDH when they are directly bound to the matrix.

Dye	*Δlog K*	*Dye*	*Δlog K*
Group 1		*Group 2*	
Procion Red HE-3B	1.62	Procion Green H-4G	1.69
Procion Red HE-7B	1.54	Procion Yellow HE-3G	1.86
Procion Yellow HE-4R	1.92	Procion Scarlet MX-G	0.97
Procion Navy H-ER	1.82	Procion Orange MX-G	0.94
Cibacron Blue F3G-A	1.33		

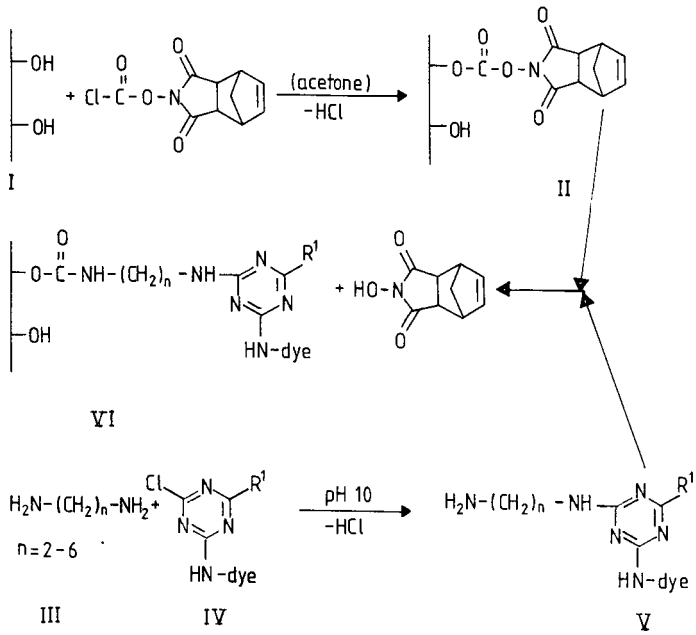

Fig. 1. Preparation of dye–spacer bead cellulose (VI) using activated bead cellulose (II) and the aminoalkyl dye (V) (see text).

In Table III the binding capacities of the adsorbents and the recoveries of the eluted enzymes are summarized. The introduction of a spacer enabled Procion Green H-4G, Procion Scarlet MX-G, Procion Orange MX-G and Procion Yellow HE-3G to bind LDH. In comparison with the affinity adsorbents involving directly attached dyes, some spacer-mediated affinity matrices provided higher binding capacity for LDH. This is not valid for the dyes Procion Red HE-3B and Cibacron Blue F3G-A, respectively. The apparent lower binding capacity of Procion Scarlet MX-G-(diaminoethyl)–bead cellulose and Procion Orange MX-G-(diaminohexyl)–bead cellulose for LDH is caused by the less specific activity of the enzyme used in the experiments.

The influence of the length of the spacer on the binding capacity and on the elution behaviour was studied in more detail using Procion Green H-4G and Procion Scarlet MX-G as affinity ligands (Table III). In the case of Procion Green H-4G, 75–90% of the adsorbed enzyme was eluted by 1 *M* KCl. With NAD^+/sulphite, differences in the effectiveness of elution as a function of the length of the spacer becomes obvious: 80–90% of the adsorbed activity was eluted in a small volume from dye-(diaminohexyl)-bead cellulose. However, applying affinity cellulose with a shorter spacer length gave a significantly lower yield and the enzyme appeared as a broad peak. In the latter case a ten-fold increase in the effector concentration in the elution buffer yielded only a two-fold increase in the amount of LDH eluted. The results revealed that the longer the spacer the lower is the concentration of NAD^+ required for elution of the enzyme and the sharper is the elution profile (not shown). This behaviour has been confirmed with spacer-mediated Procion Scarlet MX-G.

TABLE III

BINDING CAPACITY AND ELUTION BEHAVIOUR OF DYE-(SPACER)-BEAD CELLULOSE TO LACTATE DEHYDROGENASE

LDH with two different specific activities of 70 or 30 units/mg (marked by *) were applied. The percentage of the eluted activity is related to the adsorbed activity (defined as 100%). The latter was calculated from the difference between the total activity of LDH loaded onto the column and the portion unbound after washing the column with buffer B. Further experimental details are described in *Methods*. Abbreviation: Dye-C_n-BC = Dye-$NH(CH_2)_nNH$–CO–O–bead cellulose.

Adsorbent (Dye-C_n-BC)	*Binding capacity (units/g wet adsorbent)*	*Percentage of eluted enzyme activity of LDH in presence of*	
		1 M KCl	*0.05 mM NAD^+/1 mM sulphite*
Procion Red HE-3B			
-C_6-BC	30–60	n.d.	29
Cibacron Blue F3G-A			
-C_6-BC	150	n.d.	47
Procion Green H-4G			
-C_2-BC	450–620	84	35
-C_4-BC	500–640	92	51
-C_6-BC	500–680	75	84
ProcionScarlet MX-G			
-C_2-BC	500–650	5–10	31
-C_2-BC	200–300*	n.d.	30
-C_6-BC	500–770	5–10	79
Procion Orange MX-G			
-C_6-BC	200*	2–5	87
Procion Yellow HE-3G			
-C_6-BC	520	n.d.	30

In Table III, differences between Procion Green H-4G and Procion Scarlet MX-G in respect of the conditions of elution of LDH become evident. Although 1 *M* KCl is able to desorb most of the enzyme from the affinity matrix of the Procion Green type, independent of the spacer length of the ligands, LDH was almost completely retained on Procion Scarlet MX-G-(spacer)-bead cellulose under the same conditions. Decreasing or increasing the concentration of KCl did not essentially improve the yield of the enzyme. However, the enzyme was eluted by applying NAD^+/sulphite as in the case of Procion Green H-4G-cellulose (Table III) and also with buffer B containing 50% ethylene glycol, in 70% yield (not shown). Similar results were obtained with Procion Orange MX-G coupled via an aminohexyl spacer to bead cellulose. This is not surprising because of the structural similarities between the dichlorotriazinyl dyes Procion Scarlet MX-G and Procion Orange MX-G as shown in Fig. 2.

The superior properties of Procion Scarlet MX-G-(spacer)-bead cellulose have been successfully exploited for the purification of LDH from muscle extract without prepurification as demonstrated in Table IV. After centrifugation of the extract (specific activity of LDH: 5.8 units/mg protein) the clear supernatant was loaded onto a column containing dye-liganded cellulose. Then the column was rinsed with buffer B containing 1 *M* KCl to wash off non-adsorbed and non-specifically bound protein, followed by a washing step with buffer B to remove the high salt concentration. After adding NAD^+/sulphite to the elution buffer, the enzyme was desorbed in 90% yield.

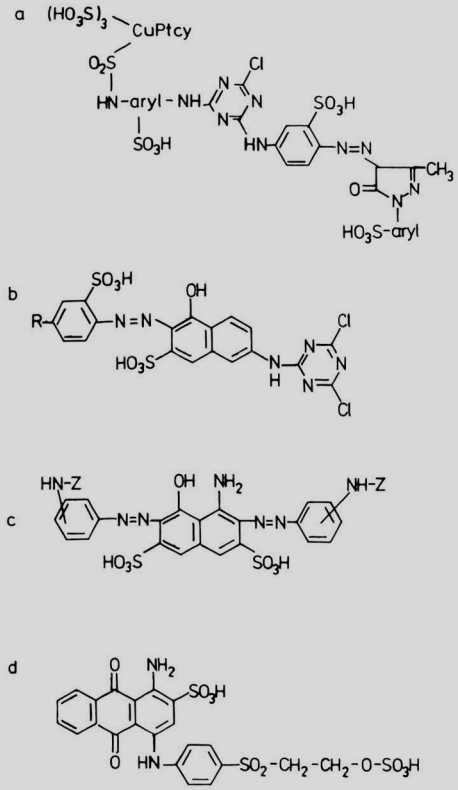

Fig. 2. Structures of reactive dyes. (a) Procion Green H-4G (Ptcy = phthalocyanine moiety); (b) Procion Orange MX-G (R = H) and Procion Scarlet MX-G (R = OCH_3); (c) Procion Navy H-ER (Z = halogenoheterocyclic reactive group[3]; (d) Remazol Brillant Blue R. Beside the well known structures of Cibacron Blue F3G-A and Procion Red HE-3B[2], the structures of other dyes used have not yet been published.

TABLE IV

PURIFICATION OF LACTATE DEHYDROGENASE FROM HEART MUSCLE BY APPLYING PROCION SCARLET MX-G-(DIAMINOHEXYL)-BEAD CELLULOSE

Experimental conditions: extract obtained from 30 g of minced muscle was centrifuged (9000 *g*, 30 min) and the supernatant was loaded onto a column (20 cm × 2 cm) packed with 14 g of dye–cellulose. After washing with buffer B containing 1 *M* KCl, and buffer B without KCl, the enzyme was desorbed by NAD^+/sulphite in buffer B. The purification is relative to the specific activity of LDH in the extract of heart muscle and in the main fraction from dye–cellulose chromatography.

Step	*Volume (ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Extract	47	1700	5.8	100	1
Dye–cellulose	14	891	200	52	34
Ultrafiltration	1.5	750	210	44	36

The main fractions contained 52% of the bound activity of LDH with a specific activity of 200 units/mg protein. In the course of this step the activity of MDH decreased from 250 to about 50% relative to the activity of LDH. The binding capacity of Procion Scarlet MX-G-(diaminohexyl)-bead cellulose for LDH from a crude muscle extract was determined to be 100–120 units/g wet weight. Starting this chromatography with prepurified LDH (see Methods), an enzyme with a specific activity of 300–400 units/mg and a contaminating activity of 0.1% MDH (relative to the activity of LDH) results.

In Fig. 3 the protein patterns before and after the dye chromatography starting with prepurified LDH (A) and with a crude muscle extract (B) have been monitored by sodium dodecyl sulphate (SDS)-polyacrylamide gel elctrophoresis. Beside the main band of LDH, a more rapidly migrating faint band appeared but to different extents in the two procedures. This is related to the difference in the specific activities of the two preparations.

The results of this study revealed that the common property of diverse triazine dyes immobilized on bead cellulose to bind LDH is caused by several interactions.

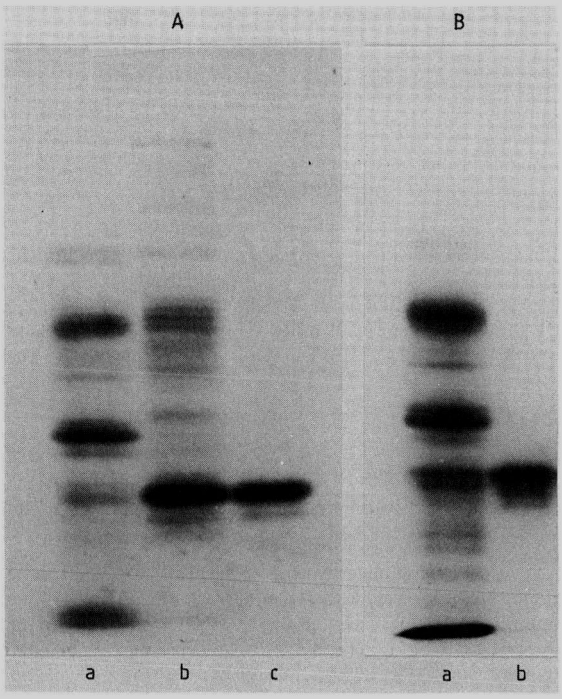

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of LDH samples at different stages of purification. (A) (a) Extract of heart muscle; (b) sample after DEAE-Sephadex chromatography (prepurified LDH); (c) sample after chromatography on Procion Scarlet MX-G-(diaminohexyl)-bead cellulose using prepurified LDH. (B) (a) Extract of heart muscle; (b) sample after chromatography on Procion Scarlet MX-G-(diaminohexyl)-bead cellulose using extract of heart muscle. Contents of protein and specific activity of LDH, respectively: (A) lane (a) 0.1 mg (4 units/mg), (b) 0.24 mg (50 units/mg) and (c) 0.02 mg (380 units/mg); (B) lane (a) 0.06 mg (6 units/mg), (b) 0.01 mg (210 units/mg). The electrophoresis was performed according to Neville[13].

Beside binding forces which are created specifically due to the nature of the dyes and certain structural domains of the enzyme, the influence of the spacer and of the matrix is evident. Work is in progress using structurally related dyes of Procion Scarlet MX-G to study the significance of the spacer for the specificity of the interaction with LDH and other enzymes.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF VARIANTS OF CHROMOSOMAL PROTEINS FROM PROKARYOTES

FRANÇOIS CHARTIER*, GILLES CREVEL, BERNARD LAINE and PIERRE SAUTIERE
URA 409 CNRS, Institut de Recherches sur le Cancer de Lille, Place de Verdun, 59045 Lille (France)
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SUMMARY

The separation of variants of chromosomal proteins exhibiting closely related amino acid compositions has been achieved using weak cation-exchange or reversed-phase high-performance liquid chromatography. The purity of the isolated proteins has been ascertained by polyacrylamide gel electrophoresis and in several cases by micro-sequencing.

INTRODUCTION

In the nucleoid of prokaryotes, acid-soluble proteins of molecular weights (M_r) ranging from 7000 to 16 000 daltons were shown to be involved in DNA condensation[1]. In several cases, these proteins are heterodimers constituted of polypeptides of closely related amino acid sequences which are referred to as variants. As an example, the number of sequence differences between variants of the DNA-binding protein II ($M_r \approx 10\,000$ daltons) ranges from only one residue in *Thermoplasma acidophilum*[2] to 27 residues in *Escherichia coli*[3]. Moreover, most of the observed changes are conservative. Highly resolving chromatographic methods are therefore required to separate variants of chromosomal proteins with a view to their characterization by amino acid composition and sequence analyses. Ion-exchange and reversed-phase high-performance liquid chromatography (HPLC) have been shown to be suitable for resolving complex mixtures of basic proteins differing in molecular mass, charge and hydrophobicity such as ribosomal proteins[4–8]. This paper deals with the application of HPLC to the separation of variants of eubacterial and archaebacterial chromosomal proteins.

MATERIALS AND METHODS

HPLC-grade acetonitrile was obtained from Carlo Erba and sequanal grade trifluoroacetic acid (TFA) from Pierce. Water for HPLC was provided by an Elgastat UHQ water apparatus. All other chemicals were proanalysis grade. Saline buffers were filtered through a 0.22-μm Millipore filter.

HPLC separations were performed with Beckman equipment consisting of

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a pair of Model 126 Altex pumps and a Model 167 variable wavelength detector controlled by a Tandon computer.

Preparation of chromosomal proteins

Proteins were prepared by affinity chromatography on a DNA-cellulose column as described in ref. 9 for eubacterial DNA-binding protein II and as in ref. 10 for the protein MC1 from the archaebacterium *Methanothrix soehngenii*. The eubacterial proteins eluted from the DNA-cellulose column were desalted on a Sephadex G-25 column equilibrated and eluted in 10 m*M* hydrochloric acid, and freeze-dried. The protein MC1 was dialysed against water and concentrated in a SpeedVac apparatus.

For cation-exchange chromatography, the proteins were dissolved in 200 μl of 10 m*M* sodium acetate buffer, pH 5.6 containing 8 *M* urea. The variants were separated on a TSK IEX CM-2SW column (250 mm × 4.6 mm) (Beckman) equilibrated in 10 m*M* sodium acetate buffer (pH 5.6) containing 6 *M* urea and eluted with a linear gradient of NaCl in the same buffer. Proteins were then desalted on a reversed-phase column as described in ref. 11.

Reversed-phase chromatography was performed on a Ultrapore C_8 column from Beckman (particle size 5 μm, pore size 300 Å, column size 250 mm × 4.6 mm). The proteins dissolved in 10 m*M* hydrochloric acid were loaded on the column equilibrated in 0.05% TFA in water and eluted with a gradient of acetonitrile in 0.05% TFA.

Analytical gel electrophoreses and amino acid analyses were performed as described in ref. 9.

RESULTS AND DISCUSSION

Cation-exchange HPLC

The separation by ion-exchange HPLC of the two variants 1 and 2 of the DNA-binding protein II from *E. coli* is shown in Fig. 1. In preliminary experiments performed at pH 7.0 with 10 m*M* sodium phosphate, the two variants were coeluted in a single fraction, whereas at pH 6.0 with 10 m*M* sodium phosphate or sodium acetate they were partially resolved (data not shown). The use of 10 m*M* sodium acetate at pH 5.6 gave the best resolution. The separation of these variants appears mainly dependent on the pH and, to a lesser extent, on the nature of the salt used as the buffer. Sequence analyses[3] indicate that the only difference in net charge between the two variants of this protein can be brought by the presence in variant 2 of one histidine residue. Since the pK' of the imidazole group is equal to 6.0, the protonation of the histidine residue probably plays an important role in the retention time of this protein. On the other hand, the presence of 6 *M* urea in the buffer is necessary to dissociate the two polypeptide chains which tightly bind together to form a stable dimer corresponding to the functional state of the protein[12].

The chromatogram obtained with the *Azotobacter vinelandii* DNA-binding protein II using the sodium acetate buffer at pH 5.6 is presented in Fig. 2. Variants 1 and 2 were eluted in fractions 1 and 2 respectively. By contrast with *E. coli* variants which are in equimolar amounts, the variant 1 is about twice as abundant as the variant 2 in *A. vinelandii*. The amino acid compositions of variants 1 and 2 present only slight differences except in the amounts of threonine and glutamic acid (Table I). The variants display the same total number of lysine plus arginine and differ by the presence of two histidine residues in variant 2.

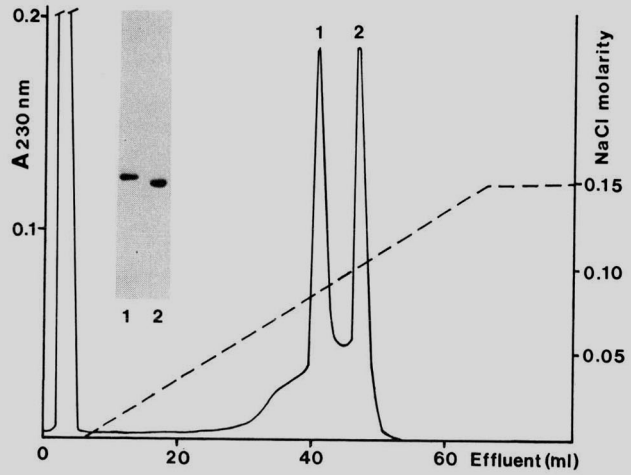

Fig. 1. Separation of the variants of the *E. coli* DNA-binding protein II (2 mg injected) on a TSK IEX CM-2SW column (250 mm × 4.6 mm). --- = Linear gradient of sodium chloride in 10 m*M* sodium acetate pH 6 containing 6 *M* urea; flow-rate 1 ml/min. Fractions of 1 ml were collected. Insert: polyacrylamide gel electrophoresis (PAGE) of variants 1 (lane 1) and 2 (lane 2) in 0.9 *M* acetic acid containing 6.25 *M* urea and 0.38% Triton X-100. Samples (3 μg) dissolved in 10 m*M* hydrochloric acid, 8 *M* urea, 0.5 *M* 2-mercaptoethanol were run at 22 mA for 3 h at room temperature in the gel containing 17% acrylamide. The gel was stained and destained according to ref. 14.

Reversed-phase HPLC

The DNA-binding protein II from *Synechococcus* PCC 7002 was desorbed from the DNA-cellulose column together with a contaminant protein of $M_r \approx 16000$ daltons. Using a C_8 Ultrapore column eluted with a linear gradient of acetonitrile in 0.05% TFA, the contaminant protein was eluted in fraction 1 whereas the DNA-

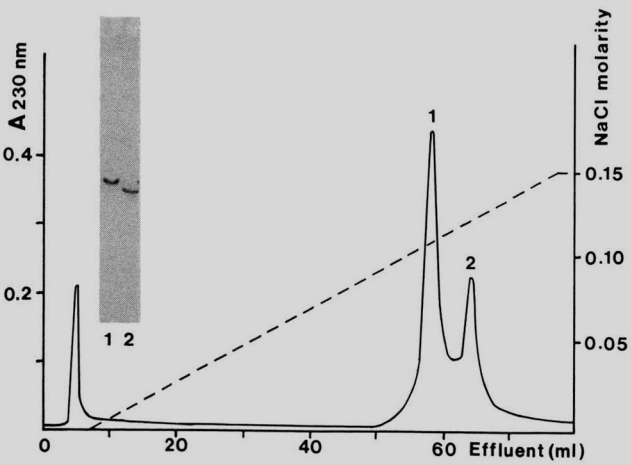

Fig. 2. Separation of the variants of the *A. vinelandii* DNA-binding protein II (2.5 mg injected) on a TSK IEX CM-2SW column (250 mm × 4.6 mm). The protein was chromatographed as indicated in Fig. 1. Insert: PAGE of variants 1 (lane 1) and 2 (lane 2) in 0.9 *M* acetic acid containing 2.5 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

TABLE I

AMINO ACID COMPOSITIONS OF VARIANTS OF DNA-BINDING PROTEIN II FROM EUBACTERIA

Results are expressed as the number of residues per mol of protein. Numbers in parentheses are the nearest integers. Values for *Synechococcus* 7002 variants are from ref. 15.

Amino acid	*A. vinelandii*		*Synechococcus 7002*	
	Variant 1	*Variant 2*	*Variant 1*	*Variant 2*
Asp	9.1 (9)	7.9 (8)	8	7
Thr	4.2 (4)	8.3 (8)	6	6
Ser	3.9 (4)	4.1 (4)	5	6
Glu	5.3 (5)	8.7 (9)	12	11
Pro	3.8 (4)	2.1 (2)	5	5
Gly	9.1 (9)	9.2 (9)	7	7
Ala	18.0 (18)	14.0 (14)	12	11
Val	8.0 (8)	6.3 (6)	9	8
Met	0.7 (1)	0.7 (1)	4	4
Ile	6.7 (7)	5.1 (5)	4	4
Leu	5.2 (5)	7.0 (7)	3	3
Phe	3.0 (3)	3.0 (3)	4	4
His	0 (0)	1.8 (2)	1	2
Lys	8.7 (9)	9.7 (10)	11	10
Arg	4.0 (4)	3.1 (3)	4	6
Total	90	91	94	95

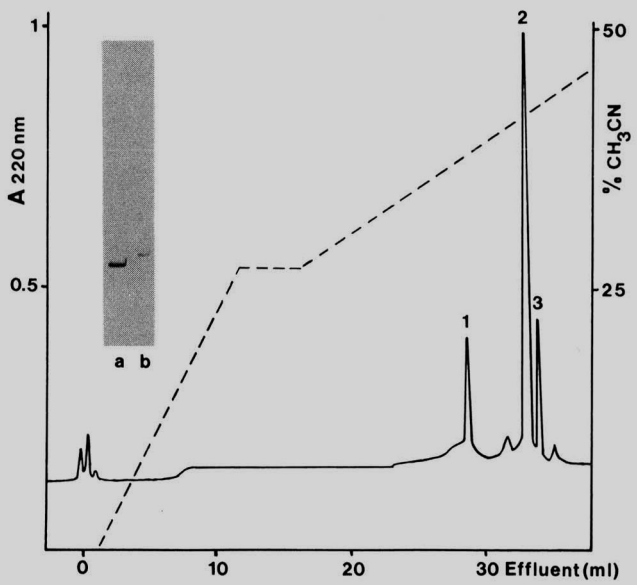

Fig. 3. Separation of variants of *Synechococcus* DNA-binding protein II (0.3 mg injected) on an Ultrapore C_8 column (250 mm × 4.6 mm). --- = Gradient of acetonitrile in 0.05% TFA, flow-rate 1 ml/min. Fractions of 0.5 ml were collected. Insert: PAGE of variants 1 (lane a) and 2 (lane b) in 0.9 *M* acetic acid containing 6.25 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

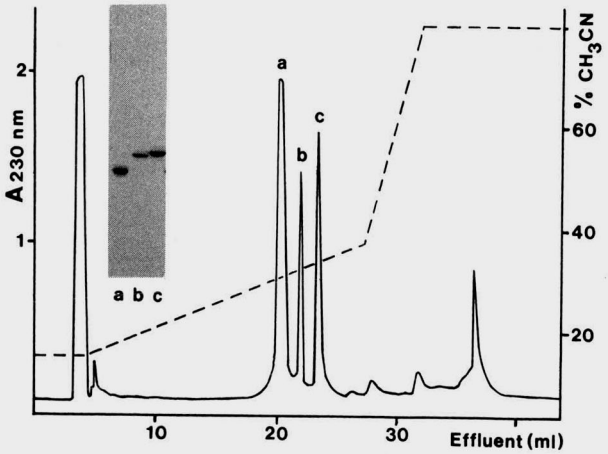

Fig. 4. Separation of variants of the protein MC1 from the archaebacterium *Methanothrix soehngenii* on an Ultrapore C_8 column (250 mm × 4.6 mm). The crude fraction eluted from the DNA-cellulose was concentrated in a Speedvac apparatus and injected. --- = Gradient of acetonitrile in 0.05% TFA; flow-rate 1 ml/min. Fractions of 0.5 ml were collected. Insert: PAGE of variants a (lane a), b (lane b) and c (lane c) in 0.9 *M* acetic acid containing 2.5 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

TABLE II

AMINO ACID COMPOSITIONS OF THE VARIANTS OF *Methanothrix soehngenii* PROTEIN MC1

Results are expressed as mol per 100 mol. n.d. = Not determined.

Amino acid	*MC1a*	*MC1b*	*MC1c*
Asp	7.2	8.7	9.1
Thr[a]	3.5	3.6	2.6
Ser[a]	0.0	2.3	2.5
Glu	10.6	12.3	13.0
Pro	6.0	6.0	6.6
Gly	9.2	9.4	8.9
Ala	10.4	11.8	8.5
Cys	0.0	0.0	0.0
Val	7.1	6.0[b]	5.8
Met	1.1	2.1	2.2
Ile	6.8	5.9[b]	7.4
Leu	4.9	5.0	5.9
Tyr	0.0	1.1	0.0
Phe	3.5	3.5	4.4
His	1.9	1.2	1.1
Lys	18.5	12.6	13.3
Arg	9.3	8.5	8.7
Trp	n.d.	n.d.	n.d.
Total	100.0	100.0	100.0

[a] Values obtained by linear extrapolation to zero hydrolysis time.
[b] 72-h Hydrolysis values.

binding protein II was resolved in two variants obtained in fractions 2 and 3 (Fig. 3). The high resolution of the column is demonstrated by the separation of these variants which exhibit similar numbers of amino acid residues with aromatic or bulky aliphatic side chains (Table I)[13].

The chromosomal protein MC1 isolated from the archaebacterium *Methanothrix soehngenii* is a complex constituted of three variants named a, b and c which were eluted from the Ultrapore C_8 column in fractions 1, 2 and 3 respectively (Fig. 4). The three variants have about the same molecular size ($M_r \approx 11\,000$ daltons). From their electrophoretic migration in acid–urea polyacrylamide gel (see insert, Fig. 4), variants b and c have a similar charge whereas variant a is more basic. The three variants were separated in a single step according to their hydrophobicities (Table II). Microsequence analyses of the three polypeptides clearly show that they are obtained in pure form and are structurally related to each other, which indicates that they represent variants of the same protein. Taking into account the low amount of protein available and the complexity of the DNA-cellulose fraction from which the protein MC1 variants were prepared, purification of the three variants in a single step shows the high efficiency of the method.

CONCLUSIONS

Variants of chromosomal proteins isolated from different prokaryotic organisms have been separated by ion-exchange or reversed-phase HPLC. The results demonstrate the efficiency of these methodologies for the separation of variants exhibiting only slight differences in amino acid compositions within each bacterial strain.

Previous chromatography of *E. coli* DNA-binding protein II on carboxymethyl-cellulose gave similar results[3] but required a much longer time (1 week *versus* 2 h) and six times as much mobile phase as does HPLC. Moreover, the sensitivity of HPLC is suitable to identify variants when low amounts of proteins are available and/or when the stoichiometry of one out of the variants is very low.

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CHROM. 21 188

SEPARATION AND MOLECULAR WEIGHT DETERMINATION OF PROSTAGLANDIN B_1 OLIGOMERS BY SIZE-EXCLUSION CHROMATOGRAPHY

I. MARTIN* and Ü. LILLE

Laboratory of Prostanoid Chemistry, Institute of Chemistry, Estonian Academy of Sciences, Akadeemia tee 15, 200108 Tallinn (U.S.S.R.)

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SUMMARY

An efficient size-exclusion chromatographic method for the simultaneous separation and molecular weight (MW) determination of prostaglandin (PG) oligomers on Sephadex G-50 with borate buffer is described. The prostaglandins 15-keto-PGB_1 and 16,16-dimethyl-15-keto-PGB_1 were used for the synthesis of oligomers. MW determinations in the monomer to octamer range is based on the linear correlation between the partition coefficient and log (MW) of the oligomers. The essential role of the borate anion between the gel matrix and the oligomers is demonstrated.

INTRODUCTION

The polymeric prostaglandins PGB_x with a molecular weight (MW) of 2200–2500 (6–7 monomeric units), synthesized from 15-keto-PGB_1, has been shown to maintain oxidative phosphorylation during hypotonic degradation in aged mitochondria isolated from rat liver[1,2]. It also stimulates the release of Ca^{2+} from the fragmented sarcoplasmic reticulum and heart mitochondria. The observed ionophoric activity of PGB_x is approximately two orders of magnitude greater than that of other monomeric prostaglandins[3]. The complexity of an oligomeric mixture due to several reactive sites in the 15-keto-PGB_1 molecule led to the synthesis of 16,16-dimethyl-15-keto-PGB_1 oligomers. Oligomers of the 16,16-dimethyl analogue of 15-keto-PGB_1 also exhibit protection from the loss of oxidative phosphorylation in isolated rat liver mitochondria[4] and the Ca^{2+} ionophoric activity[5].

The separation of physiologically active oligomers from a crude polymerization mixture by size-exclusion chromatography on Sephadex LH-20 using methanol as an eluent has been described[2,6]. However, the quality of the separation on Sephadex LH-20 and/or LH-60 with methanol is not satisfactory owing to the absence of well defined peaks on the elution curve. As a result of the poor resolution, vapour pressure osmometric measurements were required in order to determine the average MW in each of the collected fractions. The first attempts to determine the MW of

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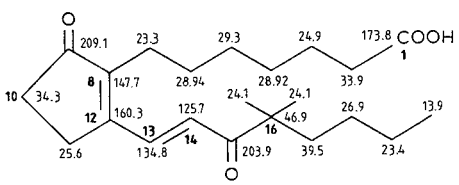
prostaglandin oligomers by gel chromatography have been reported previously[7]. In this paper, we describe a method of size-exclusion chromatography on Sephadex G-50 which gives an excellent resolution and permits the MW determination of prostaglandin B_1 (PGB_1) oligomers synthesized from 15-keto-PGB_1 and 16,16-dimethyl-15-keto-PGB_1.

EXPERIMENTAL

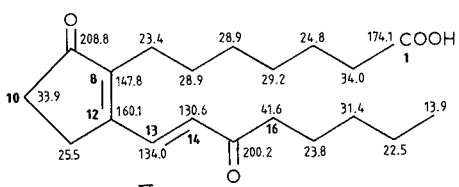
Synthesis of oligomers

Prostaglandins I and II (Fig.1) were synthesized starting from ethyl 5-oxo-1-cyclopentene-1-heptanoate[8]. The Michael addition of nitromethane to the enone unit followed by Nef reaction[9] and Wittig–Horner reaction with dimethyl 2-oxoalkylphosphonate gave the 15-ketoprostaglandins. Introduction of a double bond into the cyclopentane ring system was accomplished with copper(II) bromide according to Miller *et al.*[10]. The ^{13}C NMR chemical shifts in ppm downfield from internal tetramethylsilane standard are given in Fig. 1. The chemical shifts for II are consistent with the literature[11,12]. Assignments in I are based on the chemical shifts in II and multiplicities of the carbon-13 signals in monoresonance spectra.

Preparation and purification of dimers were accomplished according to Polis *et al.*[11]. Dimers are formed by base-catalysed Michael addition in which two nucleophilic (C-10, C-16) and two acceptor (C-13, C-14) sites of II[6] and one nucleophilic (C-10) and two acceptor (C-13, C-14) sites of I are active[5]. The presence of multiple reaction sites coupled with the formation of two new chiral centres for each new bond formed result in the formation of a complicated mixture of structural isomers further complicated by the presence of closely related stereoisomers[13]. The UV spectra of dimers of I and II showed two absorption peaks with maxima at 296 and 238 nm, observed to be the absorption maxima of the monomers (296 nm) and of the cyclopentenone unit (238 nm) formed by the disappearance of the 13,14-double bond in the Michael addition reaction[11]. Secondary ion mass spectrometric (SIMS) measurements of I dimer with glycerine, glycerine–NaCl and glycerine–$NaHCO_3$ liquid matrixes were carried out[14].



I



II

Fig. 1. Monomeric prostaglandins 16,16-dimethyl-15-keto-PGB_1 (I) and 15-keto-PGB_1 (II) as precursors of corresponding oligomers. ^{13}C NMR chemical shifts in ppm downfield from internal TMS are shown.

The abundant ions at *m/z* 725 (M + H), 747 (M + Na), 769 (M + 2Na − H) and 791 (M + 3Na − 2H) show that the I dimer with three acidic hydrogens (two carboxylic and one presumably at C-10) was obtained.

The treatment of 16,16-dimethyl-15-keto-PGB_1 (I) and/or 15-keto-PGB_1 (II) with 1 *M* 50% ethanolic KOH (2.5 mg/ml PG) for 30 min at 20°C gave a crude reaction product. After neutralization with 3 *M* HCl, the oligomers were extracted with ethyl acetate. The organic layer was washed with saturated KCl solution and oligomers were extracted into 50 m*M* borate buffer (pH 8.9). The final concentration of oligomers was about 5 mg/ml. The remaining trace amounts of ethyl acetate were removed by vacuum evaporation. The dimeric and monomeric prostaglandins were also dissolved in 50 m*M* borate buffer (pH 8.9) at a concentration of 5 mg/ml.

Chromatographic materials and equipment

Sephadex G-25, G-50, G-75, LH-20 and LH-60 gels were purchased from Pharmacia and dextran blue from Fluka. Methanol, KH_2PO_4, $KHCO_3$, H_3BO_3, KOH and KCl were obtained from Reachim; methanol was distilled before use. Doubly distilled water was used to prepare the buffer solutions. An LKB gel filtration apparatus including a Varioperpex peristaltic pump, a two-channel Uvicord III ultraviolet detector, a RediRac fraction collector, a two-channel 2210 recorder and 150 × 2.8 cm I.D. glass columns with a volume of 920 ml were used.

Chromatographic conditions

Sephadex G-25, G-50 and G-75 columns were eluted with 50 m*M* borate, 50 m*M* carbonate or 50 m*M* phosphate buffer solutions at pH 8.9, 9.0 and 8.0, respectively. Methanol was used as the eluent with Sephadex LH-20 and LH-60 columns. The sample size did not exceed 6 ml. The flow-rate was adjusted to 40 ml/h. The time interval between fractions was 6 min. The elution process was monitored by a UV detector simultaneously at 254 and 206 nm. Elution volumes (V_e) were calculated from the printed-out retention times. The void volumes (V_0) were determined by using dextran blue. The partition coefficients (K_{av}) were calculated using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_t is the total volume of the chromatographic bed[15].

RESULTS AND DISCUSSION

The elution curves of 15-keto-PGB_1 oligomers on Sephadex LH-type columns are presented in Fig. 2. The appearance of several maxima on the elution curve obtained with LH-60 instead of LH-20 suggest some improvement in resolution. Further investigations on Sephadex G-50 demonstrated that the fractions collected from the main peak on the LH-60 elution curve contain oligomers from the dimer to the hexamer.

In comparison with other Sephadex G-type media, the best results were obtained using a Sephadex G-50 column and 50 m*M* borate buffer at pH 8.9 as the eluent (Fig. 3). The distribution coefficients, K_{av}, for monomers and dimers were measured by eluting them separately. They both gave a single peak and the K_{av} values for 15-keto-PGB_1 and its dimer were 0.655 and 0.47 and for 16,16-dimethyl-15-keto-PGB_1 and its dimer 0.735 and 0.51, respectively. The K_{av} values were plotted against log (MW), assuming that the third, fourth and fifth peaks on the elution curve

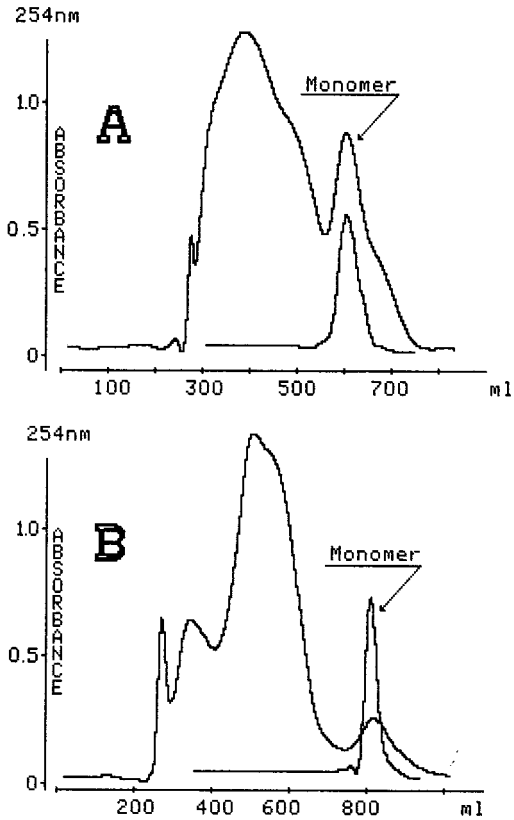


Fig. 2. Chromatography of 15-keto-PGB_1 oligomers on Sephadex LH-20 (A) and LH-60 (B) with methanol as eluent.

correspond to the trimer, tetramer and pentamer, respectively. The correlation coefficient, R, and the linear regression coefficients, k_1 and k_2, were obtained by least-squares fitting. Their values in the equation $K_{av} = k_1 - k_2 \log (MW)$ are presented in Fig. 4. A linear correlation between K_{av} and log (MW) with correlation coefficient $R = 0.999$ confirms that the third peak corresponds to the trimer, the fourth peak to the tetramer, etc. This permits the simultaneous separation and MW determination of oligomers. MW determinations up to the octamer are possible after extrapolation of the linear relationship between K_{av} and log (MW).

The resolution on Sephadex G-50 was strongly affected when borate buffer was replaced with sodium phosphate buffer (*cf.*, Figs. 3 and 5). Similar results were obtained by using carbonate buffer. This suggests that the borate anion plays a critical role in the separation process. We propose that, under our conditions, complexation of the borate and Sephadex occurs. There is other evidence that the borate anions give complexes with dextrans as it gives rise to undesirable phenomena in the traditional gel filtration of proteins[16]. Interestingly, the addition of 0.2 *M* NaCl to the borate buffer in an effort to increase the ionic strength leads to the disappearance of individual peaks on the elution curve (Fig. 6). The good linear correlation ($R = 0.999$) between K_{av} and

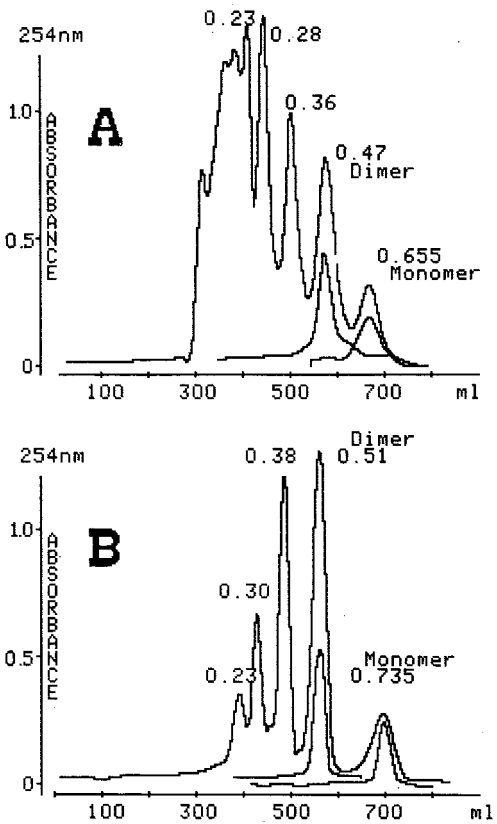


Fig. 3. Chromatography of 15-keto-PGB_1 oligomers (A) and 16,16-dimethyl-15-keto-PGB_1 oligomers (B) on Sephadex G-50 with 50 m*M* borate buffer (pH 8.9) as eluent. Numbers at the peaks are K_{av} values.

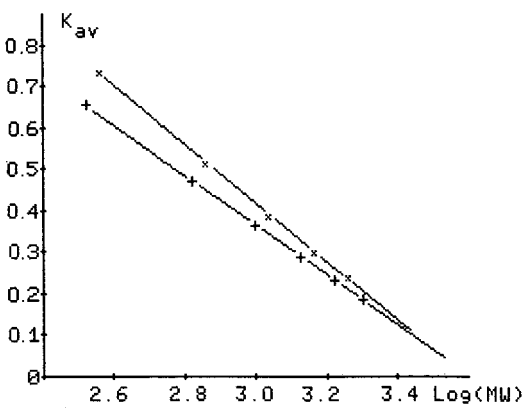


Fig. 4. Relationship between partition coefficient (K_{av}) and molecular weight (MW) of 15-keto-PGB_1 (+) and 16,16-dimethyl-15-keto-PGB_1 (×) oligomers on Sephadex G-50 with 50 m*M* borate buffer (pH 8.9) as eluent. Coefficients k_1 and k_2 in the equation $K_{av} = k_1 - k_2 \log(MW)$ were obtained by least-squares fitting. ×: $k_1 = 2.58 \pm 0.03$; $k_2 = 0.72 \pm 0.01$; $r = 0.9997$. +: $k_1 = 2.20 \pm 0.02$; $k_2 = 0.61 \pm 0.01$; $r = 0.9998$.

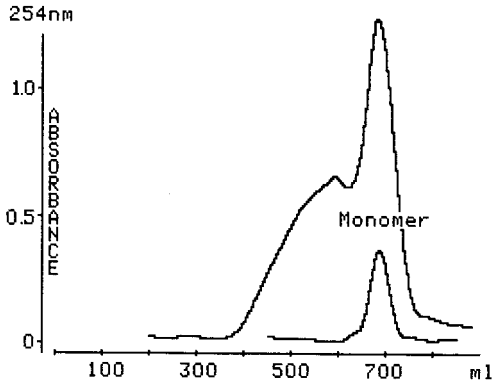


Fig. 5. Chromatography of 15-keto-PGB_1 oligomeric mixture on Sephadex G-50 with 50 m*M* phosphate buffer (pH 8.0) as eluent.

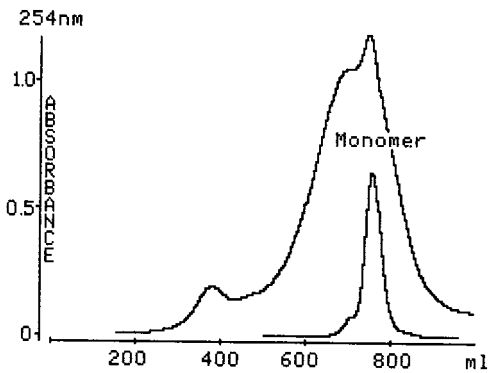


Fig. 6. Chromatography of 15-keto-PGB_1 oligomers on Sephadex G-50 with 50 m*M* borate buffer–0.2 *M* NaCl (pH 8.9) as eluent.

log (MW) suggests that only the molecular sieving process take place with borate buffer. Replacing the buffer anion or adding NaCl results in a decrease in resolution and therefore we conclude that borate buffer with a low molarity is required to prevent secondary, perhaps hydrophobic, interactions between the prostaglandin oligomers and the gel matrix.

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CHROM. 21 168

PYROLYSIS GAS–LIQUID CHROMATOGRAPHY OF N,N-DIMETHYLALKYLAMINE N-OXIDES AND THEIR MIXTURES

FERDINAND DEVÍNSKY*
Department of Inorganic and Organic Chemistry, Laboratory of Bioorganic Chemistry, Faculty of Pharmacy, Kalinčiakova 8, 832 32 Bratislava (Czechoslovakia)
and
JOHN W. GORROD
Chelsea Department of Pharmacy, King's College London (KQC), University of London, Manresa Road, London SW3 6LX (U.K.)
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SUMMARY

A rapid quantitative and small scale method based on direct injection pyrolysis gas–liquid chromatography is described for the determination of N,N-dimethylalkylamine N-oxides. The method is suitable for determination of individual N-oxides as well as of their homologous compositions with or without the presence of parent tertiary amines in water or methanol solutions. The sensitivity of the method is 10–15 nmol of compounds injected. The unsymmetrical (1-methyldodecyl)dimethylamine N-oxide forms two isomeric alkenes 1-tridecene and *trans*-2-tridecene in a ratio of 1.68, in agreement with the predicted value.

INTRODUCTION

Non-aromatic amine oxide surfactants have wide technological and basic scientific applications in physical and biological systems[1,2]. A rapid and quantitative small scale analytical method is required for determination of these compounds not only as individual substrates but often in mixtures containing two or more members of homologous series and/or of the parent N,N-dimethylalkylamines.

Such a method is essential for determining small amounts of amine oxides from an environmental point of view, as well as in studies of their biological activity or their biological formation, *e.g.*, in *in vitro* tests, as the results obtained are clearly dependent upon the purity of the substrates used. Since amine oxides are usually prepared by oxidation of the corresponding tertiary amines, they can contain them as impurities.

Analytical methods used to date for determination of individual amine oxides and their homologous mixtures are summarized in a recent review[3]. Some of these methods are tedious and ill suited to small scale analysis, because of low sensitivity (titration methods) or require relative large amounts of compound (IR, ^{1}H NMR) or

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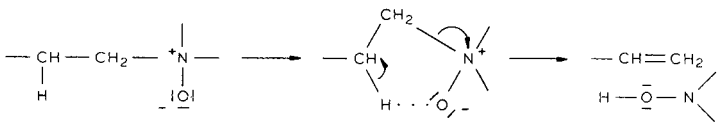
are unable to detect individual members in mixtures of homologues (reductometric methods and methods using ^{18}O-labelling).

The heat sensitivity of non-aromatic amine oxides make them suitable for pyrolysis gas–liquid chromatography (GLC) which is a method sensitive enough to determine small amounts of compounds and at the same time separate the reaction products (alkenes) from the parent amines. This method is, however, limited to amine oxides possessing at least one β-hydrogen and is not suitable for N-methyl six-membered heterocycloalkylamine N-oxides[4].

It is well know, *e.g.*, refs. 4 and 5 that non-aromatic tertiary amine oxides containing one or more β-hydrogens undergo cleavage upon heating to produce an alkene and N,N-dialkylhydroxylamine (Cope reaction). The reaction is a stereoselective *syn* process and a five-membered E_i mechanism operates:



The reaction requires mild conditions (100–150°C), however the conversion of alkylamine N-oxide into alkene at these temperatures is usually not quantitative[6]. Solvents play a decisive rôle: with tetrahydrofuran or dimethyl sulphoxide the reaction can proceed even at room temperature[7]; hydroxylic solvents capable of hydrogen bonding, such as alcohols and water, stabilize the amine oxide.

To overcome these difficulties, different techniques have been devised, *e.g.*, use of a separate pyrolytic unit attached to the inlet port of a gas chromatograph and heated to 650°C[8] or direct injection with injector temperatures of 220[9], 300[10] or 150–340°C[11], or use of ionic exchangers before GLC analysis[12].

In this communication we present the results of direct injection pyrolysis GLC of an homologous series of N,N-dimethylalkylamine N-oxides (alkyl: hexyl to hexadecyl); mixtures of amine oxides and in the presence of parent N,N-dimethylalkylamines.

EXPERIMENTAL

Materials

N,N-Dimethylalkylamines, $C_nH_{2n+1}N(CH_3)_2$ (n = 6, 8, 10, 12, 14 or 16) and $C_{10}H_{21}CH[N(CH_3)_2]CH_3$ (2-ATD), were prepared from doubly distilled 1-alkylamines and (1-methyldodecyl) amine by reductive methylation using formic acid and formaldehyde[13]. The resulting tertiary amines were purified by double distillation from sodium wire under nitrogen using a 30-cm Vigreaux column and stored in the dark in sealed ampoules under a nitrogen atmosphere.

The corresponding N-oxides were prepared by a method described earlier[14] using a 30% aqueous solution of hydrogen peroxide in 10% molar excess. After azeotropic drying, the hygroscopic compounds were crystallized four to six times from dry acetone until pure according to thin-layer chromatography[15] (TLC), dried and stored over P_4O_{10} *in vacuo*. 1-Dodecylpyrrolidine N-oxide, 1-dodecylpiperidine N-oxide, 4-dodecylmorpholine N-oxide and 1-dodecylperhydroazepine N-oxide were prepared in the same way.

1-Alkenes were obtained as follows: 1-hexene, 1-octene, 1-dodecene from Aldrich (U.K.); 1-decene, 1-tetradecene, 1-hexadecene from Lancaster Synthesis (U.K.); *trans*-2-octene from Sigma (U.K.). Organic solvents used were of high-performance liquid chromatographic (HPLC) purity (Fison's, U.K.).

Methods

GLC was carried out on a Perkin-Elmer Model F33 gas chromatograph equipped with flame ionization detection. The precoiled glass column (1 m × 3 mm I.D.) was packed with 8% Apiezon L on Chromosorb G AW DMCS (80–100 mesh) and 10% potassium hydroxide, conditioned for 48 h before use. The part of the column inserted into the injection port (*ca.* 7 cm) was packed with non-treated support material. Gas pressures were set as follows (kN/m^2): air 126, hydrogen 140, nitrogen (carrier gas) 175, except for 1-hexene (and consequently also for N,N-dimethylhexylamine N-oxide) which was 35 kN/m^2. The injection port temperatures were 200, 250, 300, 375 or 450°C. For the final analysis it was maintained at 450°C. The column temperature was programmed from 50 to 220°C at 4°C/min with an initial hold of 10 min. The samples (1 ml of different concentrations in methanol or water) were directly injected into the gas chromatograph. The peak areas were determined by a standard integration method. Relative retention times (RRTs) were calculated according to ref. 8 and are based on a time of 100 for 1-decene and a time of zero for the injection of the sample.

RESULTS AND DISCUSSION

The estimated lowest amounts required for quantitative analysis according to the sensitivity of the method (under the described experimental conditions) were 10–15 nmol of the injected compounds. The detectable amounts were about 5 nmol, however, these concentrations were useful only for qualitative purposes. For the lower members of the homologous series (n = 6 and 8), *i.e.*, tertiary amines and 1-alkenes formed by pyrolysis of the parent amine oxides, the peaks were very close to the solvent front when using methanol. In the case of aqueous solutions there was no interaction with the solvent.

Table I summarizes the retention times and relative retention times for 1-alkenes formed by pyrolysis of the parent amine oxides and N,N-dimethylalkylamines. Included are also the results for the *trans*-2-octene standard and the 1- and *trans*-2-tridecene pyrolysis products. The tridecenes were formed from (1-methyldodecyl) dimethylamine oxide in 62.7% (1-tridecene, t_R = 71 min) and 37.3% (*trans*-2-tridecene, t_R = 64 min) yield, respectively, and were well separated at 95°C. At 150°C the two peaks collapse to one symmetrical peak with a retention time of 8.0 min.

Thermal degradation of N,N-dimethylalkylamine oxides with a straight chain alkyl substituent gives 1-alkenes and N,N-dimethylhydroxylamine. Though it is generally believed that this reaction requires mild conditions (100–150°C), it has been previously shown[6] that at 100°C the pyrolysis of N,N-dimethyldodecylamine oxide yields only 11–21% of 1-dodecene and 52–53% of deoxygenated product (N,N-dimethyldodecylamine). Increasing the temperature to 120°C improved the yield of 1-dodecene to 86.5%, however, still *ca.* 12% of tertiary amine was formed. Our results (Table II) shows that the quantitative pyrolysis of N,N-dimethylalkylamine

TABLE I

RETENTION TIMES, t_R, AND RELATIVE RETENTION TIMES (RRT) OF 1-ALKENES C_mH_{2m} (A), FORMED BY PYROLYSIS OF N,N-DIMETHYLALKYLAMINE N-OXIDES AND OF N,N-DIMETHYLALKYLAMINES $C_nH_{2n+1}N(CH_3)_2$ (B)[a]

A			*B*		
m	t_R *(min)*	*RRT*	*n*	t_R *(min)*	*RRT*
6[b]	2.8	11	6	9.1	34
8[b]	16.0	60	8	21.4	81
8[c]	21.0	–	–	–	–
10	26.6	100	10	30.8	116
12	35.4	133	12	38.8	146
14	42.6	160	14	45.9	173
16	49.3	185	16	58.5	221
13[d]	64.0 (62.7%)	–	2-ATD[e]	5.8	–
13[f]	71.0 (37.3%)	–	–	–	–

[a] Injection port temperature 450°C, column temperature programmed from 50 to 220°C at 4°C/min with an initial hold of 10 min.

[b] Nitrogen pressure = 35 kN/m^2.

[c] *trans*-2-Octene standard; conditions same as in footnote *b*.

[d] 1-Tridecene (oven temperature 95°C).

[e] (1-Methyldodecyl)dimethylamine (oven temperature 210°C).

[f] *trans*-2-Tridecene (oven temperature 95°C).

oxides to 1-alkenes requires temperatures well above 250°C. This finding is not surprising in the light of the high sensitivity of aliphatic amine oxides to some metal ions which catalyse their deoxygenation to tertiary amines. In contact with the metal surface of the injection port at relatively low temperatures, the amine oxides can easily be reduced to their parent tertiary amines.

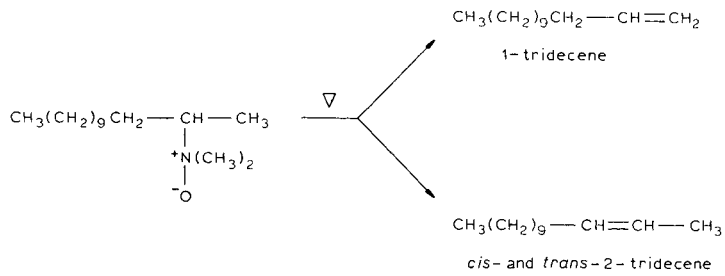
TABLE II

INJECTION PORT TEMPERATURE DEPENDENT DEOXYGENATION OF N,N-DIMETHYLALKYLAMINE N-OXIDES, $C_mH_{2m+1}N(O)(CH_3)_2$, TO N,N-DIMETHYLALKYLAMINES

For conditions see Experimental. Values are percentages.

m	*Temperature (°C)*				
	200	*250*	*300*	*375*	*450*
6	1.2	0.2	0.1	0[a]	0
8	1.2	0.4	0.1	0	0
10	1.0	0.6	0.2	0	0
12	1.6	0.5	0.2	0.02	0
14	1.8	0.7	0.4	0	0
16	1.3	0.4	0.2	0	0
2-ATDNO	1.5	0.5	0.3	0.02	0

[a] 0 represents less than 0.02% conversion to the amine.



We included in our experiments an unsymmetrical long chain amine oxide (1-methyldodecyl)dimethylamine oxide (2-ATDNO) to study the formation of different isomeric alkenes. Using high column temperatures (210°C) for rapid analysis of mixtures containing this compound, we observed a sharp and symmetrical peak of non-resolved alkenes. Because of the possibility that this amine oxide forms different reaction products we carried out a more detailed analysis of the pyrolysis of 2-ATDNO.

The reaction theoretically follows Hofmann's rule and the main product is expected to be a 1-alkene. In reality this is the case; 1-tridecene was formed in 62.7% yield, other isomers giving an total yield of 37.3%. From the generally accepted mechanism of amine oxide pyrolysis (five-centre *cis* intramolecular elimination) the *cis* isomer is more likely to occur. The orientation of the double bond is statistical and is determined by the number of β-hydrogens available. However, steric effects in long chain molecules play an important rôle and can influence the direction of elimination by the need to minimize steric interactions in the transition state or to relieve steric interactions in the ground state. In this case the Zaitsev product (2-alkene) should be formed. Our results (Table I) show that the orientation in pyrolytic elimination of 2-ATDNO is statistically, as well as sterically, dependent. The ratio of 1-alkene and 2-alkene formed (1.68) is very close to the 3:2 distribution predicted statistically using the number of β-hydrogens available.

The transition state requires that eclipsing of the 1,2-alkyl group be at a maximum in order to obtain the *cis*-olefin, while production of the *trans* isomer would involve only hydrogen–alkyl eclipsing. This effect may influence the course of the reaction and suggests that the effect is greater when one of the eclipsed groups is ethyl or larger[16]. In our case the eclipsing group is dodecyl which would indicate that the formation of *trans*-2-tridecene is preferred. In fact we did not detect any *cis*-olefin. These results are in agreement with the findings of Cope *et al.*[16] for N,N-dimethylisobutylamine N-oxide which upon pyrolysis yielded 67.3% 1-butene, 11.7% *cis*- and 21% *trans*-2-butene, and for (1-ethylpropyl)dimethylamine oxide which yielded 29% of *cis*- and 71% of *trans*-2-pentene, respectively.

Fig. 1 illustrates the separation of 1-alkenes (formed by pyrolysis of mixtures of N,N-dimethylalkylamine oxides) and different N,N-dimethylalkylamines. Samples (1 μl) containing equal amounts of N,N-dimethylalkylamine N-oxides and N,N-dimethylalkylamines in water were injected at an injection port temperature of 450°C. The samples were prepared by dissolving homologous amine oxides in water and adding tertiary amines to this solution so that the concentration of each compound in the final mixture injected was $1 \cdot 10^{-5}$ g. After sonication, clear solutions were obtained.

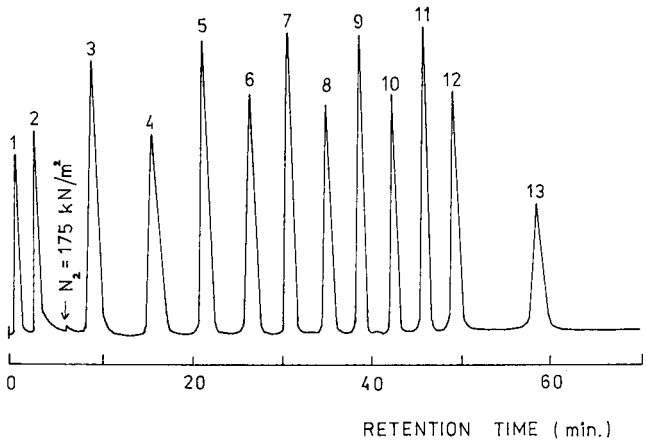


Fig. 1. Separation of pyrolysis products of N,N-dimethylalkylamine N-oxides (1-alkenes and N,N-dimethylhydroxylamine) and appropriate N,N-dimethylalkylamine standards. Conditions: injection port temperature 450°C; column temperature programmed from 50 to 220°C at 4°C/min with a 10-min initial hold; carrier gas, 175 kN/m^2 nitrogen; 1 μl of an aqueous solution containing à $1 \cdot 10^{-5}$ g of N,N-dimethylalkylamine N-oxides and N,N-dimethylalkylamines, respectively. Peak identities: 1 = N,N-dimethylhydroxylamine; 2 = 1-hexene; 3 = C_6dimethylamine; 4 = 1-octene; 5 = C_8dimethylamine; 6 = 1-decene; 7 = C_{10}dimethylamine; 8 = 1-dodecene; 9 = C_{12}dimethylamine; 10 = 1-tetradecene; 11 = C_{14}dimethylamine; 12 = 1-hexadecene; 13 = C_{16}dimethylamine.

2-ATD and 2-ATDNO were not included in this mixture. The retention times of the 1-alkenes formed were identical with those of standard compounds. The same results were obtained irrespective of whether aqueous or methanolic solutions were used.

The peak with t_R = 1 min was obtained for the product of pyrolysis of respective N-oxides and was comparable to that for standard N,N-dimethylhydroxylamine.

In the case of N-dodecylheterocycloalkylamine oxides (pyrrolidine, piperidine, morpholine, perhydroazepine), 1-dodecene was formed exclusively, clearly as the result of the stereochemistry of the pyrolyzed compounds.

CONCLUSIONS

The results indicate that pyrolysis GLC can be used for qualitative as well as quantitative determination of nanomolar quantities of individual as well as mixtures of homologous N,N-dimethylalkylamine oxides and in the presence of the parent tertiary amines. The method does not require, *e.g.*, a separate pyrolysis unit and is sensitive enough even for a complex mixture containing six amine oxides and six parent tertiary amines with an alkyl chain length variation from C_6 to C_{16}. Under the given experimental conditions no deoxygenation of amine oxides to tertiary amines occurs and 1-alkenes are formed quantitatively.

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DETERMINATION OF TRACE ANIONIC IMPURITIES IN CONCENTRATED INORGANIC ACIDS BY RECYCLE ION CHROMATOGRAPHY

MITSUNORI MURAYAMA*, MASAO SUZUKI and SHOJI TAKITANI
Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162 (Japan)
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SUMMARY

A simple, sensitive and rapid method for determination of trace anions (chloride, nitrite, bromide, nitrate and sulphate ion) in concentrated inorganic acids (hydrochloric, nitric, phosphoric and sulphuric) has been developed. Each sample is diluted 100–400-fold in water and neutralized with sodium hydroxide. The determination is made by recycle ion chromatography to separate an objective trace anion from the coexisting anion of the sample acid. The detection limit of each anion was 0.3–0.9 ppm in the sample acid.

INTRODUCTION

Almost all of the pure chemicals have to be analyzed for the contents of inorganic impurities. Metallic impurities are determined sensitively by atomic absorption spectrometry[1,2] and simultaneously by inductively coupled plasma–atomic emission spectrometry[3,4], but an universal determination method for anionic impurities is not available. Methods such as turbidimetry[5–7], spectrophotometry[8–10], ion-selective electrode potentiometry[11,12] and other electrochemical methods[13] have been developed for the determination of trace anions, but many of them can detect only one or a few anions, and in relatively clean samples.

Ion chromatography (IC), introduced by Small *et al.*[14], is one of the most effective methods to determine trace inorganic anions, owing to its high sensitivity, rapidity and ease of operation. Many anions are determined by IC simultaneously. However, because of the low capacity of the ion exchanger used as a packing (less than 0.1 mequiv./g), anions often cause overload, and peaks become broad at relatively low concentrations (100–1000 ppm). Thus it is difficult to determine trace anions in the presence of large amounts of other anions, as the peak of an objective anion is obscured by the broad peak due to overloading. More selective detectors, *e.g.*, UV absorbance[15,16], electrochemical[17,18] are available, but these cannot be used so widely for determinations of various anions.

On the other hand, recycle IC[19–21] is a convenient method to separate a small peak which is hidden by a large peak. This paper describes a simple, sensitive and rapid

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method for the determination of trace anions in concentrated inorganic acids by recycle IC.

EXPERIMENTAL

Reagents

Deionized water (Millipore RO-Q system) was used throughout the present study. A 1.0 *M* sodium hydroxide solution (normality factor 1.004–1.009) from Kanto was used. Other reagents and sample acids used were of reagent grade. Sample acids conformed to the Japan industrial standard (JIS)[22].

Recycle IC conditions

The flow diagram of the recycle ion chromatograph system A was the same as shown in our previous report[21]. The system was equipped with a sample loop (50 μl), an HPIC-TAC-1 concentrator column, an HPIC-AG4A guard column (50 mm × 4 mm), an HPIC-AS4A separator column (250 mm × 4 mm), an anion micro-membrane suppressor (AMMS) and a conductivity detector. The objective anion was collected on the concentrator column at the back of the detector.

Fig. 1 illustrates the recycle ion chromatograph system B. This system is equipped with a 1.5-ml loop (760 cm × 0.5 mm I.D.) instead of a concentrator column as in system A, and the collection point was between the separator and the suppressor.

Sample preparation

Each sample was diluted according to Table I and neutralized with 1.0 *M* sodium hydroxide. Hydrochloric acid and nitric acid were neutralized with an equimolar volume of sodium hydroxide, phosphoric acid and sulphuric acid required a double equimolar volume of sodium hydroxide to be neutralized.

Determination of bromide and nitrate ions

The prepared sample solution was subjected to the recycle system A. A fraction containing an objective anion was collected on a concentrator. This column and connection were washed with 2 ml of water. The concentrated anion was reinjected into the separator. Times to start and stop the collection were the same as the beginning

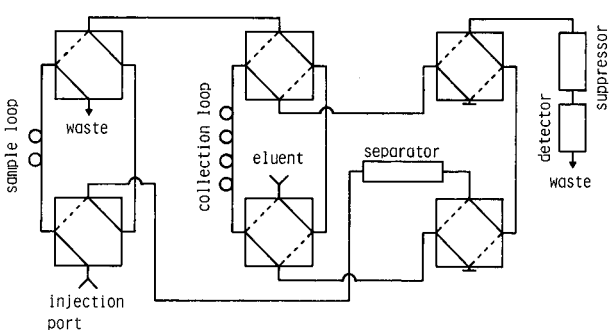

Fig. 1. Schematic diagram of recycle system B.

TABLE I

DILUTION RATIO OF SAMPLE ACIDS

Objective anion	*Dilution ratio (v/v)*			
	HCl	HNO_3	H_3PO_4	H_2SO_4
Chloride, nitrite	–	200	400	400
Bromide, nitrate	200	–	400	200
Sulphate	100	200	400	–

and ending of an objective anion peak. Standard solutions were used to construct the calibration graph and to define the collection time of an objective anion.

Determination of sulphate ion

Recycle system A was used as above. The system-peak height of sulphate was determined previously. The peak height of an objective sulphate ion was obtained by subtracting the system-peak height from the peak height of the sample.

Determination of chloride and nitrite ion

The prepared sample solution was subjected to recycle system B. A fraction containing an objective anion was collected in a 1.5-ml loop, then reinjected into the separator. Times to start and stop the collection were 12 s earlier than those of the beginning and ending of an objective anion peak.

RESULTS AND DISCUSSION

Eluent for recycle IC

Since the aim of this study was to determine many anions in various inorganic acids, the most suitable eluent for simultaneous separation of the main acid anions was selected. Table II shows the effect of the eluent composition on the retention times of

TABLE II

EFFECT OF THE ELUENT COMPOSITION ON THE RETENTION TIMES OF ANIONS

Eluent component (mM)			*Retention time (min)*			
NaOH	Na_2CO_3	$NaHCO_3$	*Chloride*	*Nitrate*	*Phosphate*	*Sulphate*
50	0	0	2.2	4.8	–[a]	8.6
30	0	0	2.7	6.4	–[a]	20.0
10	1	0	2.1	4.7	46.0	10.6
5	3	0	1.8	3.5	13.8	5.4
0	3	0	1.9	3.8	5.0	5.4
0	2	0.5	2.0	4.0	7.2	9.5
0	2	1	2.0	4.0	6.2	8.8
0	1	4	2.2	4.5	7.2	12.9

[a] Did not elute within 1 h.

chloride, nitrate, phosphate and sulphate ion. The eluent selected, 2.0 m*M* sodium carbonate–1.0 m*M* sodium bicarbonate, gave short retention times and high resolution. Increasing the concentration of this eluent decreased the retention time, but the resolution was lowered.

When using recycle system A, an objective anion was retained on the concentrator column together with anionic components of the eluent, and upon reinjection gave a peak together with system peaks. The sodium carbonate–bicarbonate eluent gave two system peaks comprising carbonate and sulphate[21]. The carbonate is likely to orginate from the eluent and sulphate from the scavenger that passed through the suppressor. We thought that a sodium hydroxide eluent would give a system peak only of sulphate (from scavenger). However, it was difficult to avoid contamination of volatile anionic compounds, and some system peaks were observed (mainly carbonate, chloride and nitrite). Furthermore, since 10 m*M* sulphuric acid was required to suppress the sodium ion in the sodium hydroxide eluent, the resulting system-peak height of sulphate was 10-fold higher than that obtained with the sodium carbonate–bicarbonate eluent. Thus we selected the latter. The large carbonate peak was reduced by washing the concentrator column with water before reinjection[19,21], but interference in the determination of chloride and nitrite was still present.

Suppressor and scavenger for recycle IC

The system peak from sulphate was minimized by the use of an AMMS and 2.5 m*M* sulphuric acid as a scavenger. In our previous study[21] an anion fibre suppressor (AFS) and 50 m*M* dodecylbenzenesulphonic acid (DBS) as a scavenger were used. Since the AMMS had an higher efficiency of cation suppression, and caused smaller diffusion of analyte than did the AFS, the former was used in this study. Sulphuric acid was used as a scavenger because the AMMS did not suppress cations sufficiently with DBS as a scavenger.

Recycle IC system

The recycle system B was developed to cancel any system peak. As system A concentrated an objective anion together with other anions on the top of the concentrator, eluent was pumped in the reverse direction to that for the collection. The system B collected separated anions with the same matrix as the eluent. Eluent was pumped in the same direction as that used for the collection.

Times to start and stop the collection in system A were the same as the beginning and ending of an objective anion peak, because the analyte was collected just behind the detector cell. In system (B), since the objective anion was collected just behind the separator column, and it took 12 s to flow from the back of the separator to the detector, collection times were 12 s earlier than the beginning and ending of an objective anion peak.

The peak heights of reinjected anions in recycle systems A and B were 95–98 and 30–35%, respectively, of the peak height obtained by conventional IC. The low recovery in system B resulted from diffusion caused by passing the separator twice without any concentration procedure.

Determination of trace anions in inorganic acids

Fig. 2a shows a chromatogram of diluted phosphoric acid (1/400). A broad peak

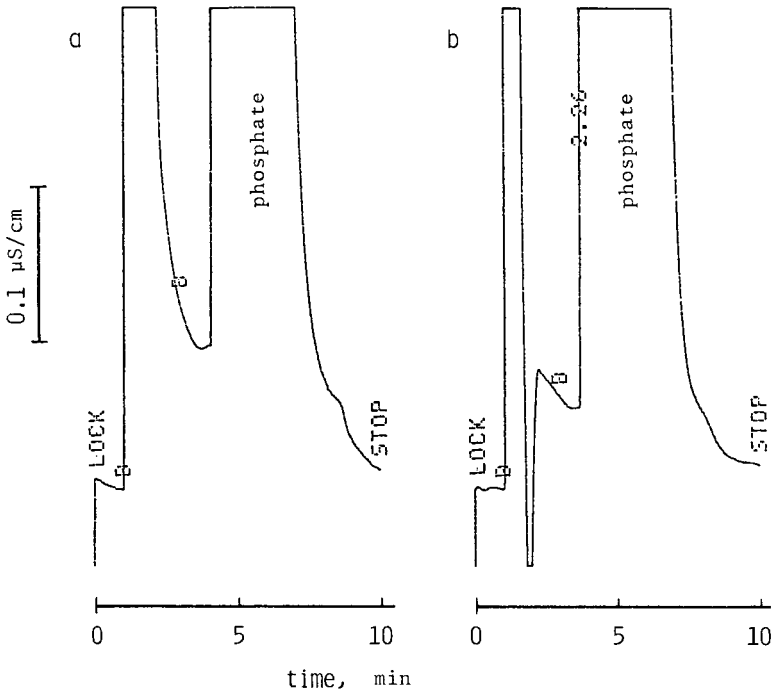

Fig. 2. Chromatograms of diluted orthophosphoric acid (1/400): (a) without neutralization; (b) neutralized with sodium hydroxide.

appeared just behind a water dip and interfered with the determination of trace chloride and nitrite even though recycle system B was used. We believe that this broad peak is due to carbonate that is extruded by a large amount of phosphate. The same phenomenon was observed for other acids. This broad peak disappeared upon neutralization of sample acids with sodium hydroxide as shown in Fig. 2b. The neutralization was not needed for the determination of anions by recycle system A, but was necessary to avoid damage of the separator column by acidic samples.

Fig. 3 shows an example of chromatograms in which 30 ppm of bromide in concentrated hydrochloric acid were determined by recycle system A. Fig. 4 shows an example of the determination of 6.0 ppm of chloride and 7.8 ppm of nitrite in concentrated phosphoric acid by recycle system B. Both systems were effective in separating and determining hidden peaks of analyte behind a large peak of sample acid.

The results of the determination of trace anions in hydrochloric, nitric, phosphoric and sulphuric acids are shown in Tables III–VI. Chloride and nitrite were determined simultaneously by recycle system B. Bromide and nitrate were determined simultaneously, and sulphate was determined separately by recycle system A. The recoveries of each anion were in the range of 95–103% with a relative standard deviation (R.S.D.) of 0.4–5.2%.

To determine sulphate by recycle system A, the system-peak height of sulphate should first be determined. Since the system-peak height had high reproducibility, the

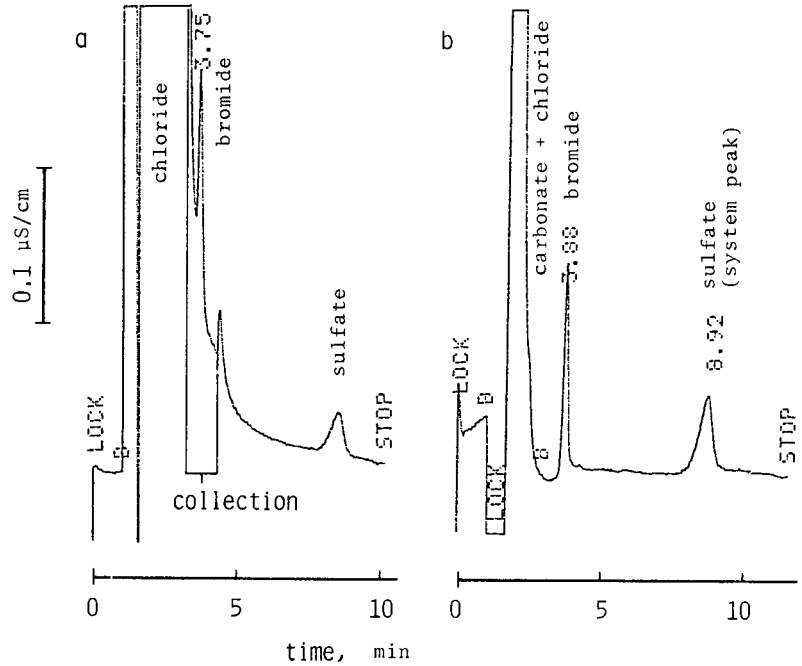

Fig. 3. Determination of bromide ion (30 ppm) in concentrated hydrochloric acid by recycle system A. The chromatograms show (a) collection, (b) reinjection.

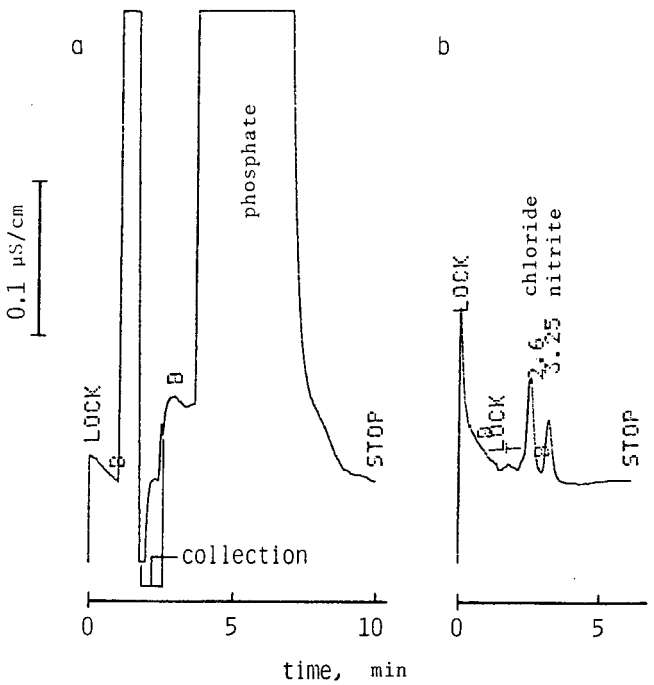

Fig. 4. Determination of chloride ion (6.0 ppm) and nitrite ion (7.8 ppm) in concentrated orthophosphoric acid by recycle system B. The chromatograms show (a) collection, (b) reinjection.

TABLE III

RECOVERY OF TRACE ANIONS IN CONCENTRATED HYDROCHLORIC ACID

Objective anion	*Concentration (ppm)*		*Recovery (%)*	*R.S.D. (%)*	*n*
	Added	*Found*			
Bromide	0	19.24	–	0.82	4
	6.45	25.77	101.2	0.54	6
Nitrate	0	0.10	–	5.22	4
	5.0	5.12	100.4	1.05	6
Sulphate	0	1.97	–	4.93	4
	10.0	12.30	103.3	2.02	6

TABLE IV

RECOVERY OF TRACE ANIONS IN CONCENTRATED NITRIC ACID

Objective anion	*Concentration (ppm)*		*Recovery (%)*	*R.S.D. (%)*	*n*
	Added	*Found*			
Chloride	0	1.62	–	4.73	4
	3.0	4.58	98.7	3.17	5
Nitrite	0	0.00	–	0	4
	3.89	3.78	97.2	2.81	5
Sulphate	0	1.89	–	4.28	4
	10.0	12.20	103.1	1.69	6

TABLE V

RECOVERY OF TRACE ANIONS IN CONCENTRATED PHOSPHORIC ACID

Objective anion	*Concentration (ppm)*		*Recovery (%)*	*R.S.D. (%)*	*n*
	Added	*Found*			
Chloride	0	0.00	–	0	3
	3.0	2.92	97.3	2.83	5
Nitrite	0	0.00	–	0	3
	3.89	3.85	99.0	2.53	5
Bromide	0	0.00	–	0	4
	6.45	6.49	100.6	2.01	6
Nitrate	0	0.00	–	0	4
	5.0	5.12	102.4	1.74	6
Sulphate	0	12.48	–	4.37	4
	10.0	22.76	102.8	0.39	6

TABLE VI

RECOVERY OF TRACE ANIONS IN CONCENTRATED SULPHURIC ACID

Objective anion	*Concentration (ppm)*		*Recovery (%)*	*R.S.D. (%)*	*n*
	Added	*Found*			
Chloride	0	0.91	–	4.45	4
	3.0	3.75	94.7	2.93	5
Nitrite	0	0.00	–	0	4
	3.89	3.78	97.2	2.19	5
Bromide	0	0.00	–	0	4
	6.45	6.55	101.6	1.13	6
Nitrate	0	0.00	–	0	4
	5.0	4.84	96.8	1.54	6

lower determination limit was less than that obtained by system B. Sulphate was determined by system B without any system peak, but the recovery was *ca.* 30%.

We could not determine data for phosphate because the recovery of phosphate by recycle system A was 70–75% even for standard phosphate in the absence of any other anion. Furthermore, the recovery of phosphate by conventional IC was reduced by the coexistence of large amounts of other anions.

Table VII shows the detection limit of anionic impurities in concentrated inorganic acids by recycle IC. The value for sulphate was defined as three times the standard deviation of the system-peak height, and those for other anions were defined at a signal-to-noise ratio of 3. Each value was lower or equivalent to the limit of anionic impurities according to JIS[22]. An objective anion would be separated completely from very large amounts of coexisting anions by repeated recycling, and thus the detection limits might be improved.

TABLE VII

DETECTION LIMITS OF TRACE ANIONS IN CONCENTRATED ACIDS BY RECYCLE ION CHROMATOGRAPHY

Sample acid	*Detection limit (ppm)*				
	Chloride	*Nitrite*	*Bromide*	*Nitrate*	*Sulphate*
HCl	–	–[a]	0.50	0.41	0.32
HNO_3	0.25	0.49	–[a]	–	0.54
H_3PO_4	0.42	0.82	0.70	0.57	0.91
H_2SO_4	0.39	0.77	0.32	0.26	–

[a] No data.

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CHROM. 21 171

Note

Chemometric approach to explain the liquid chromatographic retention of some chiral indoles on swollen microcrystalline triacetylcellulose

PER ERLANDSSON
Technical Analytical Chemistry, Chemical Centre, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
ROLAND ISAKSSON*
Division of Organic Chemistry 3, Chemical Centre, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
INGEMAR NILSSON
Hässle Research Laboratories, Department of Organic Chemistry, S-431 83 Mölndal (Sweden)
and
SVANTE WOLD
Research Group for Chemometrics, Institute of Chemistry, University of Umeå, S-901 87 Umeå (Sweden)
(First received September 7th, 1988; revised manuscript received December 8th, 1988)

During the last decade triacetylcellulose[1] (TAC) has been used successfully as a chiral stationary phase for liquid chromatography to resolve a great number of chiral compounds of different classes. However, little is known about the mechanisms that govern the interaction between the stationary phase and the optically active solute. It is not possible to predict either resolution or retention orders with the present knowledge of these mechanisms.

It has been suggested by Staudinger and Döhle[2] and Hesse and Hagel[1] that the solutes enter cavities between the laminae in the crystalline cellulose to form inclusion complexes. Several experimental findings support this idea, *e.g.*, the difference in retention between benzene and 1,3,5-tri-*tert.*-butylbenzene[3]. The unsubstituted benzene is supposed to fit between the laminae and is retained whereas 1,3,5-tri-*tert.*-butylbenzene is too bulky to enter between these laminae and is therefore not retained. Also, Blaschke *et al.*[4] reported a dependence of the resolution on the size of the solute. Drugs which are not retained are not resolved. Blaschke *et al.* concluded that the size of these solutes is too large to allow their entry into the cavities of the cellulose.

Hesse and Hagel[1] and Francotte *et al.*[5] have found that the tertiary structure of the cellulose is important for selectivity. It was found that, compared with the corresponding crystalline TAC, amorphous TAC more or less completely loses its resolving power, presumably because of a loss of the chiral cavities in the amorphous material. The idea of chiral cavities is not conclusive, however. Okamoto *et al.*[6] found that the retention order of the enantiomers of Troegers base on microcrystalline TAC were reversed when columns prepared by coating dissolved TAC on silica were used.

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The native structure of cellulose, cellulose I, is lost in the preparation of these columns and is presumably replaced with another kind of cellulose, the morphology of which is not yet known. We have also shown[7] that it is possible to separate the enantiomers of even larger molecules on TAC than those studied by Blaschke *et al.* A possible explanation of this could be that partial inclusion in such cavities is sufficient to afford stereoselectivity. Another possible explanation is that the entire surface of the cellulose is utilized in the chiral recognition process.

Recently, Schulze and König[8] reported enantiomer separations on silica gel to which monosaccharides were covalently bonded. Glucose, a monosaccharide, is the structural unit of cellulose. These findings, together with the great variety of structures of compounds resolved into enantiomers on TAC, indicate that several different chiral recognition mechanisms have to be considered.

Multivariate statistical (chemometric) methods for the evaluation of data in chemistry[9] have been extensively used in studies of the quantitative structure–activity relations (QSAR) of drugs and other biologically active compounds. The same approach has also been used to study the relationship between molecular structure and chromatographic behaviour and has been termed quantitative structure–retention relationships (QSRR)[10]. Among the multivariate methods, multiple linear regression (MLR), an extension of ordinary least squares, has been most commonly used. Partial least squares in latent variables (PLS), developed by Wold and co-workers[11,12], is based on the projection of the original multivariate data matrices down on smaller matrices (T) with orthogonal columns.

$$X = TP' + E$$

$$Y = TQ' + F$$

Here the $n \times p$ matrix X is projected down on the $n \times A$ matrix T (score matrix) by the $p \times A$ projection matrix P leaving the residuals E. Analogously, Y ($n \times q$) is projected on T by the $q \times A$ projection matrix Q leaving the residuals F. P and Q are often called loading matrices. The calculations also gives an auxiliary matrix W (PLS weights), which is similar to P. For a new case (here a compound), the values of t_a ($a = 1,2, \ldots, A$) —one additional row in the matrix T — are computed from its x-vector and the matrices P and W. This t-vector gives predicted values of y for this case (compound) as tQ'. The projections are chosen to give a maximum correlation between the descriptor (X) and the response matrices (Y), under the condition that X and Y are well approximated by TP' and TQ', respectively. Determination of the significant number of model dimensions (A) is made by cross-validation[11,12]

PLS has several advantages over MLR. MLR cannot handle co-linearities in X whereas PLS concentrates the systematic variation in X into fewer factors (columns in T) than variables in the original matrix X. The PLS projection can be calculated regardless of the number of variables in X, *i.e.*, the number of descriptors may be large than the number of objects (compounds). Projections involving many variables are stable provided that the number of model dimensions (latent variables) extracted is less than about one third of the number of compounds[13]. PLS has been extensively described in the literature and today has a firm statistical basis[14].

To be able to carry out a PLS study of this kind, it is necessary to have a

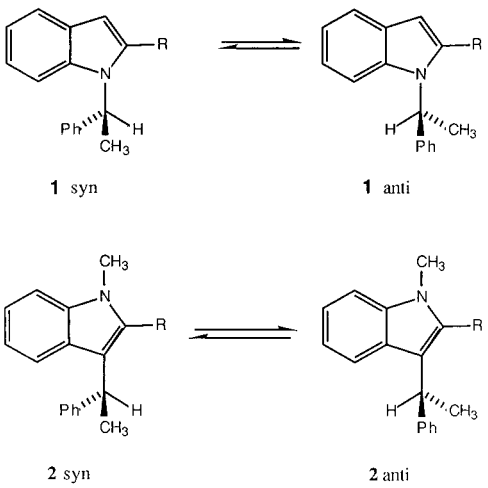

Fig. 1. Chiral indole derivatives **1** and **2**. For explanation of R, see Table I. Ph = Phenyl.

material that shows a systematic variation in both structure and chromatographic behaviour. The chiral indole derivatives[15] (Fig. 1) used in this study are good candidates, as their absolute configurations and conformations are known[16,17,18]. Further, the retention, separation and conformations are sensitive to the substitution pattern on the indole framework. In this paper, only the results for the phenethylamino derivatives are reported.

The purpose of this investigation is (a) to use PLS as a tool to analyse retention data for chiral resolutions so as to be able to predict retention and retention orders between enantiomers and (b) to find relationships between the structures of the solutes and their retention that can be used to formulate a model for chiral recognition on TAC.

EXPERIMENTAL

The syntheses of indoles **1a–e** and **2a–e** have been reported previously[15].

The separations were performed by liquid chromatography on swollen microcrystalline TAC as described[15]. The capacity factors (k') for **1b**, **1d** and **1e** were calculated by fitting the experimental chromatograms to skewed overlapping Gaussian curves.

CALCULATIONS

The three-dimensional representations of the indoles were obtained from molecular mechanics calculations (MMP2 force field)[19]. Coordinate transformations and calculations of the non-tabulated molecular descriptors were carried out with the use of the MIMIC (methods for interactive modelling in chemistry) system[20] available at the Chemical Centre in Lund. The absolute configurations were obtained by comparing CD data with those from compounds of known absolute configuration prepared from chiral precursors[16,17].

Molecular descriptors (matrix X)

Two kinds of molecular descriptors were used: (i) tabulated values[21] describing the length, breadth, Hammett σ_m and σ_p, lipophilicity and molar refractivity of R, and also the number of hydrogen bonding sites, and (ii) calculated values from the modelled three-dimensional coordinates such as coordinates for the six-membered ring in the indole part, coordinates of the indole nitrogen, coordinates for the phenyl, methyl and hydrogen connected to the asymmetric carbon, Van der Waals area and volume of the whole molecules and of substituents R, total dipole moment and its three-dimensional direction. These variables comprise the *X*-block in the PLS model, each row corresponding to one compound and each column corresponding to one descriptor.

The chromatographic behaviour of the solutes was described by the logarithm of the capacity factor, log k', and was used as response data in the *Y*-block of the PLS model.

Calculations of the PLS model were carried out with a Victor V-286 microcom-

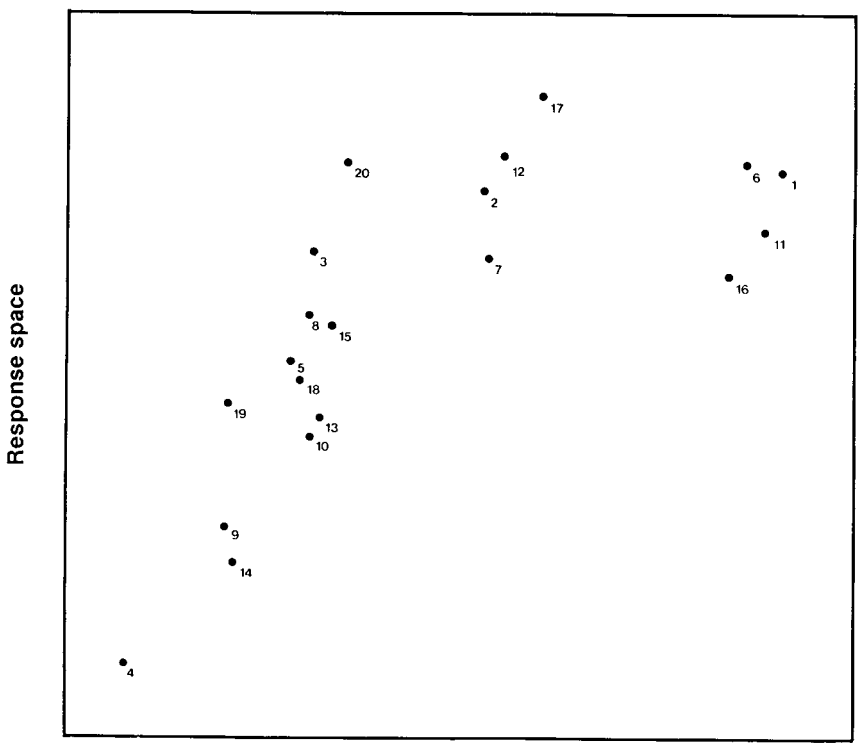

Fig. 2. Score plot of the first dimension of the PLS model. The *x*-axis is the most significant direction in descriptor space and the *y*-axis the most significant direction in response space (chromatographic retention data). 1 = **1a**-*S*; 2 = **1b**-*S*; 3 = **1c**-*S*; 4 = **1d**-*S*; 5 = **1e**-*S*; 6 = **2a**-*S*; 7 = **2b**-*S*; 8 = **2c**-*S*; 9 = **2d**-*S*; 10 = **2e**-*S*; 11 = **1a**-*R*; 12 = **1b**-*R*; 13 = **1c**-*R*; 14 = **1d**-*R*; 15 = **1e**-*R*; 16 = **2a**-*R*; 17 = **2b**-*R*; 18 = **2c**-*R*; 19 = **2d**-*R*; 20 = **2e**-*R*. Compounds 1, 6, 11 and 15 have an *anti* conformation whereas the others have a *syn* conformation.

puter and the SIMCA program package, which is available from Sepanova (Enskede, Sweden) and also from Principal Data Components (Columbia, MO, U.S.A). Details of the computational procedures and molecular descriptors will be reported in a subsequent paper.

RESULTS AND DISCUSSION

Two significant dimensions were obtained from the PLS calculations describing 80% of the variance in the retention data of the indoles. The first PLS component, illustrated by its score plot in Fig. 2, used 23.4% of the variance in descriptor data to explain 50.7% of the variance in retention data. The second PLS component used 4.9% of the variance in descriptor data to explain another 29.4% of the variance in retention data. The score plot (Fig. 2) shows that the indoles are grouped according to their conformations, *anti* and *syn*, thus implying different retention mechanisms.

The retentions of the indoles calculated from the model are listed in Table I and illustrated in Fig. 3. The retention orders of the enantiomers were correctly calculated

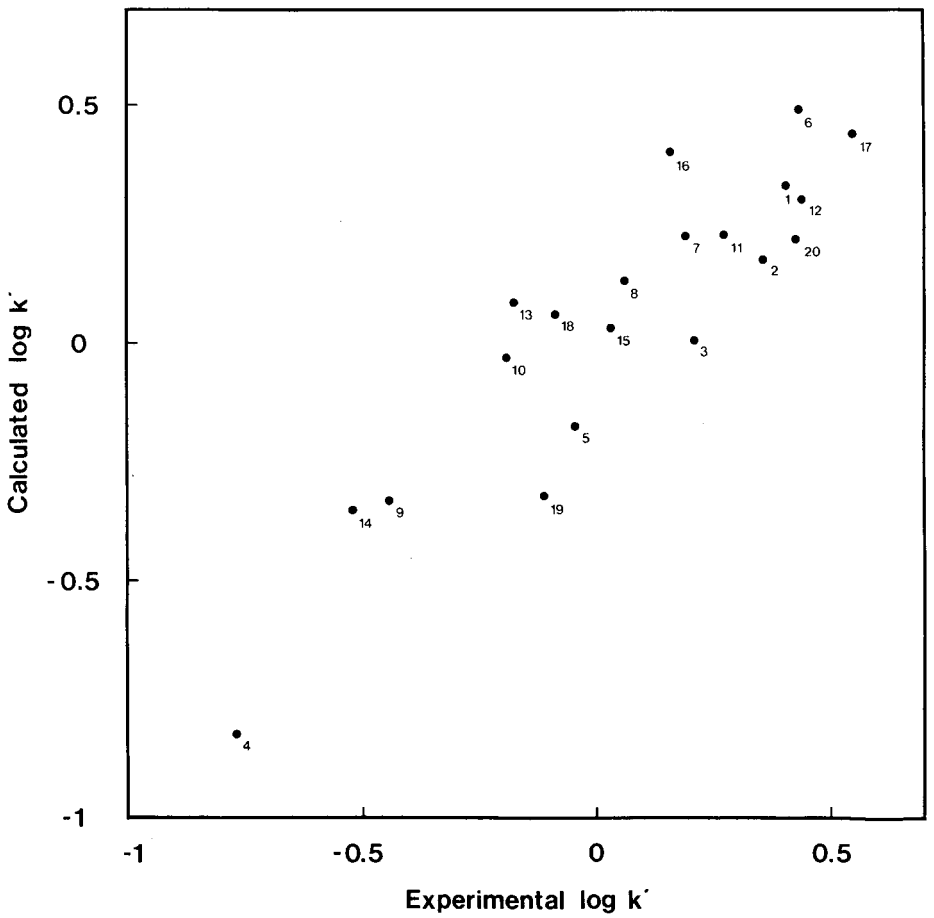

Fig. 3. Plot of predicted log k' against experimental log k' according to Table I. $R = 0.91$. Numbering as in Fig. 2.

TABLE I

EXPERIMENTAL AND PREDICTED RETENTION DATA FOR INDOLES **1** AND **2**

Compound			*Experimental values*			*Predicted values*		
No.	*R*	*Conformation*	*Log k'$_S$*	*Log k'$_R$*	*1st eluted isomer*	*Log k'$_S$*	*Log k'$_R$*	*1st eluted isomer*
1a	H	*anti*	0.4048	0.2685	*R*	0.3324	0.2251	*R*
1b	CH_3	*syn*	0.3541	0.4393	*S*	0.1758	0.2968	*S*
1c	CO_2CH_3	*syn*	0.2122	−0.1805	*R*	0.0620	0.0847	*R*
1d	CH_3COOH	*syn*	−0.7696	−0.5229	*S*	−0.8230	−0.3495	*S*
1e	$CH_3C=CH_2$	*syn*	−0.0506	0.0334	*S*	−0.1756	0.0337	*S*
2a	H	*anti*	0.4281	0.1553	*R*	0.4958	0.4034	*R*
2b	CH_3	*syn*	0.1931	0.5465	*S*	0.2221	0.4409	*S*
2c	CO_2CH_3	*syn*	0.0607	−0.0915	*R*	0.1315	0.0599	*R*
2d	CH_3COOH	*syn*	−0.4437	−0.1135	*S*	−0.3288	−0.3148	*S*
2e	$CH_3C=CH_2$	*syn*	−0.1938	0.4249	*S*	−0.0305	0.2176	*S*

in all instances, which indicates the possibility of predicting absolute configurations from retention data on TAC columns. Preliminary analysis of the PLS model shows that the most important descriptors are the coordinates of the substituents attached to the asymmetric carbon, dipole moment, volume and area of the solutes (see Table II). As the PLS model differs in its assumptions from regression, these coefficients (loadings) should not be interpreted as measures of the independent contributions of the variables of retention behaviour, but rather how much (relative) information the variables contain about the retention behaviour.

It seems as if the PLS approach described here may afford an insight into the mechanisms of retention and chiral recognition on TAC columns.

TABLE II

LOADINGS (P_{ka}) OF THE FIVE MOST IMPORTANT VARIABLES IN EACH COMPONENT OF THE PLS MODEL.

A high absolute value of a loading indicates a high contribution to the model. The maximum loading is 1 or −1.

PLS component	*Parameter*	P_{ka}
First (a = 1)	Area of R	−0.2814
	Volume of R	−0.2812
	Molar refractivity of R	−0.2806
	Breadth of R	−0.2762
	Length of R	−0.2685
Second (a = 2)	x Coordinate of the phenyl group attached to chiral carbon	−0.3832
	Total dipole moment	−0.3257
	y Component of dipole moment	0.2522
	x Coordinate of R	0.2429
	y Coordinate of R	0.2237

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CHROM. 21 177

Note

Cluster analysis in the comparison of two-dimensional chromatograms

DANIELA HEIMLER*
Dipartimento di Scienza del Suolo e Nutrizione della Pianta, Università degli Studi di Firenze, Piazzale delle Cascine 28, Florence (Italy)
and
VIERI BODDI
Istituto di Patologia Generale, Università degli Studi di Firenze, Viale G.B. Morgagni 50, Florence (Italy)
(Received December 5th, 1988)

Two-dimensional chromatography, notwithstanding its undoubted advantages from a theoretical point of view, is the least studied separation technique owing to the difficulty in interpretating the experimental results. The technique has two main drawbacks: the possibility of analysing only one sample on one sheet without the simultaneous spotting of test compounds[1] and the difficult interpretation of a two-dimensional chromatogram on the basis of R_F values obtained by one-dimensional developments. Even the quantitative analysis of two-dimensional chromatograms is difficult, as the spots are not arranged in vertical strips but occupy the whole layer. Nevertheless two-dimensional chromatography can separate very complex mixtures which are difficult to resolve by means of other techniques[2].

This paper describes a method which allows the use of chromatographic data in order to calculate similarity criteria without having quantitative data. The problem of sample classification on the basis of chromatographic results is generally carried out by means of pattern recognition techniques using the quantitative data from gas chromatography or high-performance liquid chromatography[3].

EXPERIMENTAL

Flavonoids aglycones of elm and iris leaves were examined. The crushed leaves (1 g) were treated with 25 ml of boiling hydrochloric acid for 30 min. The flavonoids were extracted with 10 ml of ethyl acetate, which was evaporated to dryness under vacuum. The residue was dissolved in 2 ml of methanol and 2 μl of this solution were spotted on Sil C_{18}-50 plates (Macherey, Nagel & Co.) and eluted in the first direction with *n*-hexane–ethyl acetate–acetic acid (72:27:1) and in the second direction with 1 *M* acetic acid in 50% methanol. The spots were sprayed with a 1% methanolic solution of ethanolamine diphenylborate and a 5% ethanolic solution of polyethylene glycol. The spots were observed under UV light (360 nm). Under these conditions the flavonoids give fluorescent spots of different colours. The spots were characterized by their positions on the layer by means of two coordinates, obtained by dividing the

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distance of the spot from the origin lines by the distance of the two solvents from the same lines, and by their colour under UV light.

For elm leaves we considered only those compounds which migrate in both eluents, because compounds which remain at the starting point with the first eluent and migrate with the second eluent are difficult to identify owing to their incomplete separation. In this way every spot in all of the chromatograms were assigned to a definite group and the results in Table I were obtained. It should be noted that only in a few instances could the spots be identified[4]. The aim of this work, however, was to compare several two-dimensional chromatograms, regarded as "fingerprints" of different plants, in order to ascertain whether the thin-layer chromatographic (TLC) profile of phenolic compounds could be of help in the determination of differences among populations, provenances and species (that is, intra- and inter-specific differences).

The elm leaves were obtained from the germoplasm collection of the Centre for Forest Pathology of the National Research Council of Florence. The data for the two-dimensional chromatograms are reported in Table II.

The iris leaves belong to spontaneous species and were sampled in the Giardino dell'Iris in Florence. In this instance we also considered the compounds lying on the y-axis (that is, those compounds which migrate with the first eluent but remain at the origin with the second), as they are better characterized than in the case of elm leaves. The data are reported in Tables III and IV.

TABLE I

COORDINATES AND COLOURS UNDER UV LIGHT OF ALL THE SPOTS OBSERVED IN THE TWO-DIMENSIONAL CHROMATOGRAMS OF ELM LEAVES

The values of the coordinates are the means of 10–32 determinations. Letters A–U represent the different spots in the chromatograms.

	Coordinates $\times 100$	*Colour*	*Name*
A	18–18	Orange	Quercetin
B	12–51	Red	–
C	13–37	Red	Myricetin
D	25–39	Red	–
E	13–50	Orange	–
F	29–74	Light blue	–
G	31–53	Light blue	–
H	39–70	Light blue	–
I	24–84	Light blue	–
L	29–61	Light blue	–
M	25–67	Yellow	Caffeic acid
N	25–75	Yellow	Caffeic acid
O	44–45	Yellow	–
P	14–48	Yellow	–
Q	19–55	Yellow	–
R	30–10	Green yellow	Kaempferol
S	23–35	Light blue	–
T	63–81	Light blue	–
U	42–82	Blue	–

TABLE II
DISTRIBUTION OF SPOTS IN THE ELM LEAVES
A–U as in Table I.

Elm leaves	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*
U. pumilia:																			
(1) S1	+	+	+			+	+	+	+	+	+					+			
(2) S12	+		+				+		+		+					+			
(3) S15	+						+	+	+		+					+			
(4) PU1	+	+	+		+		+	+	+		+	+	+			+			
(5) 73P	+	+	+			+	+	+	+		+	+				+	+		
(6) 182P	+	+	+				+	+	+		+	+	+	+	+	+			
U. parvifolia:																			
(7) PA1.1, PA1.2	+					+	+		+		+	+				+			+
(8) PA2	+		+				+		+		+	+		+		+			
(9) 157P	+	+	+	+			+		+		+	+	+			+			+
(10) NA33	+		+				+				+	+	+	+		+			
U. japonica:																			
(11) 3P	+	+	+		+		+	+	+		+	+				+			
(12) 2P	+	+	+	+	+		+	+	+		+	+				+			
(13) 127P	+	+	+		+	+	+	+	+		+	+	+			+			
(14) 23P	+	+	+	+	+	+	+		+		+	+	+			+			
(15) 57P	+	+	+	+			+		+	+	+	+	+	+		+			+
U. carpinifolia:																			
(16) C3	+	+	+				+	+			+	+	+			+			
(17) C6	+	+	+			+	+	+			+	+	+			+			
(18) 6-11	+	+	+			+	+	+			+	+				+			
U. xhollandica:																			
(19) 274, P38, 275	+		+				+		+	+	+	+	+	+		+	+		+
(20) 405	+		+	+			+		+	+	+	+	+	+	+	+			+
U. chemnoui:																			
(21) 176P.2	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+			
(22) 176P.5	+	+	+		+		+	+	+		+	+	+	+	+	+			
U. villosa:																			
(23) VII,P554	+						+		+		+	+		+		+			+
(24) *U. laevis*	+		+				+		+		+	+		+		+	+		+
(25) *U. glabra*	+		+				+		+		+	+		+	+	+			+
(26) *U. elliptica*	+		+	+			+	+	+		+	+		+		+			
(27) *U. laciniata*	+		+				+		+		+	+		+	+	+			
(28) *U. wilsoniana*	+	+	+				+	+	+	+	+	+				+		+	+

RESULTS AND DISCUSSION

The question of the comparison of qualitative data regarded as indicating the presence or absence of a compound can be solved by means of numerical indices expressed by equations which may change slightly from one case to another. The information in each line in Tables II and IV can be codified as either 0 or 1 (absence or presence of a spot). The result of the comparison of two sequences is characterized by four values: N_{11} (number of positive agreements), N_{00} (number of negative agreements), N_{10} and N_{01} (number of disagreements, that is, the presence of a spot in

TABLE III

COORDINATES AND COLOURS UNDER UV LIGHT OF ALL THE SPOTS OBSERVED IN THE TWO-DIMENSIONAL CHROMATOGRAMS OF IRIS LEAVES

	Coordinates × *100*	*Colour*
A	9–27	Green
B	12–18	Green
C	17–10	Green
D	18–64	Light blue
E	18–77	Orange
F	24–0	Red
G	25–60	Light blue
H	30–10	Light blue
I	30–83	Blue
L	51–0	Red
M	56–0	Red
N	76–0	Red
O	12–15	Green
P	86–60	Light blue
Q	18–69	Light blue
R	37–49	Light blue
S	65–28	Light blue
T	51–73	Light blue

TABLE IV

DISTRIBUTION OF SPOTS IN THE IRIS LEAVES

A–T as in Table III.

Iris leaves	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*
(1) *I. pallida*[a]				+	+	+	+		+	+		+						
(2) *I. pallida*[b]				+	+	+	+		+	+		+			+			
(3) *I. pallida*				+	+	+			+	+		+			+			
(4) *I. cengialti*	+	+	+	+		+			+	+		+		+	+			
(5) *I. florentina*	+	+	+	+	+	+			+	+	+	+			+			
(6) *I. germanica*	+		+	+	+	+			+	+		+						+
(7) *I. lutescens (Quercianella)*		+	+			+				+	+	+						
(8) *I. lutescens (Monte Marcello)*	+	+	+			+				+	+	+					+	
(9) *I. squalens*	+	+	+	+	+	+	+		+	+	+	+				+		
(10) *I. kockii*			+	+		+			+	+	+	+	+		+			
(11) *I. sambucina*	+	+	+	+	+	+			+	+		+			+			
(12) *I. aphilla*	+	+	+			+			+	+			+	+	+			
(13) *I. uinguicularis*	+	+	+	+	+	+	+	+	+	+	+	+	+					

[a] Fertile form.
[b] Sterile form.

one sequence and its absence in the other). The most general similarity index of two sequences (simple matching coefficient) is[5]

$$S_{SM} = (N_{11} + N_{00})/(N_{11} + N_{00} + N_{10} + N_{01})$$

The Jaccard–Sneath coefficient does not consider the negative agreements (N_{00}):

$$S_{JS} = N_{11}/(N_{11} + N_{10} + N_{01})$$

From the point of view of our data (TLC data), we deemed the Jaccard–Sneath coefficient to be more useful, as TLC can give information on the presence of one

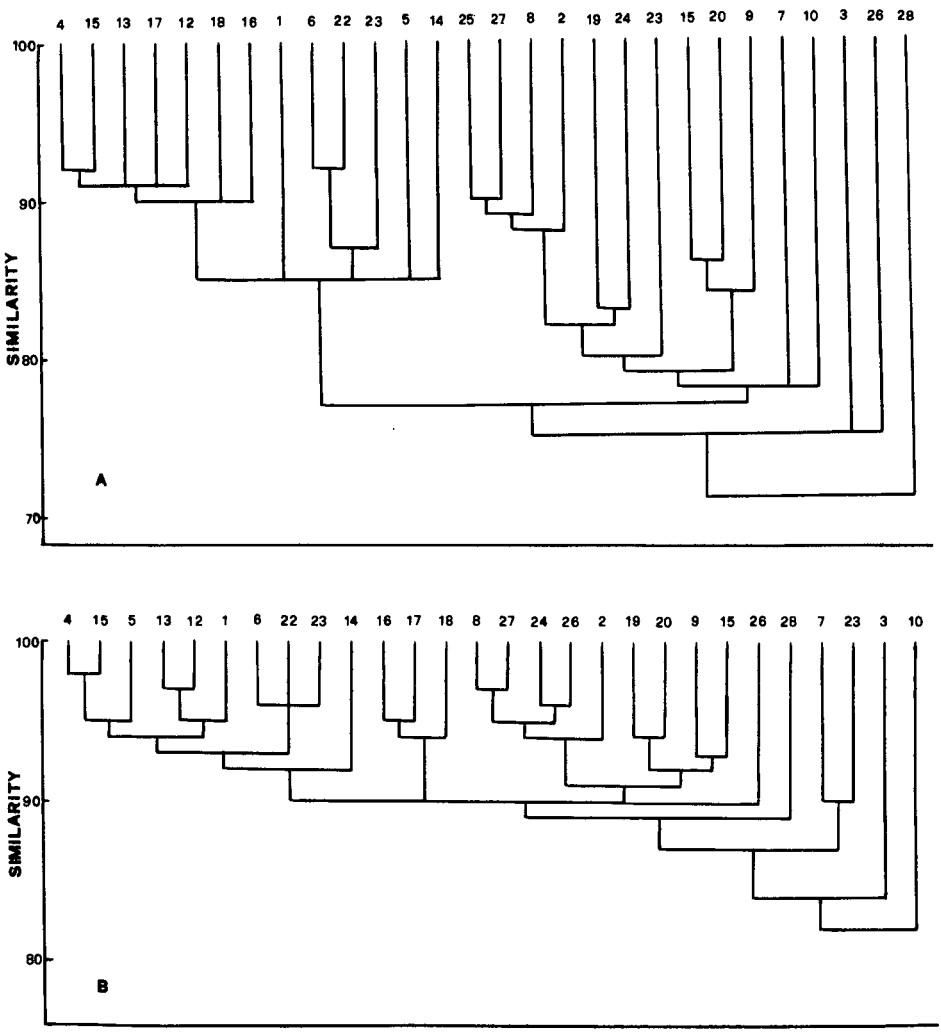

Fig. 1. Dendrograms obtained by the cluster analysis of: (A) S_{JS} coefficient and (B) S_w coefficient. The data refer to elm leaves. Numbers as in Table II.

compound but not on its absence. The similarity coefficients were calculated from all the pairs of sequences in Table II and were used for the cluster analysis. We used one of the simplest classification methods, that is, the single linkage cluster analysis[5]. The results of this kind of analysis are visualized by means of a dendrogram (see Fig. 1A). The data for 30 different two-dimensional chromatograms can be easily correlated.

A general consideration should, however, be made before discussing in detail the results in Fig. 1. The Jaccard–Sneath coefficient (S_{JS}) ascribes the same weight to each positive agreement and to each disagreement. It seemed interesting to ascribe a different weight to each spot depending on its frequency in the whole data matrix. In this way we consider the presence in one sequence of a compound which is present in a large number of sequences to be more important than the presence of one compound which rarely appears in the whole data matrix. For this reason we attributed a weight to each spot equal to the number of times that the spot appears in the whole sequences matrix. The resulting coefficient is

$$S_w = W_{11}/(W_{11} + W_{10} + W_{01})$$

where W_{11} is the sum of the weights of the spots present in both sequences and W_{01} and W_{10} are the sums of the weights of the spots present in one of the two sequences considered.

The value of a similarity coefficient S_w, such as that of Jaccard–Sneath, changes from 0 to 1. The S_w coefficient allows the introduction into each coefficient of information concerning the whole data matrix, in contrast to all the other similarity coefficients which consider only two sequences.

In order to test the validity of the S_w coefficient, Fig. 2 shows the correlation

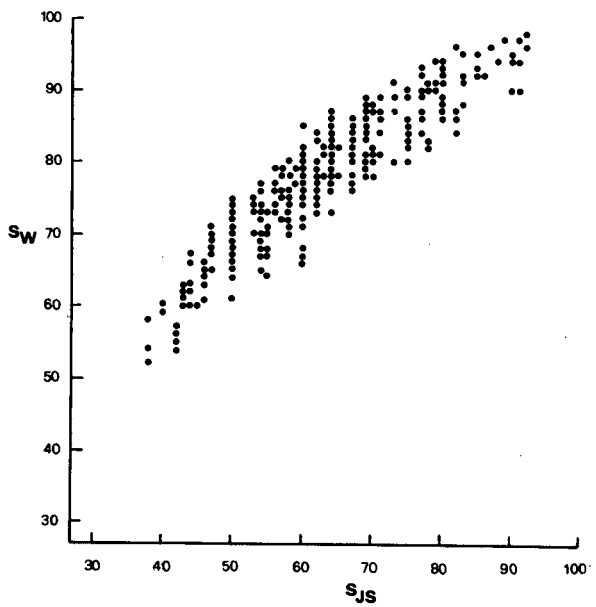

Fig. 2. Correlation between S_{JS} and S_w coefficients of the elm data set.

between the values of the S_{JS} and S_w coefficients for the same data matrix (Table II). It should be noted that the S_w coefficients generally have a higher value and exhibit a better differentiation. In fact, in many instances, one value of the S_{JS} coefficient corresponds to different values of the S_w coefficient; this occurrence could be of help in giving a better differentiation overall in those instances in which the chromatographic data are very similar. Fig. 1B shows the dendrogram obtained by the cluster analysis of the S_w coefficients. Comparison of the two dendrograms in Fig. 1A and B indicates that in Fig. 1B the similarity among the sequences due to the higher mean values of the S_w coefficients is increased with respect to Fig. 1A. However, from a general point of view, such an occurrence does not affect the dendrogram, as Fig. 1 must be considered as a whole.

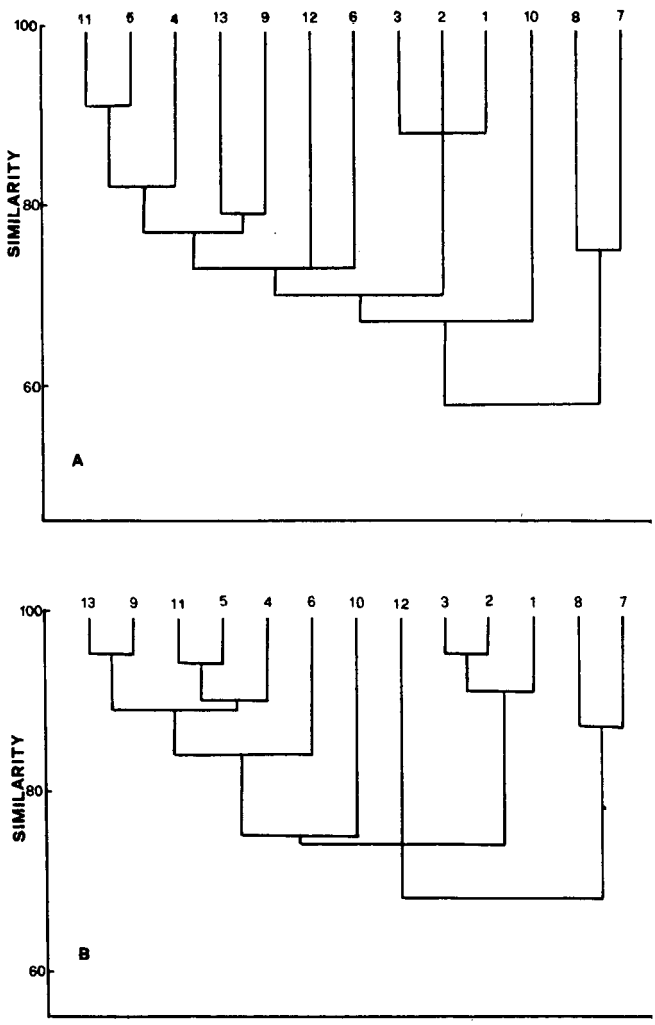

Fig 3. Dendrograms obtained by the cluster analysis of (A) S_{JS} coefficient and (B) S_w coefficient. The data refer to iris leaves. Numbers as in Table IV.

Let us now see what kind of information can be obtained from these dendrograms. *U. pumila* and *U. parvifolia* exhibit a high intra-specific variation owing to their provenance from a wide geographic area; in both instances, however, all *U. parvifolia* populations are in the cluster on the right. *U. japonica* and *U. carpinifolia* are very similar species from a botanical point of view[6]; in the dendrogram in Fig. 1A they appear in the same cluster. In Fig. 1B, however, the three populations of *U. carpinifolia* are gathered in one cluster, offering more detailed information in this instance where the differences between the samples are very small. The species *U. xhollandica* is very similar in all its populations (it should be noted that three of the four populations studied are identical) and in Fig. 1B (but not in Fig. 1A) the four populations are in one cluster.

As a further demonstration of the higher resolving power of the S_w coefficient for very similar sequences, it is interesting to consider the data for two populations of *U. japonica* (2P and 3P) which come from a restricted area of southern Japan; in Fig. 1B the two populations are linked in a more evident way than in Fig. 1A. From a botanical point of view other considerations could be made on the way in which the different species are linked, but this is beyond the aims of this paper.

As can be seen from the data in Table II, in all elm samples five spots were constantly found; in order to go deeper into the question of the interpretation of chromatographic data by means of cluster analysis, we considered the matrix in Table II without the five common columns. Apart from an expected translation towards lower values of similarity, there are no substantial differences with respect to Fig. 1A and B.

Fig. 3 shows the data relating to the iris leaves; Fig. 3A refers to the cluster analysis of the S_{JS} coefficients and Fig. 3B to that of the S_w coefficient. The only notable difference between the two dendrograms is found where the similarity between the sequences is higher, resulting in a better differentiation of clusters in Fig. 3B.

ACKNOWLEDGEMENT

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CHROM. 21 226

Note

Separation and determination of organoarsenic compounds with a microbore column and ultraviolet detection

MASAHIRO MARUO, NAOKI HIRAYAMA, HIROO WADA[a] and TOORU KUWAMOTO*
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606 (Japan)
(First received July 12th, 1988; revised manuscript received December 21st, 1988)

Ion chromatography has been widely investigated in the analysis of inorganic anions and organic anions[1–6]. Also of interest in this context is the use of a capillary column and an UV detector[7–10] in order to enhance the efficiency of separation and enable the use of small amounts of samples. We have investigated the separation and determination of small amounts of organoarsenic compounds by use of ion chromatography[15–17] with various eluents and a conductivity detector, but did not obtain satisfactory results.

In this study the separation and determination of organoarsenic acids, such as *o*-aminophenylarsonic acid (o-APA), *p*-aminophenylarsonic acid (p-APA) and *o*-nitrophenylarsonic acid (o-NPA), which have an aromatic group, were undertaken. It was found that the retention times of samples changed remarkably by changing the pH of the eluent. The effect of the eluent pH on the elution of arsenic compounds was investigated in detail by using phosphate as an eluent. Samples were completely separated at the pH values close to the pK_a values of the samples, and a concentration of at least 0.06 ppm using a sample injection of 1 μl was detectable and determined.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Tosoh double plunger pump CCPD, a Rheodyne injection valve 7520 (sample injection volume 1 μl), a Shimadzu UV detector SPD-6AV (cell volume 0.6 μl) and a stainless-steel microbore column (40–200 mm × 0.5 mm I.D.) packed with Tosoh TSKgel IC-Anion-PW (particle size 10 μm, exchange capacity 30 μequiv./ml bed).

Eluents

100 m*M* Phosphoric acid, 100 m*M* potassium hydroxide and sodium hydroxide stock eluent solutions were prepared by dissolving special grade reagents in deionized distilled water (D.D.W.), diluting to the appropriate concentration and deaerating at a water-jet pump and by supersonic vibration (Bronson).

[a] Present address: Shinwa Kako Co., Ltd., Kyoto 613, Japan.

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Standard solutions

Standard 1000 ppm (as As) sample solutions were respectively prepared by dissolving of p-APA (pK_{a1} = 2, pK_{a2} = 4.02, pK_{a3} = 8.92[18]), o-APA (pK_{a1} = 2, pK_{a2} = 3.77, pK_{a3} = 8.66[18]) and o-NPA (pK_{a1} = 3.37, pK_{a2} = 8.62[18]) in D.D.W. All other solutions were prepared from analytical reagent grade or reagent grade salts. Working standard solutions were obtained by diluting the stock solutions in the phosphate solution at the same pH as that of the eluent.

RESULTS AND DISCUSSION

Chromatography of organoarsenic species using potassium hydroxide as the eluent

The charges on the arsenic species in solution are controlled by the pK_a values of each species. Thus, the elution behaviours of these arsenic compounds have been investigated in acidic and alkaline solutions. Potassium hydroxide was selected as the alkaline eluent.

Concentration of the eluent. In potassium hydroxide solution (pH 11.0–11.6), it is estimated from the pK_a values that the charge on the arsenic acids is −2. The eluent concentration was varied in order to investigate the separation of samples. On decreasing the eluent concentration from 4 to 1 m*M*, the retention times of o-APA and p-APA were increased and the resolution between them was changed from 0.28 to 0.58 on a 120-mm column at an eluent flow-rate of 20 μl/min. p-APA and o-NPA were completely overlapped.

Flow-rate of the eluent. With 2 m*M* potassium hydroxide as the eluent, the resolution between p-APA and o-APA varied from 0.58 to 0.37 by changing the flow-rate from 10 to 80 μl/min, on a 120-mm column as shown in Fig. 1. As a result, it was concluded that a low flow-rate gives a better separation.

Column length. With 2 m*M* potassium hydroxide as the eluent and a flow-rate of 20 μl/min, the resolution between p-APA and o-APA was increased from 0.58 to 0.98 by increasing the column length from 120 to 200 mm. However, the use of the latter column was not practical because the sample was strongly retained and the pressure

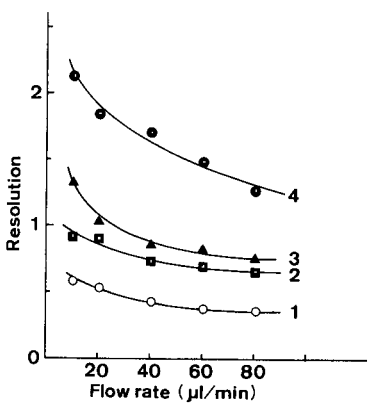

Fig. 1. Dependence of the resolution on the eluent flow-rate. 1, p-APA and o-APA in 2 m*M* KOH (pH 11.3); 2, o-NPA and o-APA; 3, o-NPA and p-APA and 4, p-APA and o-APA in 5 m*M* phosphate (pH 3.8). Column: 120 mm × 0.5 mm I.D.

was too high for elution with conventional flow-rates. Although p-APA and o-APA were separated under these conditions, they overlapped under conventional conditions. On the other hand, o-NPA and p-APA completely overlapped.

Chromatography using phosphate as the eluent

Choice of the eluent. In order to investigate the elution behaviour of organoarsenic species in acidic solutions, a phosphate eluent, adjusted to pH 3.8 by sodium hydroxide, was selected. Chloride and sulphate eluents were excluded because of difficulties in adjusting the pH, and some organic acids were excluded because of the poor separation of p-APA and o-NPA.

Concentration of the eluent. Fig. 2 shows the relationship between the retention times of samples and the concentration of the eluent at pH 3.8. The changes in the retention times of o-NPA with changes in the eluent concentration were very large compared with those of p-APA and o-APA: namely, o-NPA was eluted close to o-APA in a low concentration eluent, but close to p-APA in an high concentration eluent. From the results, it was found that 5 m*M* eluent is preferable for a good separation of p-APA, o-NPA and o-NPA.

Effect of eluent pH on the retention times of samples. By using phosphate solution as the eluent, the retention times of samples were measured in the range pH 3.4–10.3 adjusted with sodium hydroxide. Fig. 3 shows the relationship between the eluent pH and the retention times of p-APA, o-APA and o-NPA. When the pH was above 9, the charge on all samples was -2 and the samples were strongly retained. It may be concluded that o-APA has a different character and behaviour from those of p-APA and o-NPA, because it forms a six-membered ring by intramolecular hydrogen bonding between the hydrogen of the amino group and the oxygen of the arsenic group.

When the eluent pH was near 8.5, the charge on each sample was changed from -2 to -1, and the retention times of samples were decreased. When the pH was near to 7.5, the elution order of p-APA and o-NPA was reversed. The elution order was unchanged in the range pH 7.5–4.5.

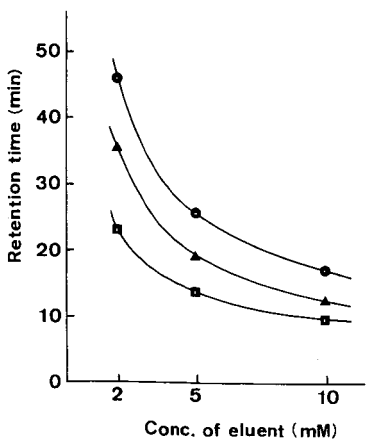

Fig. 2. Dependence of the retention time on the eluent concentration. (□) p-APA; (▲) o-NPA; (○) o-APA. Eluent: phosphate (pH 3.8); flow-rate, 20 μl/min. Column: 120 mm × 0.5 mm I.D.

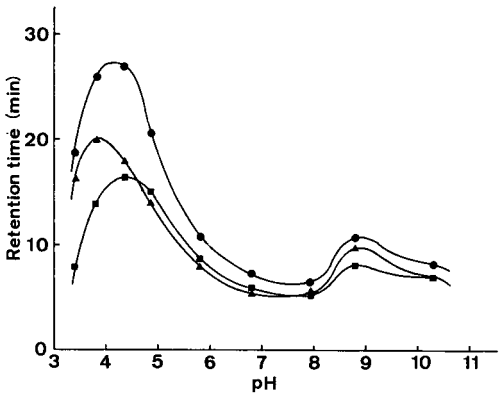

Fig. 3. Dependence of the retention time on the eluent pH. (□) p-APA; (▲) o-NPA; (○) o-APA. Eluent: 5 m*M* phosphate; flow-rate, 20 μl/min. Column: 120 mm × 0.5 mm I.D.

Approaching a pH of 3.8, the retention time of p-APA only was shortened, because the eluent pH was lower than the pK_{a2} of p-APA. Thus, the elution order of p-APA and o-NPA was reversed again. However, the retention times of o-APA and o-NPA were not shortened. Approaching pH 3.4, the retention times of all samples were shortened, and the difference in retention time between o-NPA and o-APA were small. From the results, it was found that the range pH 4.0–3.5 is suitable to determine p-APA, o-APA and o-NPA in the phosphate eluent.

It may be concluded that the separability of samples is increased when the eluent pH is near to the pK_a values of the samples.

Recommended procedure. p-APA, o-APA and o-NPA were completely separated by using the phosphate eluent adjusted to pH 3.8. A 200-mm column was selected to get a good separation, because the pressure was decreased under acidic conditions. The flow-rate was selected to be 20 μl/min in order to get high resolution and appropriate retention times (Fig. 1). Fig. 4 shows an ion chromatogram of organoarsenic compounds in 5 m*M* phosphate eluent, pH 3.8, on a 200 mm × 0.5 mm I.D. column, with a detection wavelength of 210 nm, cell volume 0.6 μl and flow-rate 20 μl/min.

Calibration graph and interference. Calibration graphs were obtained by plotting the peak heights (Abs.) against the concentrations of samples (ppm as As), The graphs

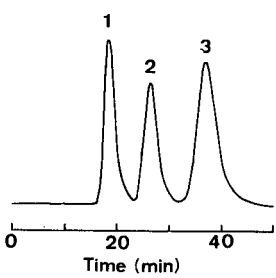

Fig. 4. Chromatogram of organoarsenic compounds. Sample: 1 = p-APA; 2 = o-NPA; 3 = o-APA. Eluent: 5 m*M* phosphate (pH 3.8); flow-rate, 20 μl/min. Detection wavelength: 210 nm. Column: 200 mm × 0.5 mm I.D.

were linear in the range 50–0.4 ppm. Detection limits obtained from the plots were 0.06 ppm for p-APA, 0.07 for o-APA and 0.08 ppm for o-NPA (three times the noise level). As(III) and As(V) did not interfere because the former was not dissociated in the pH range used and the latter did not absorb in the UV range used.

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CHROM. 21 227

Note

Programmable temperature vaporizer applications in an high-resolution gas chromatographic method for the quantitation of impurities in illicit heroin

ELISABETTA BERTOL*, FRANCESCO MARI and MARIA GRAZIA DI MILIA
Forensic Medicine Department of Florence University, Viale Morgagni 50134, Careggi, Florence (Italy)
(Received December 19th, 1988)

The problem of comparison of various heroin batches in forensic toxicology is of great importance. The delicacy of this task, the results of which are often used as evidence of illicit narcotic dealing, requires an extreme precision and accuracy of quantitative data. In the heroin mixtures available on the street, the "marker" used for comparison is the concentration of impurities or, better, the ratios between these substances. In fact, these products, *i.e.*, 6-monoacetylmorphine, acetylcodeine, papaverine, narcotine, are present, in all heroin mixtures in minor or major concentrations; some of these are unchanged in time and their concentration ratios are therefore independent of subsequent dilutions or adulterations. Only 6-monoacetylmorphine changes in concentration with time, because of the low stability of the acetyl group in the 6-position; acetylcodeine, on the contrary, is more stable, so that traces of codeine are seldom present in the mixtures. Papaverine and narcotine concentrations are the most stable of the impurities.

Gas–liquid, high-performance liquid, and most often high-resolution gas chromatography (HRGC) on capillary columns have been employed to separate these substances and good results were obtained, especially with temperature-programmed methods[1–15]. In HRGC, the traditional "split", and the "split–splitless" inlet in a single injection block, offer some advantages but some limitations also: insufficient accuracy of quantitative data; discrimination of high-molecular-weight compounds; poor linearity of the detector response, especially at low concentrations. These disadvantages can be obviated with the "on-column" capillary inlet; however this system has other inconveniences, and it is complementary and not substitutive of the "split–splitless" inlet. Ideally, an inlet for capillary columns should provide the following: improved accuracy and precision over conventional split–splitless injection; reduced discrimination of high-molecular-weight compounds; the possibility of injecting thermally sensitive samples. Our experience has demonstrated that all these aims can be achieved by employing a programmable temperature vaporizer inlet (PTV).

The PTV inlet is essentially a split–splitless capillary inlet which is temperature programmable. It eliminates the discrimination problems when analysing compounds with a large range of boiling points, with a consequent high linearity and reproducibility. The sample can be introduced from the syringe needle into the inlet

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under cold conditions, eliminating the major cause of sample discrimination and also reducing chances for sample thermolysis. After introduction, the sample is rapidly vaporized, because of the rapidity of heating of the inlet: its temperature is programmable from 50 to 400°C; the PTV inlet is heated to 200°C within 10 s, evenly across the entire inlet. The time to 90% temperature increase is about 30 s. The heating system of the injector body is programmed using an air flow; the vaporizer is a quartz liner; the splitting ratio is set as desired and programmable with an automatic valve (in split or in split–splitless mode). Rapid cooling of the injector is a further benefit of this injection system. Furthermore it is possible to obtain a pre-separation of solvent from the solute by selecting the "solvent purge" mode; here, the solvent is vented during cold injection. Once the purge is complete, the split valve is closed and the sample, remaining in the inner liner, is vaporized into the column by temperature programming in the inlet, reducing the peak profile distortion.

We applied this inlet system from some months in our studies on heroin mixtures, planning experimentally all parameters for simultaneous separation of narcotics from manufacturing and/or original products, varying the inlet and the oven programmable temperature and computing the standard deviation and coefficient of variation of for some analyses of illicit heroin samples.

EXPERIMENTAL

Equipment

A Perkin-Elmer series 8500 PTV gas chromatograph for capillary columns with a data handling facility was used. Injector: PTV capillary inlet, 45–370°C; split flow on (50 ml/min) from 1.0 min to the end of analysis. Detector: flame ionization, at 350°C. Oven: 240°C for 1.0 min; 10°C/min to 300°C; 300°C for 3.0 min. Column: DB-1 fused-silica capillary, 30 m × 0.253 mm I.D., 0.25-μm film. Carrier: hydrogen at 20 p.s.i.

Materials

Heroin, 6-monoacetylmorphine, acetylcodeine, papaverine, and narcotine were part of the reference collection of our Forensic Toxicology Laboratory. Dieldrin was obtained from Fluka (Buchs, Switzerland). All reagents, analytical grade, were obtained from E. Merck (Darmstadt, F.R.G.).

Method

The calibration graphs were constructed from alcoholic solutions of standard pure substances at known concentrations selected to correspond to those of the same substances in illicit heroin street samples. The concentration ranges were: heroin, 0.600–9.600; 6-monoacetylmorphine, 0.0625–1.000; acetylcodeine, 0.065–1.040; papaverine and narcotine, 0.125–2.000 mg/ml. Each graph was based on five concentrations. The internal standard was dieldrin, in alcoholic solution at 0.250 mg/ml.

An aliquot of each calibration solution was injected into the column. Ten determinations at different times were carried out for each concentration of each calibration solution group. The average value ($\pm$S.D.) of the ratios substance:internal standard for each calibration point was used to study the linearity of the response, the reproducibility and the precision of the quantitative data.

A linear relationship between the substance amount and the ratio substance/internal standard was observed for each calibration point. An excellent reproducibility

was obtained for each substance at each concentration: the coefficient of variation (C.V.) was in all cases very low (Table I).

On the basis of these calibration graphs, twenty illicit heroin samples, seized in the Florence area, were examined. The samples were chosen from a large number of mixtures, picking out those in which the quantitative composition as regards the heroin percentage might range from a very low to the highest concentration, with all impurities present, at various concentrations.

An 10-mg amount of each sample was homogenized and dissolved in 1.0 ml of ethanol; 1.0 ml of the internal standard solution was added and the contents of heroin, 6-monoacetylmorphine, acetylcodeine, papaverine and narcotine were calculated by reference to the calibration graphs with "data handling" according to the "internal standard method". Each heroin sample solution was examined ten times, and the average of all results, expressed as a percentage of the sample weight, was employed to calculate the standard deviation and the coefficient of variation. Fig. 1 shows a typical chromatogram obtained from an illicit heroin sample.

RESULTS AND DISCUSSION

Table II shows the composition of the twenty heroin samples chosen for the present study. The aim of the study was the evaluation of the performance of the GC capillary column method with the programmable temperature inlet. In fact the PTV

TABLE I

CALIBRATION DATA FOR EACH SUBSTANCE TESTED AT FIVE DIFFERENT CONCENTRATIONS

$n = 10$.

Substance	*Standard solutions: calibration*						*Regression equation*
Heroin	Concentrations (mg/ml)	0.600	1.200	2.400	4.880	9.600	$y = 0.59x - 0.255$
	Ratios average	0.125	0.327	1.226	2.632	5.359	($r^2 = 0.999$)
	S.D.	0.051	0.030	0.045	0.110	0.042	
	C.V. (%)	1.05	1.24	2.48	2.19	0.68	
Monoacetylmorphine	Concentrations (mg/ml)	0.0625	0.125	0.250	0.500	1.000	$y = 0.99x - 0.021$
	Ratios average	0.050	0.108	0.224	0.452	0.983	($r^2 = 0.999$)
	S.D.	0.005	0.015	0.021	0.030	0.025	
	C.V. (%)	1.85	1.28	0.80	0.79	1.83	
Acetylcodeine	Concentrations (mg/ml)	0.065	0.130	0.260	0.520	1.040	$y = 1.08x - 0.007$
	Ratios average	0.064	0.134	0.270	0.555	1.098	($r^2 = 0.999$)
	S.D.	0.002	0.001	0.003	0.003	0.004	
	C.V. (%)	2.46	1.27	1.30	0.98	1.25	
Papaverine	Concentrations (mg/ml)	0.125	0.250	0.500	1.000	2.000	$y = 0.72x - 0.034$
	Ratios average	0.070	0.151	0.319	0.665	1.420	($r^2 = 0.999$)
	S.D.	0.001	0.002	0.006	0.005	0.032	
	C.V. (%)	1.24	0.95	2.02	0.79	2.58	
Narcotine	Concentrations (mg/ml)	0.125	0.250	0.500	1.000	2.000	$y = 0.30x - 0.018$
	Ratios average	0.028	0.061	0.127	0.263	0.588	($r^2 = 0.999$)
	S.D.	0.001	0.001	0.006	0.012	0.018	
	C.V. (%)	1.55	1.80	2.80	1.55	3.85	

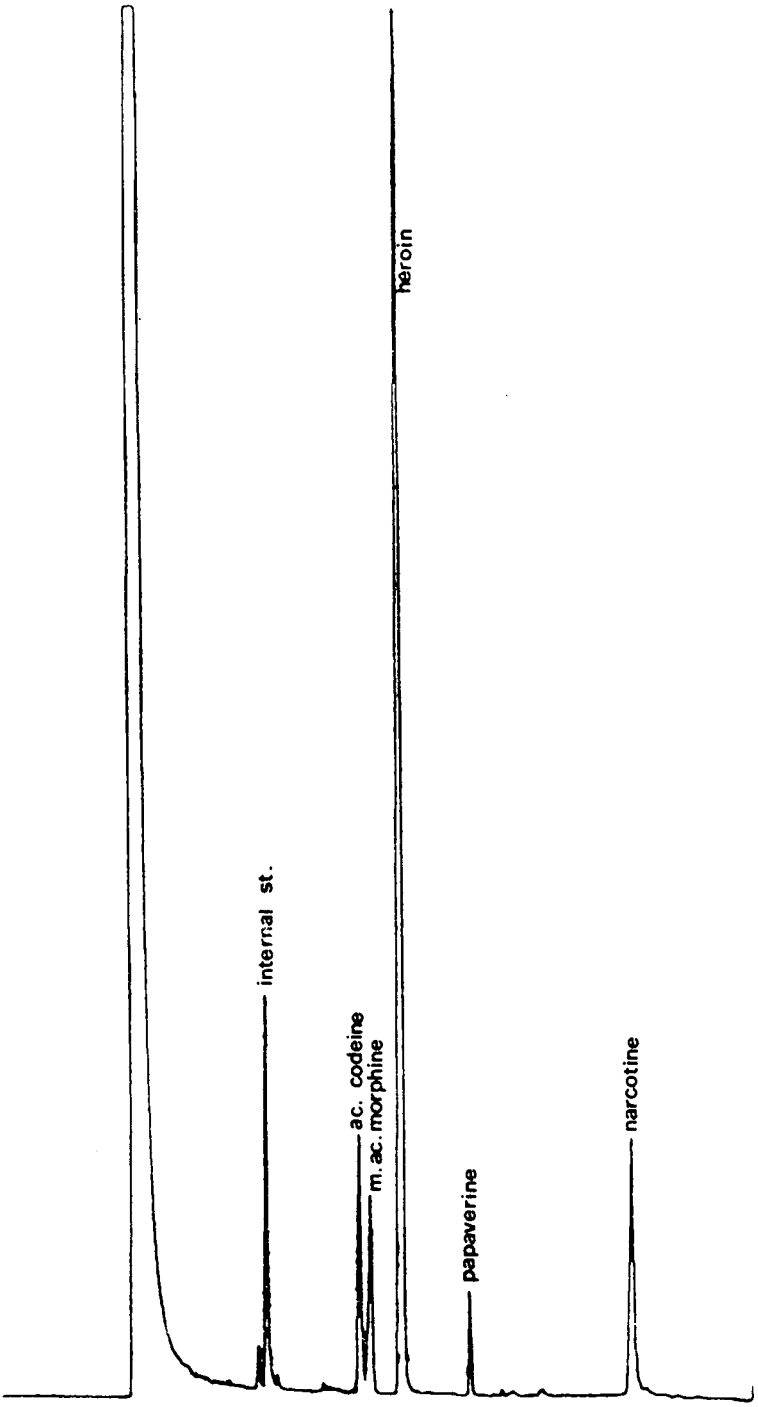

Fig. 1. Typical chromatogram obtained from an illicit heroin sample.

TABLE II

RESULTS OBTAINED FOR TWENTY STREET HEROIN SAMPLES: STATISTICAL STUDY

M = Mean; n = 10.

Sample		*Heroin*	*Monoacetylmorphine*	*Acetylcodeine*	*Papaverine*	*Narcotine*
1	M	57.092	0.027	0.924		13.307
	S.D.	0.704	0.001	0.002		0.030
	C.V. (%)	1.23	2.83	2.51		1.63
2	M	65.758	4.828	3.871	0.633	1.601
	S.D.	0.226	0.024	0.003	0.002	0.001
	C.V. (%)	0.34	2.78	0.89	1.42	2.74
3	M	69.240	0.603	1.193		12.167
	S.D.	0.217	0.003	0.001		0.002
	C.V. (%)	0.31	2.54	1.41		1.43
4	M	43.881	5.392	3.042	2.040	13.498
	S.D.	0.164	0.031	0.006	0.002	0.013
	C.V. (%)	0.38	2.38	1.95	2.01	2.66
5	M	5.942	2.542	0.497	0.632	9.863
	S.D.	0.104	0.001	0.005	0.001	0.002
	C.V. (%)	1.76	0.46	1.07	2.13	1.04
6	M	10.941	5.422	0.827	0.872	6.214
	S.D.	0.730	0.003	0.001	0.0008	0.001
	C.V. (%)	0.67	0.70	1.02	1.79	0.75
7	M	55.653	1.426	4.325	0.658	12.256
	S.D.	0.606	0.001	0.007	0.0005	0.013
	C.V. (%)	1.09	1.25	2.07	1.83	1.34
8	M	77.537	1.296	4.128	0.853	13.523
	S.D.	1.030	0.004	0.004	0.0008	0.012
	C.V. (%)	1.33	1.49	0.98	1.74	2.99
9	M	17.461	0.421	0.854	0.481	2.854
	S.D.	0.372	0.0005	0.0008	0.0007	0.0005
	C.V. (%)	2.13	2.41	0.93	2.89	0.80
10	M	34.061	0.965	2.115	1.943	11.715
	S.D.	0.311	0.004	0.001	0.001	0.006
	C.V. (%)	0.91	2.35	0.87	1.27	2.11
11	M	31.311	0.721	0.652		13.844
	S.D.	0.472	0.003	0.004		0.039
	C.V. (%)	1.51	2.33	2.35		3.35
12	M	23.373	1.459	1.295	0.658	3.112
	S.D.	0.264	0.003	0.001	0.001	0.001
	C.V. (%)	1.13	2.70	1.16	2.13	1.47
13	M	21.327	1.325	1.215	0.621	2.589
	S.D.	0.222	0.002	0.002	0.0007	0.002
	C.V. (%)	1.04	2.42	1.90	2.36	1.43
14	M	20.914	1.208	1.214	0.685	2.745
	S.D.	0.116	0.008	0.000	0.0004	0.001
	C.V. (%)	0.56	3.29	0.00	1.50	1.67
15	M	31.160	0.658	0.621		18.845
	S.D.	0.355	0.003	0.001		0.024
	C.V. (%)	1.14	2.08	3.11		3.09
16	M	58.620	10.845	7.528	6.948	25.383
	S.D.	0.691	0.055	0.008	0.002	0.085
	C.V. (%)	1.18	2.46	1.05	1.06	0.49
17	M	70.535	1.022	4.820	1.009	12.480
	S.D.	0.411	0.004	0.003	0.0005	0.015
	C.V. (%)	0.58	1.64	0.91	1.16	3.89

TABLE II (*continued*)

Sample		*Heroin*	*Monoacetylmorphine*	*Acetylcodeine*	*Papaverine*	*Narcotine*
18	M	45.653	0.711	2.854	0.481	10.835
	S.D.	0.308	0.003	0.003	0.0007	0.008
	C.V. (%)	0.68	1.52	1.36	2.83	2.18
19	M	59.438	0.398	1.320		3.911
	S.D.	0.514	0.001	0.009		0.002
	C.V. (%)	0.87	3.41	2.65		3.63
20	M	45.810	0.720	2.600	0.750	8.323
	S.D.	0.385	0.001	0.001	0.005	0.011
	C.V. (%)	0.84	2.54	0.59	1.38	2.85

quickly and simply resolves the problems of quantitative analysis: the low standard deviation and the very low coefficient of variation (always less than 4.0 and some times = 0) for each component shows the high reproducibility of the method, even when very low or high concentrations of substances were determined. This is of great importance because in street heroin samples the contents of heroin and its impurities are very variable. Furthermore, in forensic toxicology, only high reproducibility and accuracy of the quantitative data permit the comparison of samples in most cases it will be possible to ascertain whether two or more samples are different and, in many cases, to determine with reasonable certainty whether two or more samples have a common origin. The PTV inlet in combination with HRGC provided sufficient accuracy and reproducibility in quantitation for street heroin mixtures, at any concentration of heroin and all impurities that occur in the black market samples. This is very important in Forensic Toxicology where the exact chemical profile of the samples forms the basis for comparative analysis. It also makes it possible to distinguish the places of the origin of the heroin samples and their distribution routes.

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Note

High-performance liquid chromatographic analysis of naturally occurring glycosides and saponins

TORU OKUYAMA*, MITSUMASA TAKATA and KUNIO TAKAHASHI
Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy, 1-35-23 Nozawa, Setagaya-ku, Tokyo 154 (Japan)
and
TOSHIHIRO ISHIKAWA, KENNJI MIYASAKA and NAOKI KANEYAMA
Toa Nenryo Kogyo Co. Ltd., 1-1-1 Hitotsubashi, Chiyoda-ku, Tokyo 100 (Japan)
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Reversed-phase high-performance liquid chromatography (HPLC) has been efficiently applied to the separation of glycosides. We have already demonstrated that an Aquasil silica gel column with an aqueous mobile phase was effective for the separation of water-soluble glycosides by HPLC[1]. It was efficiently applied to the separation of Ginseng saponins, Bupleurum saponins, Cardiac glycosides, Paeony glycosides, Swertia herb glycosides and Pueraria glycosides. Recently, a hydroxyapatite column has been developed for the HPLC separation and purification of a wide variety of peptides, proteins, enzymes and nucleic acids[2–6]. Using this HPLC column, Kasai *et al.*[7] first demonstrated the efficient separation of glycosides and sugars. This paper describes the application of this column to the separation of monoterpene, cyanogenic and coumarin glycosides and saponins.

EXPERIMENTAL

Plant materials

The following were used: Bupleuri Radix (*Bupleurum falcatum* Linne) (Umbelliferae); *Bupleurum* root, purchased on the Chinese drug market; *B. longiradiatum* Turcz., collected in the medicinal plant garden of the high-coldish ground at Yatsugatake, Meiji College of Pharmacy; Zi-Hua Qian-Hu [*Peucedanum decursivum* Maxim. (= *Angelica decursiva* Fr. et Sav.) (Umbelliferae)]; Bai-Hua Qian-Hu [*P. praeruptorum* Dunn.]; Persicae Semen (*Prunus persica* Batsch) (Rosaceae) and Paeoniae Radix (*Paeonia lactiflora* Pallas) (Paeoniaceae).

Extraction

The pulverized plant material (5 g) was extracted with 50 ml of hot methanol and the concentrated extract was loaded on an Amberlite XAD-2 (1 g) column and eluted twice with methanol to obtain a glycoside fraction. The residue after evaporation was dissolved in 100 μl of methanol and a 5-μl aliquot was injected to HPLC.

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Apparatus and conditions for HPLC

The HPLC instrument consisted of a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), SP-8700XR solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.), Y-1000 UV detector (Senshu Scientific, Tokyo, Japan) and SIC-7000A integrator equipped with an 8-in. disk drive system (System Instruments, Hachioji City, Tokyo, Japan).

A TPS-326010 HPLC column (100 × 7.5 mm I.D.) packed with hydroxyapatite (2.2 μm) (Toanenryo Kogyo, Tokyo, Japan) and an Aquasil SS 452N column (250 × 4.5 mm I.D.) packed with aqueous silica gel (5 μm) (Senshu Scientific) were used.

The volumetric ratios of the components of the mobile phase and the linear gradient used are shown in Table I. All solvents were of HPLC grade (Nakarai, Kyoto, Japan).

TABLE I
MOBILE PHASE COMPOSITIONS

Column	*Solvent system*	*Gradient*				
		Initial state		*Final state*		*Time (min)*
		Acetonitrile	*Water*	*Acetonitrile*	*Water*	
Hydroxyapatite	A	90	10	70	30	120
	B	90	10	50	50	60
	C	95	5	85	15	60
	D	95	5	65	35	60
	E	95	5	75	25	60
		Isocratic				
		Chloroform	*Methanol*	*Water*		
Aqueous silica gel	F	60	12	1		
	G	30	10	1		

Authentic samples

All the components separated were identified by comparison with authentic samples. The authentic sample of amygdalin was a commercial product (Nakarai) and all others were isolated as described previously[8–11].

RESULTS AND DISCUSSION

The geometric location of adsorbing groups, *e.g.*, carboxyl, phosphate, amino and guanidinyl groups, on the local molecular surface of macromolecules would fit the active surface of hydroxyapatite, but the adsorbing mechanism for lower molecular mass organic compounds has not fully been elucidated.

HPLC on a hydroxyapatite column has now been used for the separation of hydrophilic plant glycosides, such as the saponins of Bupleuri Radix and Zi-Hua

Qian-Hu, the cyanogenic glycosides of peach kernel, the monoterpene glycosides of paeony root and the coumarin glycosides of Qian-Hu, Zi-Hua Qian-Hu and Bai-Hua Qian-Hu.

Saponins of Bupleurum root

The extract of *Bupleurum falcatum* contained three major triterpenoid saponins, named saikosaponin a, c and d. Kaizuka and Takahashi[1] previously reported the separation of saikosaponins using an Aquasil column. The present HPLC system on hydroxyapatite also gave excellent results, and the different *Bupleurum* species corresponded to the elution profies of saikosaponins, as shown in Fig. 1.

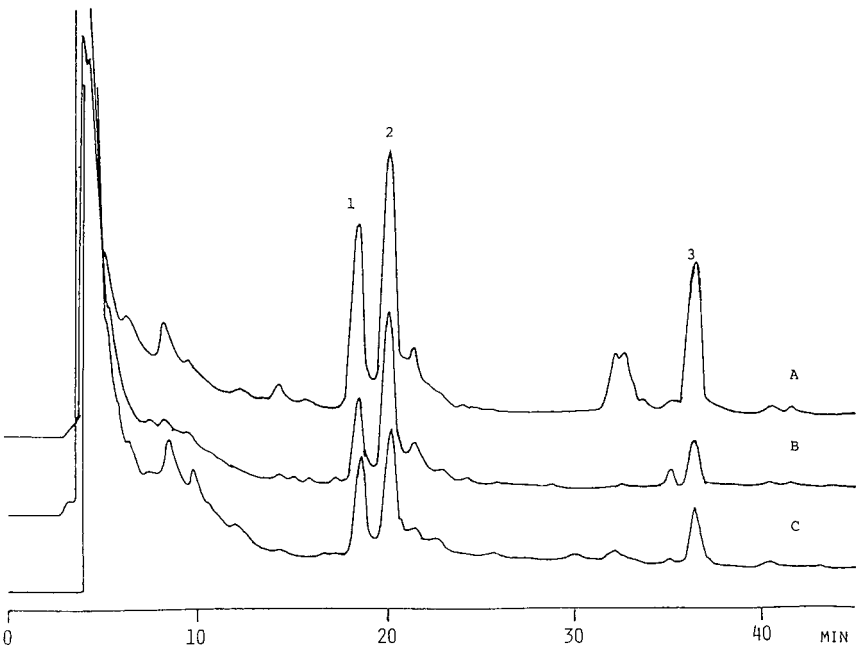


Fig. 1. HPLC profiles of XAD-2 fractions of Bupleuri Radix. (A) *B. falcatum*; (B) roots purchased on the Chinese drug market; (C) *B. longiradiatum*. Column, hydroxyapatite; eluent, solvent A (Table I); flow-rate, 1.0 ml/min; detector, UV (205 nm). Saikosaponins: 1 = d; 2 = a; 3 = c.

Saponins of Zi-Hua Qian-Hu

We isolated five bisdesmoside saponins having an oleanane skeleton as the sapogenin hederagenin from Zi-Hua Qian-Hu, as shown in Fig. 2[12]. Among these saponins Pd saponin V, 3-arabinopyranosyl-28-gentiobiosylhederagenin, was identified with an authentic sample isolated from *Akebia quinata* by Higuchi and Kawasaki[13]. The structural elucidation of the other four new saponins will be reported elsewhere. Saponins of higher molecular mass (over 1000) could not be separated well by Aquasil, whereas a good separation was achieved by using the hydroxyapatite column. Longer retention times are given by the saponins that have longer sugar residues.

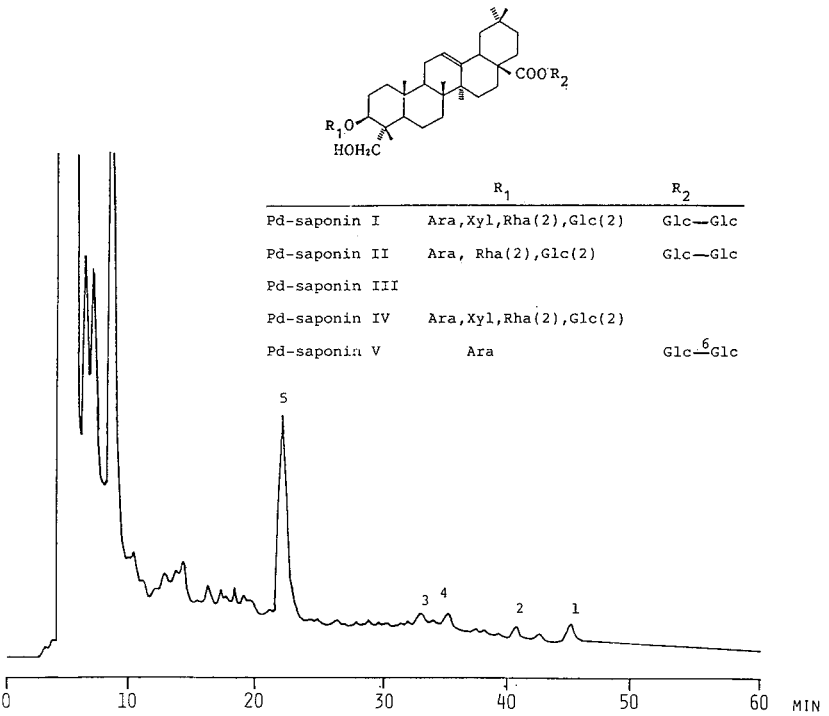


Fig. 2. HPLC profile of XAD-2 fraction of Zi-Hua Qian-Hu. Column, hydroxyapatite; eluent, solvent B (Table I); flow-rate, 1.0 ml/min; detector, UV (205 nm). Pd-saponins: 1 = V; 2 = III; 3 = IV; 4 = II; 5 = I.

Monoterpene glucosides of Paeoniae Radix

Paeoniflorin is the main glycosidic component of peony root. A good separation was obtained of the monoterpene glucoside, paeoniflorin and oxypaeoniflorin. The order of retention times was the same using both Aquasil and hydroxyapatite columns (Fig. 3).

Cyanogenic glucosides of peach kernel

Amygdalin, a cyanogenic glucoside, is a major component of peach kernel. Amygdalin is easily hydrolysed with emulsin to form benzaldehyde, hydrogen cyanide

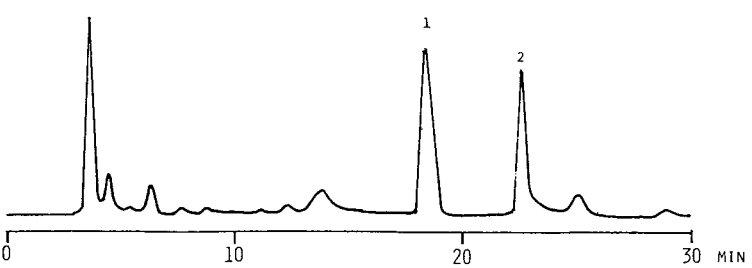


Fig. 3. HPLC profile of XAD-2 fraction of Paeoniae Radix. Column, hydroxyapatite; eluent, solvent C (Table I); flow-rate, 1.0 ml/min; detector, UV (254 nm). Peaks: 1 = paeoniflorin; 2 = oxypaeoniflorin.

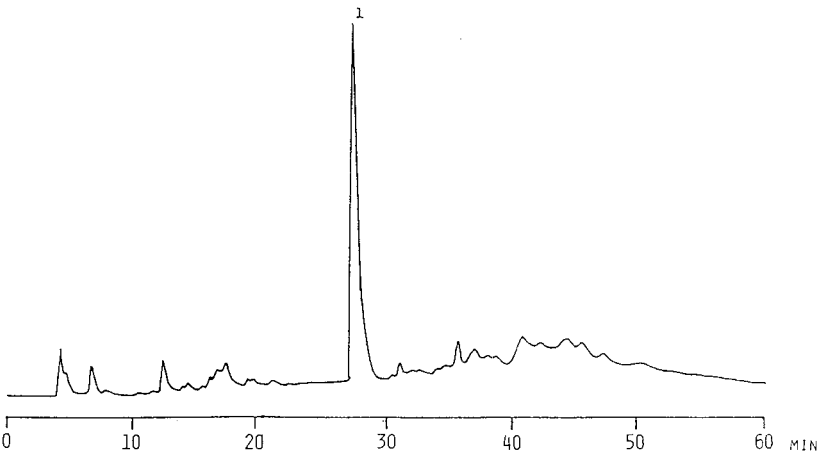
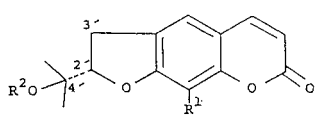
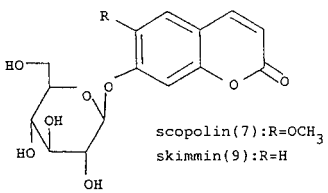


Fig. 4. HPLC profile of XAD-2 fraction of *Prunus persica* seed. Column, hydroxyapatite; eluent, solvent D (Table I); flow-rate, 1.0 ml/min; detector, UV (254 nm). Peak 1 = amygdalin.

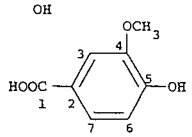
and glucose. The odour of benzaldehyde is perceptible by grinding peach kernel with water. On the hydroxyapatite column, a good separation of amygdalin was achieved, as shown in Fig. 4.



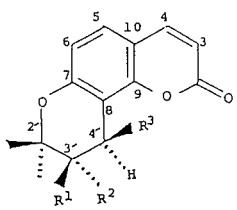


praeroside-I(1):R^1=OH,R^2=β-D-glc^{6}vanillic acid
marmesinin(4):R^1=H,R^2=β-D-glc
rutarin(6):R^1=O-β-D-glc,R^2=H
isorutarin(8):R^1=OH,R^2=β-D-glc

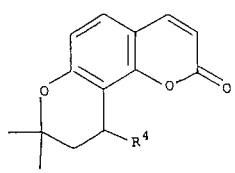
scopolin(7):R=OCH_3
skimmin(9):R=H



vanillic acid



praeroside-IV(2):R^1=O-glc,R^2=H,R^3=H
praeroside-II(3):R^1=O-glc,R^2=H,R^3=OH
praeroside-III(5):R^1=H,R^2=O-glc,R^3=OH



praeroside-V(10):R^4=O-glc

Fig. 5. Structures of coumarin glucosides from Bai-Hua Qian-Hu (Q-I type).

Coumarins of Qian-Hu

According to the ^{1}H NMR spectral data and the silica gel thin-layer chromatographic (TLC) densitometric profiles of the ethereal extracts, Qian-Hu on the drug market can be classified into four groups: Q-I, Q-II [both Chinese Bai-Hua series (*Peucadanum praeruptorum* root)], Q-III [including Chinese Zi-Hua series (*P. decursivum* root), identical with Japanese *Angelica decursiva* (Nodake root) and Q-IV (Korean *Anthriscus nemorosa* root)[14].

Coumarin glycosides of Bai-Hua Qian-Hu. From the *n*-butanol-soluble fraction of methanolic extracts derived from Bai-Hua Qian-Hu (Q-I type), the root of *P. praeruptorum*, four angular-type coumarin glycosides, praeroside II, III, IV and V, four linear-type coumarin glycosides, praeroside I, marmesinin, rutarin and isorutarin, and two simple coumarin glycosides, skimmin and scopolin, were isolated by using the Aquasil column and their structures[8,9] were established (Fig. 5).

The chromatography was performed using Aquasil or hydroxyapatite columns. The linear-type coumarin glycosides gave the same elution order on both columns. However, with the angular-type coumarin glycosides, the order of elution of praeroside II and III and also of skimmin and scopolin were the opposite on Aquasil and hydroxyapatite (Figs. 6 and 7).

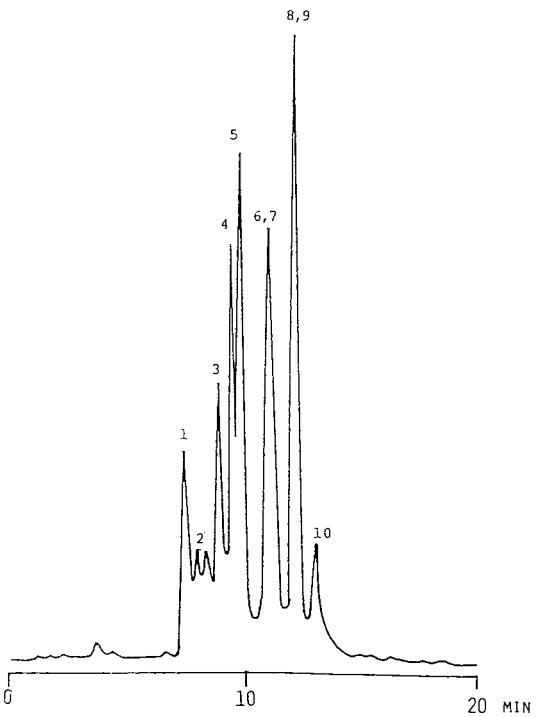


Fig. 6. HPLC profile of XAD-2 fraction of Bai-Hua Qian-Hu (Q-I type). Column, Aquasil; eluent, solvent F (Table I); flow-rate. 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = praeroside I; 2 = preaeroside IV; 3 = paeroside II; 4 = marmesinin; 5 = praeroside III; 6 = rutarin; 7 = scopolin: 8 = isorutarin; 9 = skimmin; 10 = praeroside V.

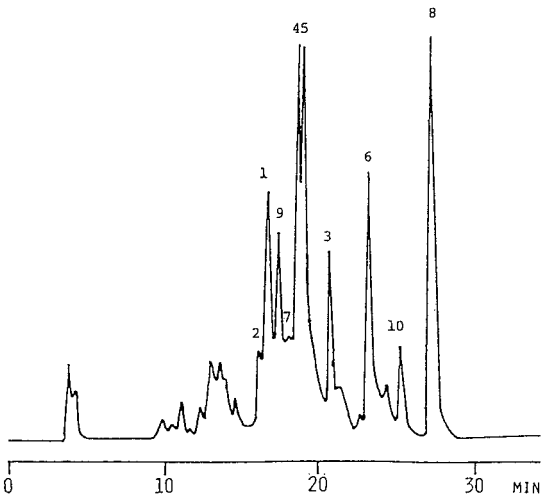
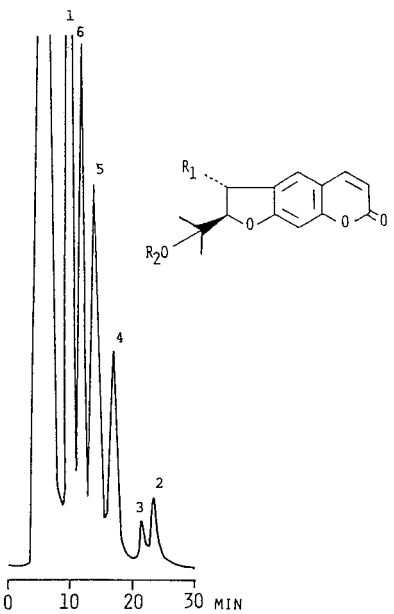


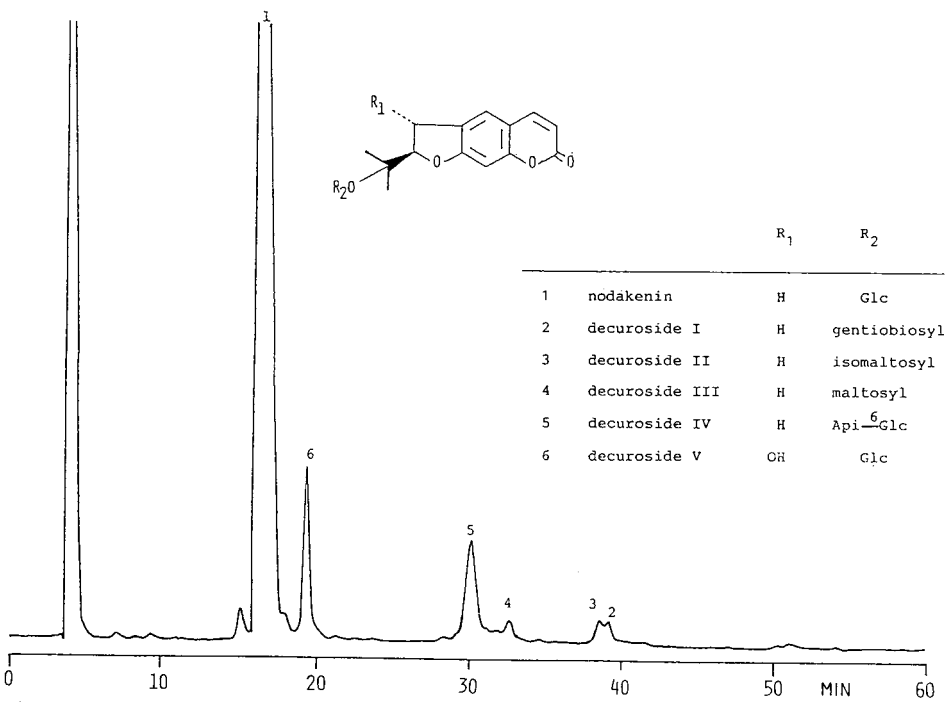
Fig. 7. HPLC profile of XAD-2 fraction of Bai-Hua Qian-Hu (Q-I type). Column, hydroxyapatite; eluent, solvent C (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = praeroside I; 2 = praeroside IV; 3 = praeroside II; 4 = marmesinin; 5 = praeroside III; 6 = rutarin; 7 = scopolin; 8 = isorutarin; 9 = skimmin; 10 = praeroside V.



		R_1	R_2
1	nodakenin	H	Glc
2	decuroside I	H	gentiobiosyl
3	decuroside II	H	isomaltosyl
4	decuroside III	H	maltosyl
5	decuroside IV	H	Api—(6)—Glc
6	decuroside V	OH	Glc

Fig. 8. HPLC profile of XAD-2 fraction of Zi-Hua Qian-Hu (Q-III type). Column, Aquasil; eluent, solvent G (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = nodakenin; 2 = decuroside I; 3 = decuroside II; 4 = decuroside III; 5 = decuroside IV; 6 = decuroside V.

Coumarin glycosides of Zi-Hua Qian-Hu. Six furocoumarin glycosides were isolated from Zi-Hua Qian-Hu, the root of *P. decursivum*[10,11], as shown in Fig. 8. Nodakenin was the main component of coumarin glycosides, and decuroside V was identical with 3′-hydroxynodakenin. Decuroside I–IV were differentiated according to their sugar moieties. An almost complete separation can be achieved using the Aquasil column (Fig. 8). Those having a disaccharide moiety including a branched sugar, apiose, had shorter retention times than those of glucobiosides. In the sugar linkage attached to the aglycone, nodakenin is different in decuroside I, II and III, and the order of retention times was III, II and I, which have α-(1–4)-, α-(1–6)- and β-(1–6)-diglucosyl moieties, respectively. The elution profile of the coumarin glycosides using a hydroxyapatite column was similar to that with an Aquasil column (Fig. 9). However, decuroside I and II could not be successfully separated on the hydroxyapatite column.



		R_1	R_2
1	nodakenin	H	Glc
2	decuroside I	H	gentiobiosyl
3	decuroside II	H	isomaltosyl
4	decuroside III	H	maltosyl
5	decuroside IV	H	Api$\frac{6}{}$Glc
6	decuroside V	OH	Glc

Fig. 9. HPLC profle of XAD-2 fraction of Zi-Hua Qian-Hu (Q-III type). Column, hydroxyapatite; eluent, solvent E (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = nodakenin; 2 = decuroside I; 3 = decuroside II; 4 = decuroside III; 5 = decuroside IV; 6 = decuroside V.

CONCLUSION

On the basis of these results, the advantages of the hydroxyapatite HPLC column can be summarized as follows. Excellent separations were achieved using a simple solvent system. Owing to the increase in the number of hydroxyl groups, the retention times increased. Concerning the sugar moieties, the diglucosal linkage shows

the order of retention times α-(1–4)- < α-(1–6)- < β-(1–6)-. The operating pressure required is lower than that in any other HPLC columns currently available. Parallel correlations exist between the elution sequence of the compounds in HPLC and their R_F values in silica gel TLC using chloroform–methanol–water as the solvent system. The HPLC column is easily regenerated by washing with 0.1 *M* sodium hydroxide solution. The column is widely applicable to the separation of naturally occurring glycosides, even high-molecular-mass compounds such as saponins.

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CHROM. 21 191

Note

The use of new chromatographic techniques for the isolation and purification of phenolic acids from *Indigofera heterantha*

AURANGZEB HASAN*
Department of Chemistry, Quaid-i-Azam University, Islamabad (Pakistan)
PETER WATERMAN
Department of Pharmacy, University of Strathclyde, Glasgow (U.K.)
and
NABILA IFTIKHAR
Department of Chemistry, Quaid-i-Azam University, Islamabad (Pakistan)
(Received December 8th, 1988)

Although standard procedures based on thin-layer, paper and column chromatography still play a major rôle in the isolation and purification of plant phenolics[1,2], the development of numerous new separation techniques has considerably increased the speed of separation and degree of resolution. The new technique available today to plant chemists include mainly high-performance liquid chromatography (HPLC), centrifugal thin-layer chromatography (CTLC), flash and vacuum liquid chromatography (VLC). HPLC is now a well established technique and has proved its usefulness in the separation of numerous types of phenolics[1,3–5]. CTLC also known as Circular Chromatotron has been successfully used for the isolation and purification of various coumarins[6,7] and Xanthones[8]. Flash liquid chromatography and VLC are two rapid techniques[9,10] which are particularly useful for fractionation of crude plant extracts. However it should be noted that all these chromatographic methods are not mutually exclusive, but complementary. Pure compounds from crude plant extracts can be obtained rapidly only by combining the various chromatographic techniques available.

Phytochemical studies of Indigofera species have shown the presence of various phenolic compounds[11–13]. In the present work, attempts have been made to isolate and purify rapidly phenolic acids from leaves and flowers of *Indigofera heterantha* (Leguminosae) by the use of newly developed chromatographic techniques.

EXPERIMENTAL

Plant material

Branches bearing leaves and flowers of *Indigofera heterantha* were collected from Murree Hills (Pakistan) in mid-May 1988.

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Extraction

The phenolic acids were extracted from air-dried leaves and flowers of *I. heterantha* by acid hydrolysis of the plant material, using the standard method of hydrolysis[14].

Isolation and purification

The following chromatographic techniques were employed for the isolation and purification of phenolic acids.

Vacuum liquid chromatography (VLC). The methanolic leaf extract was deposited on top of a TLC grade polyamide column. Elution under vacuum was carried out with benzene progressively enriched with methyl ethyl ketone and methanol. Twenty six fractions were collected in 2 h. Fractions 4 and 5 contained a single pure phenolic acid (V'), violet in colour. Another bluish violet constituent (BV') was the main compound of fractions 6 and 7 along with V'. A third blue compound (B') along with a flavonic aglycone was present in fraction 13.

Column chromatography. All the three constituents of the leaf extract, *i.e.*, V', BV' and B' were allowed to percolate rapidly through a narrow polyamide column, using the same eluting solvents as for VLC.

Thin-layer chromatography (TLC). The purity of each compound was monitored on TLC plates in four solvent systems. Each phenolic acid appeared as a single pure spot in all the systems employed. Their R_F values were noted and are presented in Table I.

High-performance liquid chromatography (HPLC). In order to check finally the purity of the isolated phenolic acids, HPLC analysis was carried out. A Shimadzu HPLC LC-6A equipped with an UV detector and an auto injector was used. Though each of the phenolic acids appeared pure on TLC plates, HPLC analysis revealed the presence of impurities in each sample (Fig. 1). Moreover, it was observed that BV' still contained V'. Using a larger HPLC column it was possible to purify all the three acids by semi-preparative HPLC (Fig. 2).

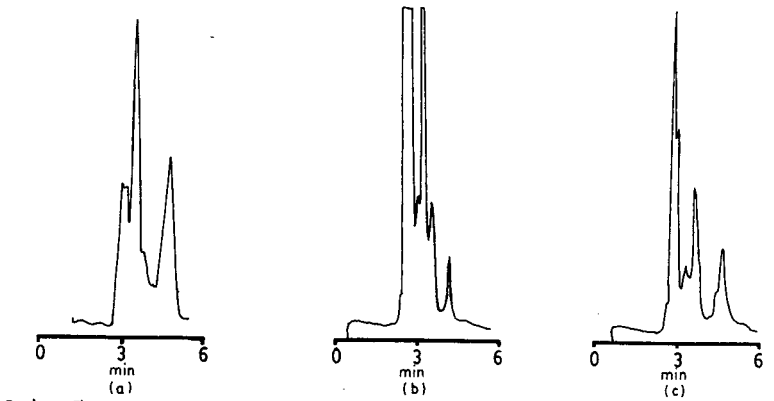
Flash chromatography. A methanolic flower extract of *I. heterantha* was deposited on top of a column-grade polyamide packed column, fitted with a solvent reservoir and a valve through which oxygen-free nitrogen was allowed to flow through the column. The flow-rate of the eluting solvents, chloroform, chloroform–methanol and

TABLE I

CHROMATOGRAPHIC AND UV SPECTRAL DATA OF PHENOLIC ACIDS IN *I. HETERANTHA*

Solvents on silica gel: 1 = acetic acid–chloroform (1:9); 2 = ethyl acetate–benzene (9:11). Solvents on cellulose: 3 = benzene–methanol–acetic acid (45:8:4); 4 = 6% aqueous acetic acid.

Compound	*TLC R_F (·100) in solvents*				*λ_{max} (nm)*		*Colour under UV*
	1	*2*	*3*	*4*	*Ethanol*	*Ethanol–sodium hydroxide*	
BV'	48	65	93	31	290	280	Blue-violet
V'	42	67	93	36	278, 314	300	Violet
B'	37	44	87	59	237, 332	312	Blue



Peak	Time	Area	Conc.
1	2.062	742351	22.3539
2	2.540	1181836	35.5878
3	2.920	238775	7.1900
4	3.998	1157943	34.8683

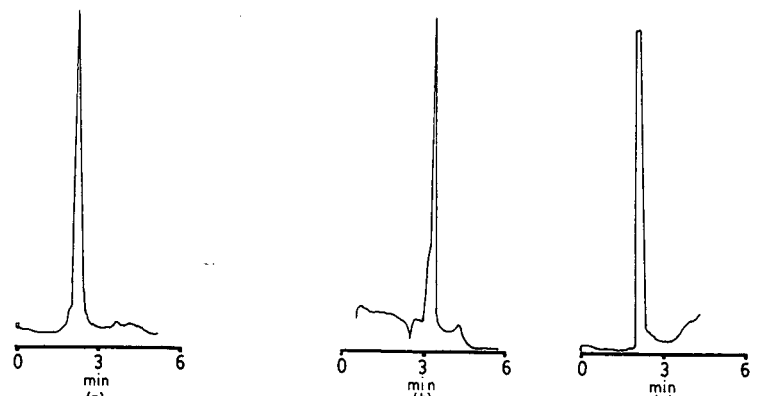
Peak	Time	Area	Conc.
1	0.298	75533	1.2621
2	2.415	4150426	69.3479
3	3.152	1204403	20.1239
4	3.520	347556	5.8072
5	4.270	207019	3.4590

Peak	Time	Area	Conc.
1	2.212	53474	2.2290
2	2.417	997150	41.5658
3	2.990	225515	9.4005
4	3.317	597274	24.8971
5	4.498	476821	19.8761
6	5.298	48734	2.0315

Fig. 1. HPLC analysis of (a) B′, (b) BV′, (c) V′. Column: Shim-pack CLC-ODS (0.15 m × 6.0 mm I.D.). Solvent: methanol–water–acetic acid (75:24:1). Flow-rate: 1.2 ml/min. Detection: fixed UV at 280 nm. Temperature: ambient.

methanol, was controlled by the flow valve. The ten fractions, which were collected under UV light at 366 nm in less than half an hour contained mainly flavonoid aglycones. However, fraction 1 was a equivalent mixture of two phenolic acids.

Centrifugal thin-layer chromatography (CTLC). Fraction 1 was applied to a 2 mm thick silica gel PF 254 rotor of a Circular Chromatotron Model 7924. The phenolic acids were eluted with chloroform enriched progressively with acetic acid. Two concentric bands PA1 and PA2 that separated by centrifugal force and under an inert



Peak	Time	Area	Conc.
1	2.613	105033	100

Peak	Time	Area	Conc.
1	3.20	41601	100

Peak	Time	Area	Conc.
1	2.423	135037	100

Fig. 2. Semi-preparative purification of (a) B′, (b) BV′, (c) V′. Column: Ultrasphere ODS (25 cm × 4.6 mm I.D.). Other conditions as in Fig. 1.

atmosphere were observed by UV light at 254 nm and were collected in separate sample tubes. Both these acids, shown to be very pure by HPLC, were co-chromatographed with the three phenolic acids obtained from leaves of *I. heterantha*. It was noted that PA1 was identical to BV′ and PA2 to B′.

On the basis of their chromatographic behaviour, colour under UV light and UV spectral data in ethanol and ethanol–sodium hydroxide (Table I), the three phenolic acids BV′, B′ and V′ were identified as protocatechuic, genestic and *p*-methoxycinnamic acids respectively.

CONCLUSIONS

The combined use of new as well as conventional chromatographic techniques is quite effective for the speedy isolation and purification of phenolic acids. The much shorter and increased resolution can improve the analysis of many more phenolic constituents.

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CHROM. 21 175

Note

Chromatographic methods for the determination of monomer, dimer and trimer fractions in dimer fatty acids[a]

T. CHANDRASEKHARA RAO, VIJAY KALE, P. VIJAYALAKSHMI, A. GANGADHAR, R. SUBBARAO and G. LAKSHMINARAYANA*
Oils and Fats Division, Regional Research Laboratory (CSIR), Hyderabad-500007 (India)
(First received February 8th, 1988; revised manuscript received December 8th, 1988)

Chromatographic techniques, namely column[1], thin-layer (TLC)[2,3], gel permeation (GPC)[4–6], high-performance gel permeation (HPGPC)[7], gas–liquid (GLC)[3,8–12] and high-performance liquid chromatography (HPLC)[12], and TLC and HPLC coupled with flame ionization detection (FID)[12,13], have been reported for the determination of dimer acids[1,4–6,12,13] and their methyl esters[2,3,7,8–12]. This paper describes improved GLC, HPGPC and TLC–FID methods for the determination of monomer, dimer and trimer contents in methyl esters of dimer acids.

EXPERIMENTAL

Materials

Commercial dimer acids were purchased from Emery Industries (Cincinnati, OH, U.S.A.). Dimer acids were also prepared in our laboratory's pilot plant by a process developed by us consisting in splitting castor oil in an autoclave[14], heating the split products and fractionation by molecular distillation into pure monomer, dimer (2.5% monomer, 94% dimer and 3.5% trimer as determined by GLC) and trimer (residue consisting of 27% dimer and 73% trimer plus higher polymers, as determined by GLC). The fractions and the residue were converted into their corresponding methyl esters using sulphuric acid as catalyst.

Methods

The GLC analysis was carried out using a Hewlett-Packard HP 5840 A instrument with dual flame ionization detectors and a stainless-steel column (61 cm × 4 mm I.D.) packed with 5% SE-30 on Chromosorb W and programmed from 170 to 330°C at 13°C/min. For HPGPC analysis a Waters Assoc. ALC/GPC 244 liquid chromatograph unit having a refractive index detector and μStyragel (styrene–divinylbenzene copolymer) columns (30 cm × 7.8 mm I.D.) of porosity 1000, 500, 500 and 100 Å connected in series was used. Tetrahydrofuran (freshly distilled over lithium aluminium hydride) was used as the eluent at a flow-rate of 1.5 ml/min. In the

[a] RRL(H) Communication No. 2136.

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TLC–FID method an Iatroscan Mark II TH-10 unit was used. *n*-Hexane–cyclohexane–diethyl ether (55:15:5, v/v/v) was used to develop the Chromarods.

RESULTS AND DISCUSSION

The methods were standardized using prepared mixtures of monomers (5–57%), dimers (28–79%) and trimers (9–28%).

The attempts of previous workers[11,12] to elute and determine monomer, dimer and trimer fractions in methyl esters of crude dimer acids by GLC using the dimer as a relative standard were unsuccessful because of the fluctuating response for the trimer fraction. This was ascribed to the retention of some of the less volatile polymers of the trimer fraction on the column[12]. Conditions were therefore chosen to elute only the monomer and dimer fractions (Fig. 1A). A known amount of methyl heptadecanoate (ACME Synthetic Chemicals, Bombay, India) was added as an internal standard to prepared mixtures of monomer and dimer fractions and the mean relative response factors of the monomer and dimer fractions with respect to the internal standard were calculated and found to be 0.79 ± 0.01 (S.D.) and 0.58 ± 0.01, respectively. Using these relative response factors, the amounts of monomer and

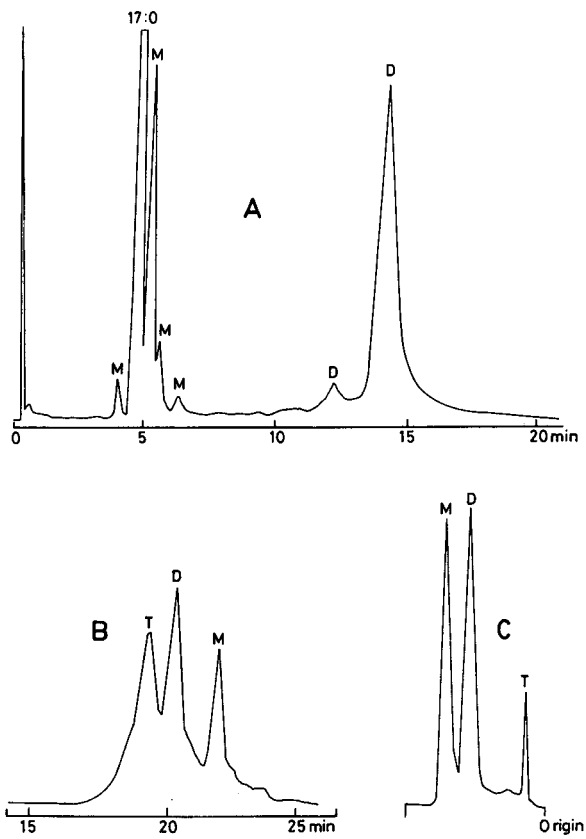

Fig. 1. Separation of dimer acid methyl esters by (A) GLC, (B) HPGPC and (C) TLC–FID. M = Monomer; D = dimer; T = trimer.

TABLE I

COMPARISON OF THE GLC, HPGPC AND TLC–FID ANALYSES OF DIMER ACID METHYL ESTERS

M = Monomer, D = dimer and T = trimer, all in % (w/w); Tr = trace.

Product[a]	*GLC*			*HPGPC*			*TLC–FID*			*Stated values*[15]		
	M	*D*	*T*	*M*	*D*	*T*	*M*	*D*	*T*	*M*	*D*	*T*
Empol 1018	Tr	81.2	18.8	Tr	80.1	19.9	Tr	80.5	19.5	Tr	83.0	17.0
Empol 1024	Tr	74.0	26.0	Tr	76.5	23.5	Tr	73.1	26.9	Tr	75.0	25.0
Empol 1040	Tr	22.4	77.6	Tr	20.1	79.9	Tr	23.0	77.0	Tr	20.0	80.0

[a] Emery Industries.

dimer fractions in prepared mixtures were calculated and the amount of trimer was found by difference. However, after analysis of 25–30 samples on the same column, baseline drift was found for the dimer peak, which affected the relative response factor of the dimer fractions.

The reported GPC methods[4–6] are time consuming. Haken and Obita[7] have described the determination of dimer acids in resinous polyamides by HPGPC, but detailed quantification was not discussed. The order of elution of monomers, dimers and trimers in the present HPGPC method is shown in Fig. 1B. The relative response factors of the monomer and trimer fractions with respect to the dimer were calculated[11] and found to be 0.58 ± 0.02 and 1.01 ± 0.03, respectively. These factors were used in the analysis of prepared mixtures.

In the reported TLC–FID method[12], dimer acids were analysed as such. To avoid tailing in TLC, methyl esters are generally preferred to acids. Of the various solvent systems tried for the separation of methyl esters of the monomer, dimer and trimer fractions by the present TLC–FID method using Chromarod S II, a mixture of *n*-hexane, cyclohexane and diethyl ether (55:15:5, v/v/v) gave best separations for efficient quantification. A typical chromatogram is shown in Fig. 1C. The monomer, dimer and trimer fractions showed equal responses and hence no correction factor was applied.

TABLE II

COMPARISON OF THE GLC, HPGPC AND TLC–FID ANALYSES OF FIVE CASTOR OIL-DERIVED DIMER ACID PREPARATIONS AS THEIR METHYL ESTERS

M = Monomer, D = dimer and T = trimer, all in % (w/w).

GLC			*HPGPC*			*TLC–FID*		
M	*D*	*T*	*M*	*D*	*T*	*M*	*D*	*T*
26.8	50.5	22.7	28.7	48.2	23.1	27.3	49.8	22.9
28.5	49.1	22.4	27.0	50.9	22.1	27.9	52.9	19.2
25.4	54.9	19.7	23.9	54.1	22.0	25.1	53.8	21.1
27.5	49.0	23.5	29.8	49.2	21.0	27.1	50.5	22.4
29.5	51.8	18.7	30.4	50.5	19.1	30.0	49.6	20.4

The maximum deviation from the actual percentage of any component in the prepared mixtures by any of the three methods was found to be 1.9% (data not shown). All three methods gave monomer, dimer and trimer contents in methyl esters of various commercial dimer acid samples obtained from Emery Industries in agreement with the stated values[15] (Table I), confirming the accuracy of the methods. All three methods gave almost the same compositions for the different batches of dimer acids prepared in the laboratory's pilot plant (Table II), confirming the reproducibility of the methods.

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Note

Resolution of neuroexcitatory non-protein amino acid enantiomers by high-performance liquid chromatography utilising pre-column derivatisation with *o*-phthaldialdehyde chiral thiols

Application to ω-N-oxalyl diamino acids

MELVIN R. EUERBY*
Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX (U.K.)
PETER B. NUNN
Department of Biochemistry, King's College London, Strand, London WC2R 2LS (U.K.)
and
LYNDA Z. PARTRIDGE
Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX (U.K.)
(First received November 2nd, 1988; revised manuscript received January 9th, 1989)

The ω-N-oxalyl derivatives of L-α,β-diaminopropanoic acid (L-β-ODAP, **1a**) and L-α,γ-diaminobutanoic acid (L-γ-ODAB, **2a**) are both natural products which were first isolated from the seeds of *Lathyrus* species[1–3], L-β-ODAP is believed to be the major causative agent of neurolathyrism, a crippling neurological disease[4–6]. The compound is a powerful convulsant[1] and is neuroexcitatory to central nervous system neurones[7–9], acting at the quisqualate and kainate receptors[8–10] [*i.e.* as distinct from N-methyl-D-aspartate (NMDA) receptors].

Pharmacological and biochemical interest in the longer chain L-ω-N-oxalyl derivatives of diamino acids, together with the possibility that the corresponding D isomers might act as important antagonists[11], prompted us to synthesise the D- and L-ω-N-oxalyl derivatives of diaminobutanoic acid (γ-ODAB, **2a,b**), ornithine (δ-OORN, **3a,b**) and lysine (ε-OLYS, **4a,b**) (see Fig. 1). The synthetic, pharmacological and biochemical properties of these compounds will be described elsewhere[12,13]. A knowledge of their optical purity was essential before a study of the pharmacological and biochemical activity could be undertaken, since L-β-ODAP activates quisqualate and kainate receptors to differing degrees and in a concentration-dependent manner[10], whereas D-β-ODAP is a weak antagonist at the NMDA receptor[14].

To date, optical purity has been ensured by the use of "optically pure" starting materials, optical rotation and rotary dispersion measurements. However, these methods are insensitive to contamination with small quantities of the minor enantiomer, either as a result of being induced by racemisation in the synthetic procedure, or being present in the "optically pure" starting materials.

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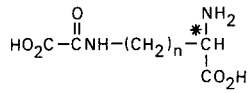

compound	n	optical configuration
1a	1	L
1b	1	D
2a	2	L
2b	2	D
3a	3	L
3b	3	D
4a	4	L
4b	4	D

Fig. 1. Structure of the ω-N-oxalyl diamino acids (asterisk denotes chiral centre).

Recently, there has been interest in enantioselective high-performance liquid chromatographic (HPLC) determination of amino acids[15–19] and related compounds[18,20–23] by pre-column derivatisation with *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives which are separable by reversed-phase HPLC, and detected using fluorometry. It was therefore decided to evaluate the use of this methodology in the development of a new enantioselective assay of ω-N-oxalyl diamino acids.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical or HPLC grade. Ultra-pure water was obtained by means of a Milli-Q system (Millipore). OPA was purchased from Sigma; N-acetyl-L-cysteine, N-acetyl-D-penicillamine and N-*tert.*-butyloxycarbonyl-S-benzyl-L-cysteine from Fluka. N-*tert.*-butyloxycarbonyl-L-cysteine was prepared as described by Buck and Krummen[18]. Synthetic ω-N-oxalyl DL, D- and L-diamino acids were prepared according to the method of Nunn *et al.*[13].

Authentic samples of L-β-ODAP and L-γ-ODAB were obtained from *L. latifolius* seeds (500 g) which were extracted with 50% (v/v) aqueous ethanol (6 × 500 ml), the resultant extracts were combined and evaporated at 40°C under reduced pressure and the resulting residue taken up in water (2000 ml). This was loaded onto a column (4 × 25 ml) of Zeo-Karb 225 (H^+) and eluted with 0.062 *M* acetic acid. Fractions were analysed for the presence of L-β-ODAP and L-γ-ODAB and were further purified as described previously[24].

Chromatographic systems

HPLC was performed using a Gilson gradient system (Anachem, Luton, U.K.) which consisted of two Model 301 single piston pumps (5-ml heads), a Rheodyne 7125 loop injector (20 μl), a Model 801 pressure module and a Model 121 fluorescence detector fitted with OPA filters (excitation at 344 nm and emission at 443 nm). The gradient was controlled by an Apple IIe computer using Gilson gradient manager software.

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF ω-N-OXALYL DIAMINO ACIDS

Duration (min)	*From (% solvent A:B)*	*To (% solvent A:B)*
0–30	90:10	75:25
30–35	75:25	65:35
35–50	65:35	65:35
50–55	65:35	40:60
55–60	40:60	40:60
60–65	40:60	90:10
65–75	90:10	90:10

Chromatograms were recorded on an LKB 2210 single-channel recorder at a sensitivity of 10 mV, a chart speed of 5 mm/min and a fluorescence sensitivity of 0.2 or 0.5 range units. A Spherisorb ODS II EXCEL, 5-μm (25 cm × 4.6 mm I.D.) column (Hichrom, Reading, U.K.), was used with a guard column (5 cm × 2 mm I.D.) packed with CO:PELL ODS sorbent (particle size 40 μm; Hichrom).

Preparation of standard ω-N-oxalyl diamino acid (1a–4b) solutions

Stock solutions of the individual enantiomers were prepared freshly each day in water at a concentration of 50 μmol/ml. Standard mixtures were prepared by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 100–300 nmol/ml for each individual component.

Mobile phases

Solvents A and B were prepared freshly every other day, filtered through a 0.22-μm membrane filter and degassed by continuous purging with helium. Solvents A and B consisted of 50 m*M* sodium acetate (pH 7.2, adjusted with dilute acetic acid) and methanol, respectively. The flow-rate was 1 ml/min and the column pressure was approximately 1600 p.s.i. at the beginning of the gradient. The gradient elution programme employed for the separation of enantiomers of γ-ODAB, δ-OORN and ε-OLYS is shown in Table I and for β-ODAP in Table II.

TABLE II

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF THE D AND L ENANTIOMERS OF β-ODAP

Duration (min)	*From (% solvent A:B)*	*To (% solvent A:B)*
0–40	100:0	91:9
40–45	91:9	40:60
45–50	40:60	40:60
50–55	40:60	100:0
55–60	100:0	100:0

Pre-column derivatisation procedure

The derivatisation reagents were freshly prepared every other day by dissolving 10 mg of OPA and the chiral thiol in 1 ml of methanol (in order to preserve the optical purity of the chiral thiols, the alkaline borate buffer was added immediately prior to derivatisation). These reagents were stored at 4°C in the dark until use. The standard and isolated ω-N-oxalyl diamino acid solutions (20 μl) were mixed with the derivatisation reagent (40 μl) and borate buffer (60 μl, pH 8.2, adjusted with 2 *M* sodium hydroxide), and incubated for 5 min at ambient temperature in the dark before immediate injection onto the column.

RESULTS AND DISCUSSION

All the ω-N-oxalyl diamino acids reacted with OPA and the chiral thiols N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP) and N-*tert.*-butyloxycarbonyl-L-cysteine (BocC) in alkaline conditions to yield highly fluorescent derivatives. The reactions occurred rapidly and quantitatively at ambient temperature in the dark, reaching their maximum fluorescence within 1–2 min, and were stable for at least 10 min. The fluorescence intensity of the derivatives was observed to be similar to that obtained with other commonly occurring amino acids (the NAP adducts had a lower fluorescent intensity than the corresponding NAC and BocC adducts). Efficient separation of the L and D enantiomers of γ-ODAB, δ-OORN and ε-OLYS was achieved by the use of the OPA–BocC reagent, with a 50 mmol sodium acetate (pH 7.2)–methanol gradient (which enhances the separation of early eluting "acidic" amino acids) and a Spherisorb ODS II EXCEL column (Fig. 2A and Table I). All

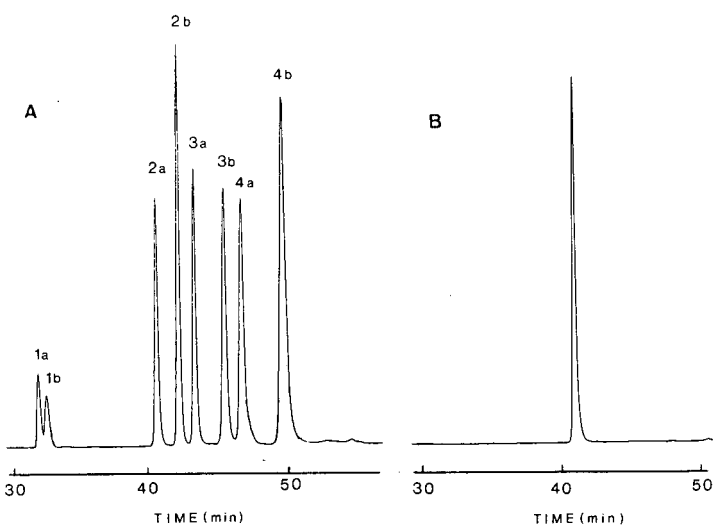

Fig. 2. HPLC of OPA–BocC derivatives of ω-N-oxalyl diamino acids on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. (a) Standard mixture of ω-N-oxalyl diamino acids. Peaks **1a,b** = L-, D-β-ODAP; **2a,b** = L-, D-γ-ODAB; **3a,b** = L-, D-δ-OORN; **4a,b** = L-, D-ε-OLYS. Each peak coresponds to 1 nmol expect for peaks **1b,2b** and **4b** which corresponds to 0.75, 1.5 and 1.4 nmol respectively. (B) "Isolated natural" L-γ-ODAB (**2a**) from *Lathyrus latifolius*.

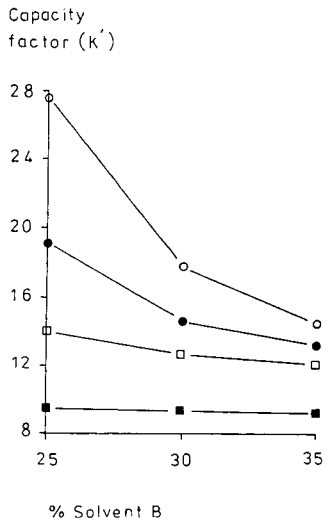

Fig. 3. The effect of methanol percentage at the plateau (35–50 min) on the capacity factors for the D enantiomers of the ω-N-oxalyl diamino acids: β-ODAP (■), γ-ODAB (□), δ-OORN (●) and ε-OLYS (○).

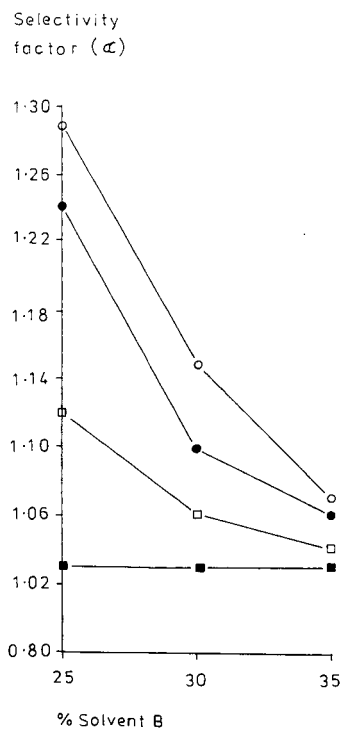

Fig. 4. The effect of methanol percentage at the plateau (35–50 min) on the selectivity factor of the enantiomers of the ω-N-oxalyl diamino acids: β-ODAP (■), γ-ODAB (□), δ-OORN (●) and ε-OLYS (○).

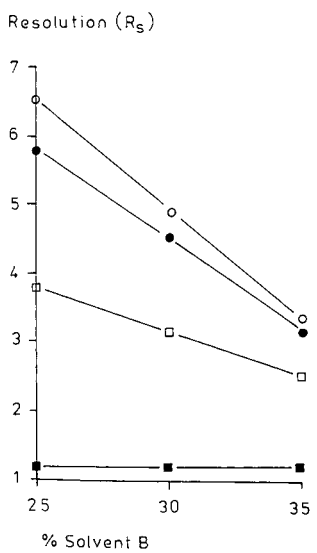

Fig. 5. The effect of methanol percentage at the plateau (35–50 min) on the resolution of the enantiomers of the ω-N-oxalyl diamino acids. Key as in Fig. 4.

enantiomers were separated in a run-time of 75 min which included wash and re-equilibrium phases. The coefficients of variation for the retention times and peak heights for all the ω-N-oxalyl compounds were below 0.7 and 1.2%, respectively. The calibration graph for the ω-N-oxalyl diamino acids showed good linearity between peak height and concentration in the range of 0.2 to 1.0 nmol per injection ($r^2 < 0.995$). As expected, the capacity and separation factor for the enantiomers of γ-ODAB, δ-OORN and ε-OLYS increased on lowering the methanol content of the mobile phase at the plateau (35–50 min), and also the resolution was observed to increase linearly (Figs. 3–5 and Table III). The capacity, separation and resolution for the enantiomers of β-ODAP remained constant (Figs. 3–5 and Table III). In an attempt to achieve better separation, other chiral thiols were investigated. OPA–NAC resulted in co-elution of the D and L enantiomer and also failed to completely resolve the enantiomers of γ-ODAB. In contrast, the OPA–NAP reagent resulted in baseline separation (separation factor and resolution of 1.37 and 1.43, respectively, for β-ODAP) using a modified sodium acetate–methanol gradient (Fig. 6A and Table II). OPA–NAP also effectively separated the enantiomers of γ-ODAB. As observed with other OPA–BocC amino acids[18,21], the L enantiomers (*S* configuration at the tertiary carbon atom bearing the primary amino group) of compounds **2–4** eluted before their corresponding D enantiomers. This is probably due to stronger hydrogen-bonds in the D diastereoisomers, resulting in a more hydrophobic molecule which would be expected to interact more strongly with the reversed-phase column and have a longer retention time than its corresponding L diastereoisomer. It has been suggested previously[18] that OPA–NAP derivatives of hydrophobic amino acids result in the D enantiomer eluting before the L enantiomer, the opposite is observed for the hydrophilic amino acids as can be seen for β-ODAP.

TABLE III

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM ω-N-OXALYL DIAMINO ACIDS AND OPA–BocC

t_0 = 3.2 min; k', α and R_s are the capacity, separation and resolution factors, respectively, for a pair of enantiomers; chromatographic conditions are as in the Experimental section.

Compound	*Methanol in buffer at plateau (%)*	k'_L	k'_D	α	R_s
β-ODAP	35	9.00	9.25	1.03	1.19
	30	9.00	9.25	1.03	1.19
	25	9.06	9.31	1.03	1.19
γ-ODAB	35	11.66	12.13	1.04	2.50
	30	11.88	12.63	1.06	3.64
	25	12.56	14.03	1.12	3.76
δ-OORN	35	12.50	13.19	1.06	3.14
	30	13.25	14.63	1.10	4.78
	25	15.47	19.19	1.24	5.73
ε-OLYS	35	13.56	14.50	1.07	3.33
	30	15.50	17.81	1.15	4.93
	25	21.38	27.50	1.29	6.53

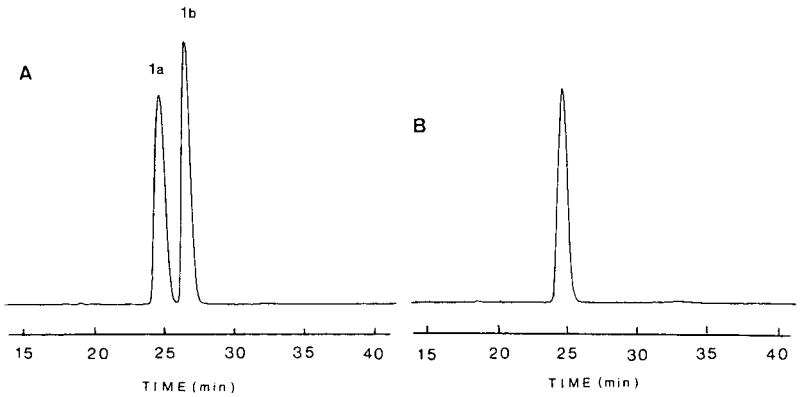

Fig. 6. HPLC of OPA–NAP derivatives of ω-N-oxalyl diamino propanoic acid (**1a,b**) on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in the Experimental section. (A) Standard mixture of D- and L-β-ODAP (**1a,b**). (B) "Isolated natural" L-β-ODAP (**1a**) from *Lathyrus latifolius*.

Samples of natural L-β-ODAP and L-γ-ODAB were isolated from an aqueous ethanolic extract of *L. latifolius* seeds. Purification and separation was achieved by the use of a Zeo-Karb 225 (H^+) resin eluting with 0.062 *M* acetic acid. This process separated the L-β-ODAP from L-γ-ODAB, the samples were further purified as described previously[24]. The isolated L-β-ODAP and L-γ-ODAB were shown to be enantiomerically pure (Figs. 6B and 2B) and the homogeneity of the assigned peaks (L-β-ODAP, t_R = 24.5 min and L-γ-ODAB, t_R = 41.1 min) was confirmed by comparison with the retention times of synthetic standards and chromatography of "spiked samples" and verifies the previously assigned L configuration in isolated materials.

The optical purity of all the synthetic ω-N-oxalyl diamino acids was shown to be in excess of 99.8%. The quantification of the minor enantiomers was achieved by comparison of peak heights in conjunction with standard additions. The limit of detection of the minor enantiomers was better than 0.1%.

The HPLC assay described represents a specific method for detecting enantiomers of ω-N-oxalyl diamino acids from natural sources and for assessing the optical purity of synthetically prepared analogues.

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CHROM. 21 258

Note

Enantiomeric purity determination of 3-aminoquinuclidine by diastereomeric derivatization and high-performance liquid chromatographic separation

IULIA DEMIAN* and DAVID F. GRIPSHOVER
A. H. Robins Co., Analytical Research Department, 1211 Sherwood Avenue, P.O. Box 26609, Richmond, VA 23261-6609 (U.S.A.)
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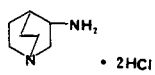
3-Aminoquinuclidine dihydrochloride (I) (1-azabicyclo[2,2,2]octan-3-amin) (CAS Reg. No. 6530-09-2) is an important intermediate in the synthesis of several pharmaceuticals[1]. For this reason the determination of its enantiomeric purity is of practical significance. No reference could be found in the literature on the optical resolution or optical purity determination of compound I.



I

Strong bases, such as 3-aminoquinuclidine are difficult to separate into enantiomers by direct chiral separation methods. Diastereomeric derivatives of compound I, on the other hand, were found to give relatively straightforward separations. In this work four different diastereomeric derivatization schemes have been used for high-performance liquid chromatographic (HPLC) separation of enantiomers of compound I.

EXPERIMENTAL

Materials and methods

TLC plates coated with silica gel 60 and RP-C_{18} (0.25 mm, F_{254}) were purchased from EM Science (Gibbstown, NJ, U.S.A.) and Whatman (Clifton, NJ, U.S.A.). Detection was by short-wavelength UV light. A Varian 5500 HPLC system equipped with a variable-wavelength UV detector, Model 200, a 6-port valve injector with a 10-μl loop, and a SP 4270 Spectra-Physics (Piscataway, NJ, U.S.A.) integrator was used. The columns used were 15 cm × 4.6 mm I.D. stainless steel packed with Zorbax C_8 and Zorbax Sil of 5 μm particle size from DuPont (Wilmington, DE, U.S.A.). ^{1}H NMR spectra were taken on a Varian 60 MHz EM 360 L spectrometer. Mass spectra were obtained on a Varian MAT Model 44 spectrometer. Infrared spectra were obtained in KBr pellets on a Nicolet Model 5 DX instrument. Optical rotation was measured using a Perkin-Elmer 241 polarimeter.

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The solvents used were spectroscopic grade. The dimethylformamide (DMF) and dioxane were further dried and purified by being passed through Sep-Pak Alumina B cartridge from Waters Assoc. (Milford, MA, U.S.A.). The non-chiral reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.).

The following chiral reagents were used: *S*(−)-1-phenylethyl isocyanate (PEIC) from Fluka (Buchs, Switzerland); *R*(−)-1-naphthylethyl isocyanate (NEIC) (Aldrich); *R,R*-(+)O,O-dibenzoyltartaric acid (DBTA) (Aldrich); *S,S*-(−)O,O-dibenzoyltartaric acid (DBTA) (Aldrich); 2,3,4,6-tetra-O-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) (Polyscience, Warrington, PA, U.S.A.).

Anhydrides of *R,R*(+)- and *S,S*(−)-O,O-dibenzoyltartaric acid, although commercially available, were prepared by a modified literature procedure[2], as follows. The corresponding acid was dissolved in excess acetic anhydride and heated at reflux for 30 min. After cooling, the white solid was filtered and triturated with light petroleum (b.p. 35–60°C), then dried. Further purification by recrystallization from xylenes is optional. The *R,R*-(+)-O,O-dibenzoyltartaric acid anhydride (*R,R*-DBTAAN) had a melting point of 196°C and $[\alpha]_D^{20} = +152°$; the *S,S*-DBTAAN 191°C and −157°.

Derivatization procedures

The structures of the chiral reagents and the corresponding diastereomeric derivatives of compound I are listed in Table I, together with pertinent literature references.

All of the derivatizations required the free-base form of compound I, which was prepared by treating a methanolic solution of compound I with 2 moles of sodium methoxide. After a few minutes the methanol was removed under a stream of nitrogen and the residue dissolved in dry DMF (for the disubstituted urea and thiourea derivatives) or in dry dioxane (for the derivatization with DBTAAN). Filtration of the inorganic material was not necessary.

Disubstituted urea and thiourea derivatives

Samples of 1–2 mg in the free-base form in 1-ml dry DMF were treated with derivatization reagent added via syringe or as a solid (GITC) in one portion in a 10% molar excess. After Vortex stirring the reaction mixture was allowed to stand at room temperature for 30 min. Samples of the reaction mixtures were then diluted with the mobile phase and analyzed by HPLC.

Dibenzoyl tartaric acid monoamides

Samples of 1–2 mg in the free-base form in 1-ml dry dioxane were treated with a 10% molar excess of DBTAAN reagent as a dioxane solution added in one portion. After Vortex stirring the mixture was allowed to stand at room temperature for 30 min. A suspension was formed which was dissolved in the mobile phase. Samples of the reaction were analyzed by thin-layer chromatography (TLC) as shown in Table II. No detectable amounts of compound I were observed.

All derivatization products had ^{1}H NMR and chemical ionization mass spectra consistent with the structures inferred from synthesis. Statistical analysis of experimental data was performed with RS1 (BBN) software run on a DEC-VAX minicomputer.

TABLE I

CHIRAL DERIVATIZATION REAGENTS AND DERIVATIVES

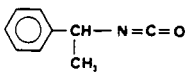
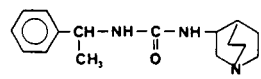
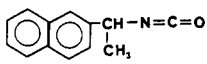
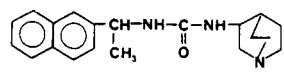
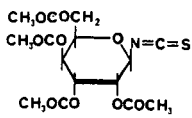
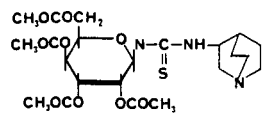
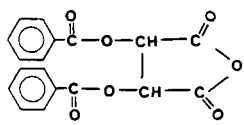
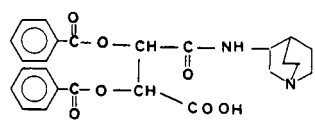
Chiral reagent	*Derivative*	*Reference*
 PEIC	 PUQ	3, 4
 NEIC	 NUQ	6
 GITC	 GTUQ	5, 7
 DBTAAN	 DBTAQ	6

TABLE II

TLC SEPARATION OF THE DIASTEREOMERIC DERIVATIVES OF COMPOUND I

Chiral reagents	*Diastereomers*	*TLC plate*	*Mobile phase*	*Detection method*	$R_F \times 100$ *of diastereomers*	
					R	*S*
PEIC	PUQ	Silica gel	Ethylacetate–isopropanol–10% ammonium hydroxide (45:35:20)	UV (254 nm)	45	42
NEIC	NUQ	Silica gel	Ethylacetate–isopropanol–10% ammonium hydroxide (45:35:20)	UV (254 nm)	46	45
GITC	GTUQ	Silica gel	Ethylacetate–isopropanol 10% ammonium hydroxide (45:35:20)	UV (254 nm)	51	51
DBTAAN	DBTAQ	RP-C_{18}	Methanol–water (65:35)–0.1% ammonium acetate	UV (254 nm)	50	66

RESULTS AND DISCUSSION

TLC separation of the diastereomeric derivatives of compound I are listed in Table II. Normal-phase separations on silica gel were performed except in the case of DBTAQ where the separation was done on reversed-phase (C_{18}) plates. The HPLC separations are listed in Table III.

All diastereomeric derivatives were strong UV absorbers; the detection was made by monitoring absorbance at 254 nm. As indicated by the separation factors (α) listed in Table III, the separation of the diastereomeric derivatives of compound I is satisfactory in all instances. Any of the derivatization schemes can be used for the optical purity determination of compound I. However, the best separation is achieved using DBTAQ.

The DBTAAN reagents were prepared from both isomers of the O,O-dibenzoyltartaric acids, (*R*,*R*) and (*S*,*S*). This allows one to cause either isomer to elute first.

Mixtures of (*R*)-I and (*S*)-I in the range of enantiomeric ratios from 0.001 to 0.01 were analyzed by DBTAAN derivatization and separation under the conditions listed in Table III. Typical chromatograms of (*R*,*R*)-DBTAQ diastereomers are shown in Fig. 1. The minor peak is clearly observed at 0.1% minor enantiomer. Plots of enantiomeric ratios (ER) *vs.* enantiomeric peak area ratios were linear. The data were fit to eqn. 1 by linear regression and the statistics of this fit are shown in Table IV.

$$ER = k[A_1/A_2] \quad (1)$$

where $ER = C_1/C_2$, concentration ratio of the two derivatives; k is the slope; A_1 and A_2 are the peak areas of the two enantiomers derivatives.

The k values are respectively 0.801 and 1.234 and are reciprocals as they should be. The same k values were also obtained by analysis of racemic I.

TABLE III

HPLC SEPARATION OF DIASTEREOMERIC DERIVATIVES OF COMPOUND I

Chiral reagent	*Diastereomers*	*Mobile phase and flow-rate*	*Column*	*k'(R)*	*k'(S)*	α^a
PEIC	PUQ	Ethyl acetate–methanol–conc. ammonium hydroxide (85:10:5), 2 ml/min	Silica gel	2.04	1.77	1.15
NEIC	NUQ	Ethyl acetate–methanol–conc. ammonium hydroxide (85:10:5), 2 ml/min	Silica gel	1.98	1.60	1.23
GITC	GTUQ	Methanol–acetonitrile–water (25:15:60)–(0.1% triethanolamine, 0.15% acetic acid), 2 ml/min	RP-C_8	10.65	9.24	1.15
DBTAAN	DBTAQ	Methanol–acetonitrile–water (25:15:60)–(0.1% triethanolamine, 0.15% acetic acid), 2 ml/min	RP-C_8	2.50	1.60	1.56

[a] $\alpha = k'(R)/k'(S)$, where k' is the capacity factor.

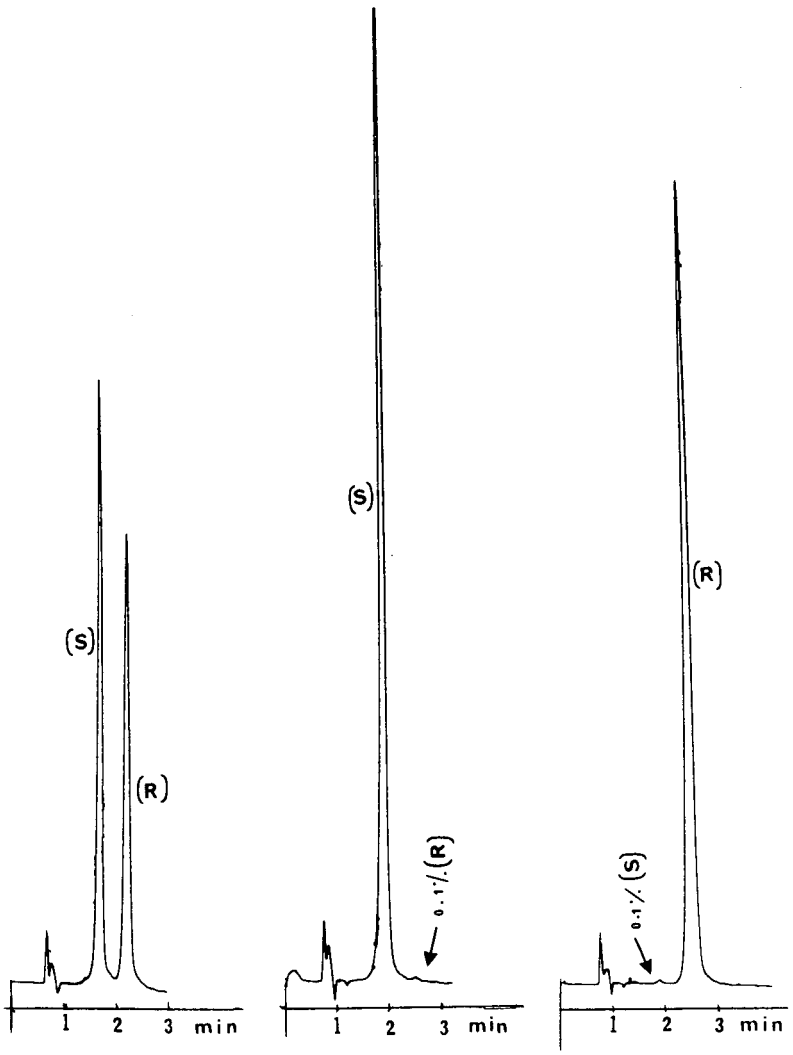


Fig. 1. HPLC separation of (*R*)-I and (*S*)-I mixture derivatized with (*R,R*)-O,O-dibenzoyltartaric acid anhydride. Column: 150 × 4.6 mm, I.D., Zorbax C_8; mobile phase: 0.15% acetic acid in water (adjusted with triethylamine to pH 4.2)–methanol–acetonitrile (60:25:15); flow-rate: 2 ml/min; detection: UV 254 nm.

TABLE IV

LINEAR REGRESSION STATISTICS OF (*R,R*)-DBTAQ ISOMERS

	Minor enantiomer	
	S	*R*
Slope, *k* (eqn. 1)	0.801 ± 0.044	1.234 ± 0.036
Fischer ratio, *F*	325	1200
Significance level, *p*	<0.001	<0.001
Square of the correlation coefficient, r^2	0.967	0.991
Number of points, *n*	12	12

In conclusion four different diastereomeric derivatization schemes were applied to mixtures of 3-aminoquinuclidine enantiomers followed by HPLC separation of the diasteromers. The best separation was achieved in the case of the O,O-dibenzoyltartaric acid derivatives which allowed detection of the minor enantiomer down to 0.001 enantiomeric ratio.

ACKNOWLEDGEMENTS

We wish to thank Dr. Young S. Lo and Mr. Dwight Shamblee for the supply of racemic and enantiomeric forms of 3-aminoquinuclidine, and improving the procedure for the DBTAAN synthesis. Thanks are also due Mr. Butch Johnson and Mr. John Forehand for recording and interpreting the NMR and mass spectra.

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CHROM. 21 246

Note

Determination of 2-acetyl-4(5)-tetrahydroxybutylimidazole in beers by high-performance liquid chromatography with confirmation by chemical derivatization

JAMES F. LAWRENCE* and CATHIE MÉNARD
Food Research Division, Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Ontario K1A 0L2 (Canada)
(Received December 29th, 1988)

2-Acetyl-4(5)-tetrahydroxybutylimidazole (THI) is a compound formed during the production of ammonia type caramel which is used as a colouring agent for beers. The substance has been found to cause lymphocyte depression in rats fed vitamin B_6-deficient diets[1]. As a result, international specifications now include a maximum concentration of 40 μg/g of THI in ammonia caramels (based on total solids content of the sample).

Two publications on the determination of THI in caramel have appeared in the past three years[2,3]. Both of these employ high-performance liquid chromatography (HPLC) either directly[2] or after derivatization of THI with 2,4-dinitrophenylhydrazine[3]. These methods were concerned with mainly caramel itself and not with determination in beers. Since caramel colour can be diluted by up to 1000 times in beer, methodology should be capable of quantitating 40 ng/ml THI in commercial samples. The present work includes method development for THI in beers that enables the detection of as low as 10 ng/ml THI with confirmation by chemical derivatization followed by analysis of the product under different HPLC conditions.

EXPERIMENTAL

Reagents

THI was dissolved in deionized water and diluted to yield a concentration of 10 μg/ml. Aliquots of this were used for direct HPLC analysis, for sample spiking and for derivatization studies. Pyridine "plus" (Applied Science Labs., State College, PA, U.S.A.) and acetic anhydride (J. T. Baker, Phillipsburgh, NJ, U.S.A.) were used as received. All other solvents and chemicals were analytical reagent-grade materials.

Cation-exchange columns

About 100 g each of Amberlite CG-50(H) type 1 (BDH, Toronto, Canada) and Dowex 50W-X8 (100–200 mesh) (Bio-Rad, Richmond, CA, U.S.A.) were allowed to soak in deionized water overnight. Each batch was rinsed several times with water, then a slurry poured to a bed height of 10 cm into separate 1 cm I.D. glass columns

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containing a glass wool plug. Each column was rinsed with at least 20 ml of water before addition of the samples.

HPLC

Separations were carried out employing a Beckman Model 114 pump, a Model 210 sample injection port (20 μl or 50 μl loop size) and a Micromeritics Model 788 variable-wavelength detector at 287 nm or 300 nm and 0.005 a.u.f.s. A Supelcosil LC-18 (5 μm) column (Supelco, Bellefonte, PA, U.S.A.) (15 cm × 4.6 mm I.D.) was used for the separations. For direct analysis of THI, two mobile phases were evaluated. These were; 0.005 *M* KH_2PO_4 (adjusted to pH 4.5 with 10% H_3PO_4) used for routine analysis and 0.02 *M* KH_2PO_4 (pH 4.0) used for confirmation. All mobile phases were filtered (0.45 μm) and degassed before use. The flow-rate was 0.7 ml/min. THI eluted around 6 min. For quantitation, peak height measurements were used.

A step gradient to 5% acetonitrile in mobile phase was initiated at 3 min and appeared in the chromatograms at 7 min. The step gradient was continued for 7 min to remove late eluting peaks before the next injection.

For determination of acetylated THI, the same HPLC system was used with a change in the mobile phase composition and the absorbance wavelength set to 287 nm. Three mobile phases were evaluated for the derivative. They all consisted of 0.02 *M* KH_2PO_4 (pH 6.0) containing either 12% (v/v) tetrahydrofuran, 32% methanol or 20% acetonitrile. The flow-rate was 1.0 ml/min. No step gradient was employed for the derivative.

Sample extraction

A 50-ml volume of degassed beer was passed through the Amberlite weak cation-exchange column and the eluate collected. The column was washed with 75 ml water and the eluate collected. The total eluate was then added to the Dowex strong cation-exchange column. The eluate was discarded. The column was then washed with 100 ml water which was also discarded. The THI was eluted with 100 ml 0.3 *M* hydrochloric acid and the eluate collected in a 500-ml round bottom flask. The solution was evaporated completely to dryness at 45°C for 30 min. The residue was dissolved in 5 or 10 ml water for HPLC analysis.

Acetylation

A 1.0-ml aliquot of the sample extract was passed through a C_{18} disposable cartridge (Sep-Pak, Waters Assoc., Milford, MA, U.S.A.) and the eluate collected. The cartridge was then washed with 3 ml water which was combined with the first eluate. This was diluted to exactly 4.0 ml then a 1-ml aliquot was transferred to a 3-ml Reactivial (Pierce, Rockford, IL, U.S.A.) and evaporated to dryness at 50°C under a stream of nitrogen. To the residue were added 10 μl of pyridine and 150 μl of acetic anhydride. The vial was capped and the contents were mixed and heated for 10 min at 90°C. After the reaction, the contents were evaporated just to dryness at room temperature under a stream of nitrogen. The evaporation was continued for an additional 5 min after dryness. The residue was dissolved in 1.0 ml water. Then, 1.0 ml chloroform was added and the mixture shaken gently. After separation, most of the chloroform was removed to a clean Reactivial and the extraction repeated with two 0.5-ml volumes of chloroform. The combined chloroform extracts were dried with

a small quantity of anhydrous sodium sulfate and then transferred to a clean vial and evaporated just to dryness at room temperature under nitrogen. The final residue was dissolved in 1 ml of HPLC mobile phase for analysis. This required allowing the solution to sit at room temperature for several hours or overnight.

RESULTS AND DISCUSSION

Direct analysis of THI in beer

The original method we employed for the direct determination of THI in caramel[2] was not suitable for routine determination of the compound in beers, due to the presence of too many coextractives. As a result, the two-column ion-exchange cleanup described by Kröplien[3] for caramel colours was employed with a reduced volume of beer (50 ml instead of 200 ml). The elution scheme was chosen after a number of optimization studies were carried out to obtain the best recovery with the least amount of interfering coextractives. Fig. 1 shows typical chromatograms for blank and spiked beer samples using 0.005 *M* KH_2PO_4 (pH 4.5) as the mobile phase and 300 nm as the absorption wavelength. At 7 min, a step gradient to 5% acetonitrile in mobile phase was incorporated to remove late eluting peaks. This shortened analysis time appreciably since otherwise some coextractives would still elute after 40 min.

The detection limit for most beers was estimated to be about 10 ng/ml although in some cases as low as 2 ng/ml could be detected when more sample was injected. Any sample extracts showing values above 10 ng/ml were reanalysed with a slightly different mobile phase (0.02 *M* KH_2PO_4, pH 4.0). It was found that this mobile phase

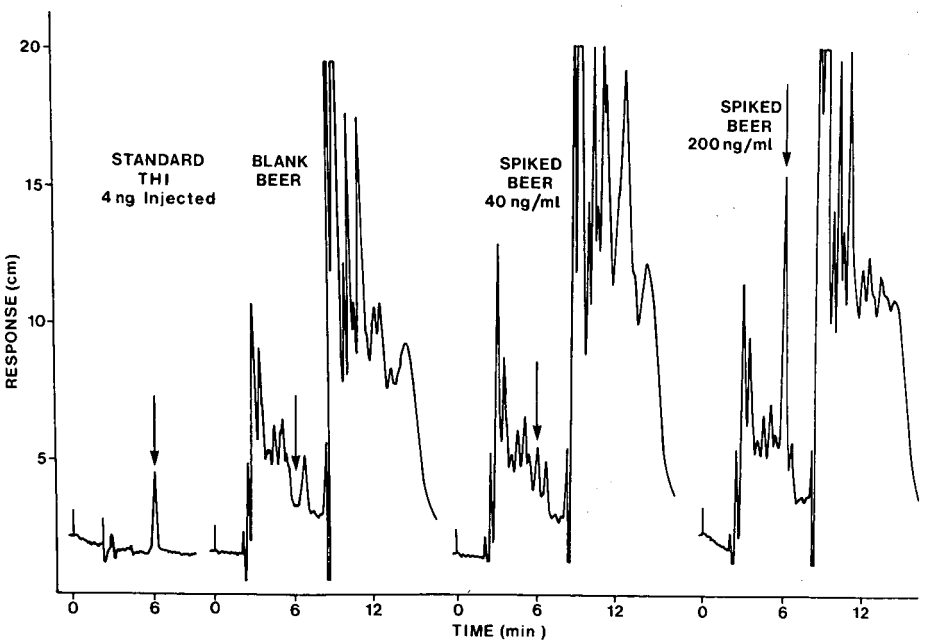

Fig. 1. Chromatograms of a pale beer spiked to contain 40 and 200 ng/ml THI. Mobile phase, 0.005 *M* KH_2PO_4 (pH 4.5). Wavelength, 300 nm. Sample extract dissolved in 10 ml mobile phase.

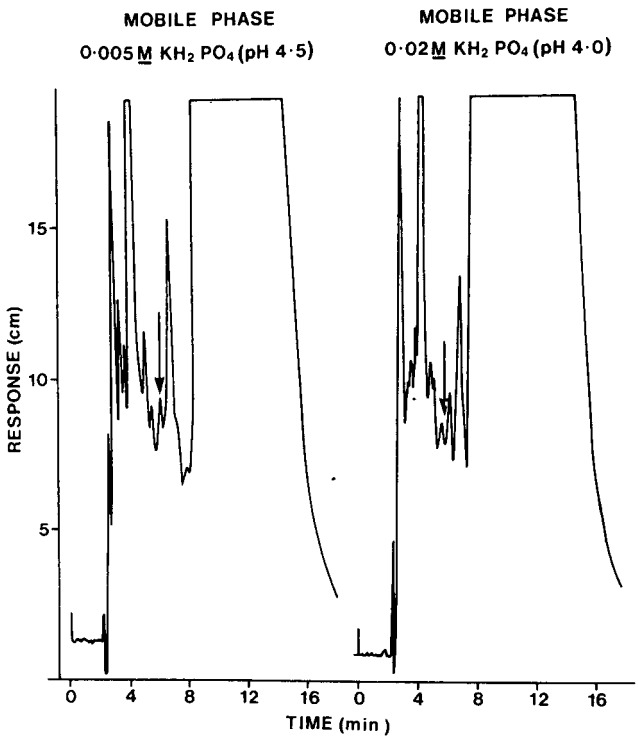

Fig. 2. A comparison of results obtained for a stout extract using the two different mobile phases studied. Wavelength, 300 nm. Sample extract dissolved in 5 ml mobile phase.

provided enough selectivity difference to act as a confirmation. In several instances values above 15 ng/ml were found to be less than 10 ng/ml with the second mobile phase. The lower value was always taken as being more accurate. Fig. 2 compares an extract of a stout with the two mobile phases. As can be seen, in one system a peak is observed corresponding to 9 ng/ml while in the second system no peak is observed with a retention time equivalent to THI although two peaks are close. With some other beers the opposite situation was observed.

We found that although 287 nm was the absorbance maximum for THI, 300 nm was chosen because of the increased selectivity obtained. The result was improved chromatograms in spite of the slightly reduced sensitivity to THI.

Recovery of THI added to different beer samples ranged from 57–70% at spiking levels of 20, 40, 60 and 200 ng/ml. The repeatability coefficient of variation of triplicate determinations each at 12 and 40 ng/ml were 12% and 18%, respectively.

In a limited survey of 18 domestic and 15 imported beers only two dark beers showed levels consistently above 10 ng/ml with the two mobile phases employed. Both of these samples produced extracts which contained much more coextractive material than the others including even stout, and thus had to be diluted in order to have the chromatograms appear on-scale.

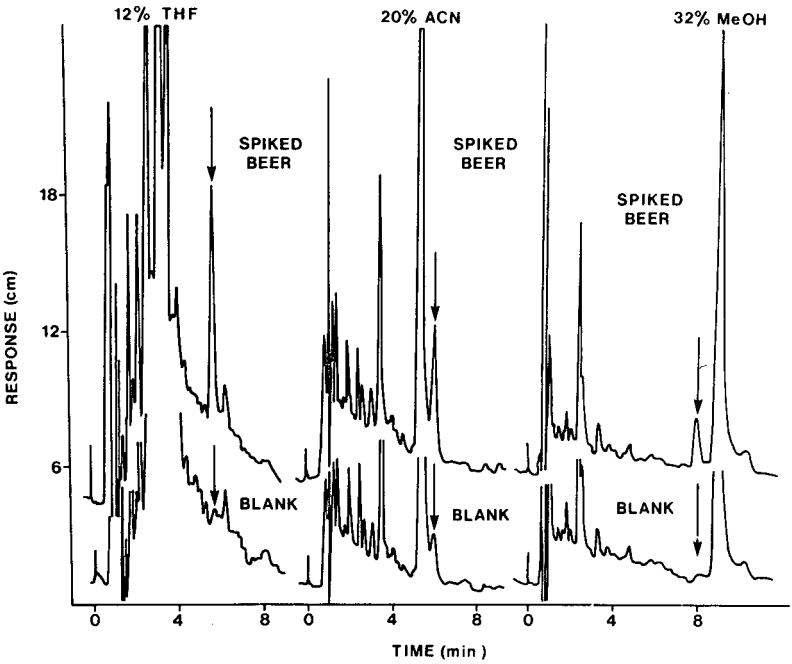

Fig. 3. Comparison of chromatograms obtained with three different mobile phases for the same sample extracts (blank and spike, 200 ng/ml) after acetylation. Reaction and chromatography conditions described in the text. Wavelength, 287 nm. Injection volumes, 20 μl for acetonitrile (ACN) and methanol (MeOH) and 50 μl for the tetrahydrofuran (THF) mobile phase.

Confirmation by acetylation

In order to confirm positive THI results obtained by direct HPLC we decided to attempt to acetylate the –OH and –NH moieties using acetic anhydride and pyridine as has been reported for sugars[4]. After a number of studies where quantities of reagents, temperature and time were varied, we obtained the reaction conditions described in the experimental section. Under those conditions a single peak was produced that was much less polar than THI but exhibited a very similar UV spectrum and the same sensitivity at the absorbance maximum of 287 nm. These characteristics would be expected if only the addition of acetyl groups were involved. Mass spectrometry (probe, electron impact) of the derivatives did not yield an unequivocal structure but it appears that three acetyl groups are added to THI as a result of the derivatization.

The Sep-Pak C_{18} cleanup was required to improve the yield of the reaction when applied to real samples. It removed coloured (pale yellow) material that turned brown during the reaction and resulted in low recoveries and interferences. After the reaction, the mixture was evaporated to dryness and then shaken with water and chloroform. This was found necessary to remove traces of pyridine which eluted as a broad tailing peak causing some difficulty in quantitating the derivative. It was observed that after the final chloroform evaporation, the THI derivative was difficult to re-dissolve in mobile phase for HPLC analysis. It required continuous vortex stirring for 15 min in order to have recoveries greater than 80%. Also, we found that just allowing the

solution to sit overnight was adequate to ensure dissolution of the derivative. After this time, the solution was stable for at least two weeks.

Three mobile phases were evaluated for the analysis of acetylated THI in the beer samples. Fig. 3 compares chromatograms obtained with each for a derivatized beer (porter) extract spiked with 200 ng/ml THI. At this spiking level the THI is clearly observed although the chromatographic patterns are quite different. The mobile phase containing tetrahydrofuran was chosen for routine use. The others were also useful, but occasional interfering peaks appeared with some samples that were not observed or were smaller with the tetrahydrofuran mobile phase (compare blanks for example, in Fig. 3). The lower detector response to THI with the methanol containing mobile phase appears to be the result of peak broadening due to increased retention time as well as perhaps other chromatographic effects. This was consistent for all samples and standards analysed with that system.

The detection limit using the acetylation was estimated to be about 10 ng/ml in the beers studied. Lower levels could be detected depending upon the particular sample and the quantity injected. Yields of derivative from reactions carried out in triplicate at 50 and 200 ng/ml THI in beer were 81 and 88% respectively compared to a pure standard carried through the same reaction. The corresponding coefficients of variation were 24% at 50 ng/ml and 5% at 200 ng/ml THI. The linear range for the acetylation reaction extended from the detection limit to at least 200 ng/ml THI. Higher concentrations were not evaluated.

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CHROM. 21 202

Note

Capillary gas chromatography of *Delphinium* diterpenoid alkaloids

GARY D. MANNERS*
Western Regional Research Center, Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710 (U.S.A.)
and
MICHAEL H. RALPHS
Poisonous Plant Research Laboratory, Agricultural Research Service, Logan, UT (U.S.A.)
(First received September 5th, 1988; revised manuscript received December 19th, 1988)

Tall larkspur (*Delphinium barbeyi*) and duncecap larkspur (*Delphinium occidentale*) are poisonous plants indigenous to the Western mountains of the U.S.A.[1]. These plants are poisonous to cattle and cause annual losses as high as 12% on mountain grazing ranges[2]. Alcohol extracts of *D. barbeyi* and *D. occidentale* were found to have a median lethal dose (LD_{50}) of 4.5 and 40.4 mg/g body weight respectively when administered subcutaneously to rats[3].

Diterpenoid alkaloids have been established as toxic components occurring in the larkspurs. Five diterpenoid alkaloids (anthranoyllycoctonine, delpheline, deltaline, dictyocarpine and lycoctonine) have been identified in *D. barbeyi*[4–6] while deltaline, deltamine, dictyocarpine, dictyocarpinine, delcosine, 14-dehydrodelcosine, browniine, 14-dehydrobrowniine, glaucerine, glaucenine, glaucedine, hetisine, hetisione, delsoline, delpheline, 6-acetyldelpheline, occidentaline and occidentalidine and have recently been described in *D. occidentale*[7]. The toxic character of various diterpenoid alkaloids, including anthranoyllycoctonine, lycoctonine, deltaline and delcosine, has been reviewed[8]. Toxicological data for the majority of diterpenoid alkaloids reported in *Delphinium* sp. is lacking and the correlation of specific larkspur diterpene alkaloids with the mortality of cattle has not been described.

While the specific toxicants occurring in tall and duncecap larkspur have not been identified, livestock grazing management based upon total alkaloid occurrence has been formulated[9]. It is recognized that more efficient management of livestock on larkspur infested grazing lands could be achieved if the identity and phenological variation of specific toxic alkaloids were available.

Capillary gas chromatography (GC) has been shown to be a sensitive and efficient method for the analyses and detection of quinolizidine alkaloids obtained from lupin[10] and for the screening of drug abuse alkaloids[11]. We now describe a capillary GC method for the analysis of underivatized diterpenoid alkaloids occurring in larkspur. This analytical method provides a rapid quantification of specific diterpenoid alkaloids occurring in larkspur and will allow the correlation of the phenological occurrence of these alkaloids with observed rangeland livestock toxicity.

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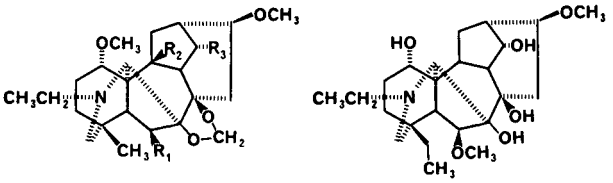

Scheme 1. Delpheline (1), R_1 = OH, R_2 = H, R_3 = OCH_3; deltamine (2), R_1 = OH, R_2 = OH, R_3 = OCH_3; deltaline (3), R_1 = $OOCCH_3$, R_2 = OH, R_3 = OCH_3; 14-acetyldictyocarpine (4), R_1 = $OOCCH_3$, R_2 = H, R_3 = $OOCCH_3$; dictyocarpine (5), R_1 = $OOCCH_3$, R_2 = H, R_3 = OH; dictyocarpinine (6), R_1 = OH, R_2 = OH, R_3 = OH. Structure on the right shows delcosine (7).

EXPERIMENTAL

Apparatus and operation conditions

A Hewlett-Packard 5890A gas chromatograph equipped with a hydrogen flame ionization detector and an HP on-column injector was used for all analyses.

A 30 m × 0.32 mm I.D. flexible fused-silica SE-30 capillary column (J&W Scientific) was used with the following operating conditions: detector temperature 300°C; initial temperature 85°C (0.1 min.), 40°C/min to 175°C, 5°C/min. to 300°C, final temp. 300°C (20 min); helium carrier gas flow-volume, 2.13 ml/min.

Alkaloid standards and reagents

Deltaline, dictyocarpine, deltamine, and delcosine standards were kindly supplied by Dr. S. William Pelletier. These four diterpenoid alkaloids were also isolated and purified from extracts of *D. occidentale* and *D. barbeyi* in our laboratory and served as standards for analytical analyses. Dictyocarpinine obtained from *D. occidentale* and 14-acetyldictyocarpine and delpheline obtained from *D. barbeyi* in our laboratory were also used as analytical standards. Chemical structures for the alkaloids are shown in Scheme 1. The hydrocarbon standard mixture was obtained from J&W Scientific. Solvents used were reagent grade and used without purification.

TABLE I

MEAN WEIGHT RESPONSE FACTORS AND RELATIVE RETENTION TIMES OF DITERPENOID ALKALOIDS

Alkaloid	*Mean wt. response factor (ng/count)*	*Observed retention time (min)*	*Rel. retention time*[a]
Delpheline (1)	0.012	24.22	$C_{28.93}$
Deltamine (2)	0.026	24.85	$C_{29.58}$
Deltaline (3)	0.011	25.34	$C_{29.91}$
14-Acetyldictyocarpine (4)	0.013	25.92	$C_{30.35}$
Dictyocarpine (5)	0.023	26.90	$C_{31.31}$
Dictyocarpinine (6)	0.021	26.90	$C_{31.31}$
Delcosine (7)	0.004	29.16	$C_{32.67}$

[a] Retention times relative to C_{28}–C_{34} hydrocarbons.

External standard calibration of capillary GC

A standard stock solution (acetone containing approximately 600 n/μl of each alkaloid) was prepared and analyzed to determine the retention times of the individual alkaloids (Table I). Aliquots of the stock solution of the standard alkaloid solutions were combined to produce a mixed alkaloid standard.

The mixed standard was used to calibrate the gas chromatograph. Samples of 1 μl of sequentially diluted aliquots of the mixed standard solution were applied to GC to establish an external standard concentration *vs.* response curve for each of the alkaloids. Weight response factors of the individual alkaloids were obtained (Table I). The response data was accumulated and stored using a Hewlett-Packard ChemStation (Model 5859A) computer interfaced to the GC system.

A standard mixture of alkanes (C_{28}–C_{34}) was added to the standard alkaloid mixture and the combined standards were applied to the gas chromatograph. Retention times for the standard alkaloids relative to the standard alkanes were established (Table I).

Quantitative analysis of alkaloids in larkspur extracts

A total alkaloid extract was obtained from ground, air-dried, whole plant material of *Delphinium occidentale* and *Delphinium barbeyi* through the modification of a procedure used by Pelletier *et al.*[12]. The ground plant material (10 g) was exhaustively extracted (80% ethanol) in a soxhlet apparatus and the resulting extract was concentrated (*in vacuo*) to a syrup. The syrup was dissolved in chloroform (200 ml) and extracted with 10% hydrochloric acid (100 ml). Two additional extractions with 10% hydrochloric acid (50 ml) were performed and the acidic extracts were combined. The chloroform extract was discarded. The acid extract was made basic (pH 8) with 20% sodium hydroxide, cooled and extracted with ether (three times with 100 ml). The ether extract was dried (magnesium sulfate) and concentrated (*in vacuo*). The remaining basic solution was further extracted with chloroform (three times with 100 ml). The chloroform extract was dried (magnesium sulfate) and concentrated (*in vacuo*). The ether and chloroform solutions were combined and concentrated to dryness under a stream of nitrogen. The combined extracts were considered to contain the total alkaloids of the plant extract.

The total alkaloid extract was dissolved and diluted to 50 ml in acetone. A suitable aliquot from the solution was diluted to afford a sample solution containing approximately 200 n/μl. A 1-μl sample of this solution was injected for analysis. The sample was compared to the calibrated external standard data base to establish the quantities of known alkaloids present (Table II).

RESULTS AND DISCUSSION

The capillary GC separation of the standard alkaloid mixture is shown in Fig. 1. The figure shows six peaks for the seven standard alkaloids applied to the chromatographic column. It was found that the diterpenoid alkaloids dictyocarpine and dictyocarpinine have identical retention times in the chromatographic system and are therefore unresolved. The five remaining standard alkaloids are clearly resolved. Calibration curves for all of the standard alkaloids were linear over the range of concentrations used. The minimum range of detectability for the standard alkaloids

TABLE II

DITERPENOID ALKALOID COMPOSITION OF *D. OCCIDENTALE* AND *D. BARBEYI*

Values are given as % of total alkaloid extract.

Alkaloid	*D. occidentale*	*D. barbeyi*
Delpheline (1)	0.1	2.6
Deltamine (2)	0.5	2.3
Deltaline (3)	33.0	37.5
14-Acetyldictyocarpine (4)	0.6	8.2
Dictyocarpine/dictyocarpinine (5,6)	19.5	4.2
Delcosine (7)	0.2	>0.1

was 2–12 ng. Correlation coefficients, r (response ratio *vs.* amount of alkaloid), were from 0.999 to 1.000 for all calibrations. Dictyocarpine and dictyocarpinine were independently calibrated and found to be closely comparable in detectable response. Therefore, a mixed standard containing delpheline, deltamine, deltaline, 14-acetyldictyocarpine, dictyocarpine (representing the unresolved mixture) and delcosine was used to calibrate the GC system for analysis of alkaloid mixtures from larkspur.

Total alkaloid extract yields for the two larkspur species varied according to the phenological growth stage of the samples. Yields (based upon air dry plant weight) ranged from 0.5 to 5% for *D. occidentale* and from 0.4 to 3% for *D. barbeyi*. Figs. 2 and 3 show the GC separation of total alkaloids extracts obtained from early growth leaf

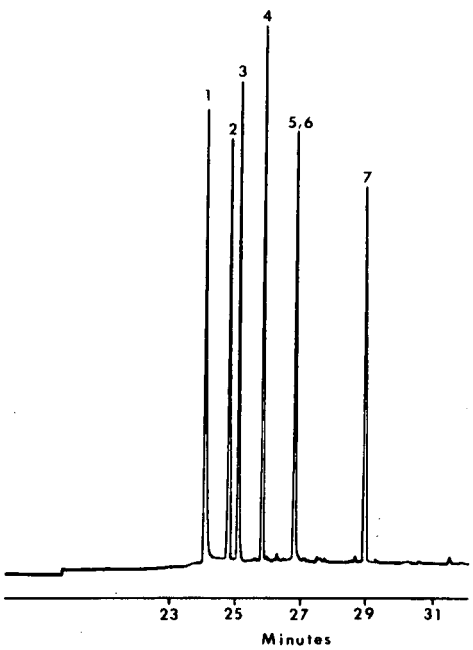

Fig. 1. Capillary GC trace of seven diterpenoid alkaloids. Peaks: 1 = delpheline; 2 = deltamine; 3 = deltaline; 4 = 14-acetyldictyocarpine; 5,6 = dictyocarpine and dictyocarpinine; 7 = delcosine.

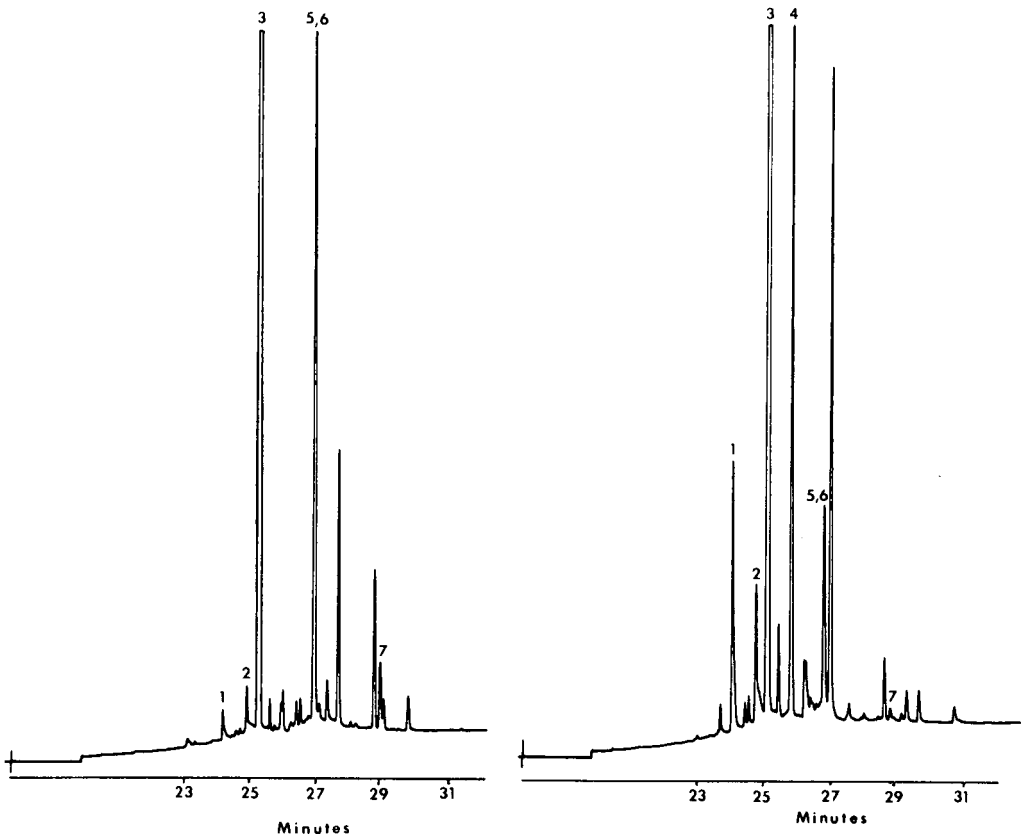

Fig. 2. Capillary GC trace of total alkaloid extract of *Delphinium occidentale*. Peaks: 1 = delpheline; 2 = deltamine; 3 = deltaline; 5,6 = dictyocarpine and dictyocarpinine; 7 = delcosine.

Fig. 3. Capillary GC trace of total alkaloid extract of *Delphinium barbeyi*. Peaks: 1 = delpheline; 2 = deltamine; 3 = deltaline; 4 = 14-acetyldictyocarpine; 5,6 = dictyocarpine and dictyocarpinine; 7 = delcosine.

material of *D. occidentale* and *D. barbeyi* respectively. Results of the quantification of the known diterpenoid alkaloids in these extracts is presented in Table II. The results clearly indicate that the seven standard alkaloids comprise over 50% of the total alkaloid extract obtained from *D. occidentale* and *D. barbeyi* although the occurrence of dictyocarpinine in *D. barbeyi* is negligible (established by thin-layer chromatography) and the occurrence of 14-acetyldictyocarpine and delpheline is very low in *D. occidentale*. Each of the larkspur species shows the occurrence of an unidentified constituent (*D. occidentale*, 27.83 min; *D. barbeyi*, 27.14 min) which appears in negligible yield in the other species. Isolation and identification of these compounds and the other unidentified constituents present in the alkaloid extract of these larkspur species is currently underway in our laboratory. The identification of 14-acetyldictyocarpine in *D. barbeyi* is the first reported natural occurrence of this diterpenoid alkaloid.

CONCLUSION

The results of this investigation show that diterpenoid alkaloids occuring in extracts of *Delphinium* can be detected (2 ng minimum) and quantified by capillary GC. The quantification of alkaloids from *D. occidentale* and *D. barbeyi* extracts illustrates the speed and sensitivity of this technique and its potential for the accurate assessment of the phenological variation of *Delphinium* sp. diterpenoid alkaloids in relation to their toxicity to rangeland cattle.

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CHROM. 21 197

Note

Determination of 4-*tert.*-butylphenol and 4-*tert.*-butylcatechol in cosmetic products by reversed-phase high-performance liquid chromatography

L. GAGLIARDI, A. CIMORELLI and G. CAVAZZUTTI
Istituto Superiore di Sanita', Rome (Italy)
L. MONTANARELLA
Varian, Rome (Italy)
and
D. TONELLI*
Istituto di Scienze Chimiche, Universita' di Bologna, Via San Donato 15, 40127 Bologna (Italy)
(Received December 12th, 1988)

The commercial cosmetic creams producing depigmentation of the skin can contain active principles at levels of 1–2%. In conformity with the Directive of the European Communities Commission 87/137 EEC, Italy has issued departmental order 24.11.87 n. 53 (Annex II) that states which skin-lighteners can be used and their permitted levels.

We have started a study on the identification and determination of depigmenters in cosmetic products to test their compliance with EEC regulations, considering, first of all, hydroquinone and some of its ethers[1]. Among the substances excluded from use as depigmenters are 4-*tert.*-butylphenol (I) and 4-*tert.*-butylcatechol (II). To our knowledge, there are no reports in the literature concerning the determination of such phenolic compounds in cosmetic creams, whereas they have been determined by a variety of methods, especially for air- and water-pollution studies. The environment contains numerous mono- and dihydroxybenzenes derived from both natural and man-made sources. Of the developed methods, gas chromatography (GC) of the mono- and dihydroxy compounds[2,3] or of their derivatives[4] has been most widely used, especially for quantitative measurements at ppb (μg/l) levels, since the late 1950s, but these compounds have been determined also by thin-layer chromatography (TLC)[5,6] and spectrophotometry[7,8]. Advancements in the practice of high-performance liquid chromatography (HPLC) have revealed many possibilities for the determination of phenolic compounds by this technique. Several studies have been done using normal and reversed-phase HPLC with fluorescence, UV and electrochemical detection[9–12].

We report here a simple analytical method based on reversed-phase HPLC with isocratic elution for the determination of compounds I and II which is suitable for the routine analysis of vanishing creams in order to test their compliance with the EEC regulations.

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EXPERIMENTAL

Apparatus

A Model 5000 liquid chromatograph (Varian, Zug, Switzerland) equipped with a Valco AH 60 injection valve, a Varian Polychrom 9060 photodiode array detector and a Varian 4290 integrator was used. The analytical column was a 5-μm ODS Ultrasphere (150 mm × 4.6 mm I.D., Beckman).

Reagents

All reagents were of analytical reagent grade. Compounds I and II were obtained from Merck (Darmstadt, F.R.G.), 4-benzyloxyphenol, used as the internal standard (I.S.), from Fluka (Buchs, Switzerland). Acetonitrile was of solvent-for-liquid chromatography grade. All solvents and solutions for HPLC analysis were filtered through a Millipore filter, pore size 0.5 μm, and vacuum degassed by sonication before use.

Chromatographic conditions

The HPLC conditions were as follows: mobile phase, acetonitile-water containing acetic acid at 1% (40:60); flow-rate, 2 ml/min; injection volume, 10 μl; detection wavelength, 278 nm; detector sensitivity, 0.16 a.u.f.s.

Calibration graphs

Standard solutions were prepared by dissolving the appropriate amounts of compounds I and II in 100 ml of the mobile phase containing 0.1 mg/ml of I.S. These solutions and the set of solutions produced by serial dilutions were processed using the HPLC conditions described above. The ratios of the peak areas of I and II relative to the peak area of the I.S. were plotted *versus* the amounts injected.

Extraction from the cosmetic sample

About 2 g of a cosmetic cream were treated with 50 ml of methanol containing the I.S. at 0.1 mg/ml and the mixture heated at 50°C in a water-bath with shaking until sample dissolution was complete. After cooling and centrifugation, 10-μl aliquots of the solution were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a standard solution of compounds I, II and I.S. A good resolution was obtained. The most important parameters of the compounds investigated are summarized in Table I. Retention times were reproducible under the experimental conditions used. In an actual analysis, unknown peaks are identified using the retention times, but a more definite identification can be obtained by estimating the purity parameter (Varian) format values[13]. Table I also reports the response factors relative to the I.S., calculated from the weight ratio.

Calibration graphs were constructed from six consecutive injections. The equations obtained by linear regression analysis were $y = 1.203x - 0.011$ ($r^2 = 0.9998$) for compound I and $y = 1.470x + 0.075$ ($r^2 = 0.9994$) for II. Linearity was observed up to 10 μg injected for each compound. The reproducibility of the analysis was very

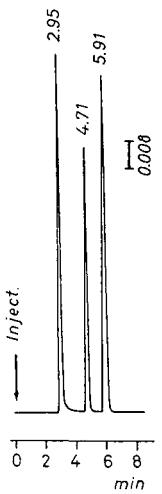

Fig. 1. Chromatogram of a standard mixture of compounds I, II and I.S.

TABLE I

RETENTION TIMES, PURITY PARAMETER FORMAT VALUES AND RELATIVE RESPONSES

Each value is the mean of six determinations.

Compound	*Retention time (min)*	*λ_m (239–311) (nm)*	*Relative response*
I	2.95	275.63	1.17
I.S.	4.71	279.70	1.00
II	5.91	273.54	1.59

TABLE II

RECOVERIES OF COMPOUNDS I AND II FROM VANISHING CREAMS

Each value is the mean of five determinations.

Cream	*Amount of I added (%, w/w)*	*Recover (%)*	*S.D.*	*Amount of II added (%, w/w)*	*Recovery (%)*	*S.D.*
A	1	98.0	1.2	1	100.0	1.8
	2	99.7	1.5	2	99.0	1.1
B	1	97.8	1.0	1	98.7	2.1
	2	99.0	1.9	2	99.4	1.8
C	1	98.4	1.9	1	101.2	1.5
D	1	98.8	1.5	1	97.9	1.7
	2	98.9	2.0	2	98.7	1.9

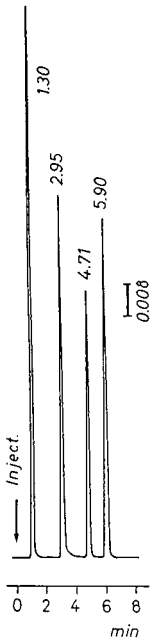

Fig. 2. Chromatogram obtained for a commercial cream containing hydroquinone and spiked with I and II at concentrations of 2% (w/w).

good, the average coefficient of variation being less than 1.7%. The detection limits, calculated as twice the noise level, were approximately 20 ng.

The applicability of the proposed method for the determination of compounds I and II in cosmetic samples was demonstrated by studying their analytical recoveries from four different creams bought on the market. The samples were tested for the presence of forbidden depigmenters. Once verified that the only skin-lightener present was hydroquinone, the creams were spiked with weighed amounts of compounds I and II and subjected to the extraction procedure described above. Five samples of the same tube of cream were analyzed. The recoveries obtained are shown in Table II. Good recoveries and precision are observed. Fig. 2 shows a chromatogram obtained for a sample of cream. The peak with a retention time of 1.3 min corresponds to hydroquinone. Peak identities were confirmed by determining the purity parameters which were in excellent agreement with the values reported in Table I.

In conclusion, the analytical method reported here for the determination of compounds I and II in cosmetic vanishing creams meets the following requirements: simplicity, rapidity of sample preparation and analysis, reproducibility and accuracy.

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Note

Separation of simple guanidines on cation-exchange columns using indirect ultraviolet detection

THOMAS A. WALKER
Chemical Products Discovery, The NutraSweet Co., 601 E. Kensington Road, Mt. Prospect, IL 60056 (U.S.A.)
(First received December 6th, 1988; revised manuscript received December 28th, 1988)

Indirect photometric or "vacancy" chromatography (IPC) is an analytical technique where ionic analytes are separated on an ion-exchange column and are then detected through a photometric process[1,2]. IPC, which was described by Small and Miller, Jr. in 1982[1], consists of an UV-absorbing counterion in the mobile phase that competes with UV-transparent, injected analyte ions for the ion-exchange sites. As the UV-transparent analyte elutes off the column, it replaces the UV-absorbing counterion in the effluent. This replacement leads to a decrease in absorbance at the detector and produces a negative peak.

IPC has become a commonly used method for the analysis of inorganic and organic UV-transparent ions where a strong cation- or anion-exchange column is used[3–6]. Indirect UV detection has also been extended to reversed-phase[7] and ion-interaction chromatographic separations[8,9] as well as for separations where low-capacity ion-exchange columns are used[10,11]. The separation and indirect UV detection of organic analyte cations on low-capacity cation-exchange columns, however, has not been studied to the extent that other chromatographic systems have.

This paper describes the separation and indirect UV detection of several simple guanidines on a low-capacity polymeric cation-exchange column and on a silica-based strong cation-exchange column. Quantitation of the guanidines was also studied on the low-capacity cation-exchange column.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A). HPLC-grade water was obtained by passing deionized water through a Millipore water purification unit. Benzyltrimethylammonium chloride and the guanidines were obtained from Aldrich (Milwaukee, WI, U.S.A). All chemicals were reagent grade.

Apparatus

The liquid chromatographic (LC) apparatus used consisted of a WISP Model 710B Autosampler, Waters Model 590 high-performance liquid chromatography

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(HPLC) pump, a Kratos Model 783 variable-wavelength UV detector, and a Linear Model 500 strip-chart recorder. The columns used in this study were: a 150 × 4.1 mm I.D. Hamilton PRP-X200 low-capacity cation-exchange column available from Hamilton (Reno, NV, U.S.A.) and a 250 × 4.6 mm I.D. Whatman Partisil 10 SCX (Cherry Hill, NJ, U.S.A.). The PRP-X200 column is a spherical, 10 μm poly(styrene–divinylbenzene) sulfonate packing with a cation-exchange capacity of 35 μequiv./g. Flow-rates of 1.0 ml/min were used, unless noted otherwise. Aqueous analyte samples of approximately 1 mg/ml were used. Sample aliquots of 10 μl were used. Inlet pressures of 500–600 p.s.i. were observed.

RESULTS AND DISCUSSION

Several groups have used low-capacity cation-exchange columns for the separation of inorganic and organic analyte cations[10–15]. Cantwell and co-workers[12–14] has shown that a dual retention mechanism of cation exchange and adsorption account for the retention of organic analytes that contain both a fixed charge site and a hydrophobic center. Differences in elution orders for organic cations have been observed when comparing separations on polymer-based low-capacity cation-exchange columns and silica-based strong cation-exchange columns[10]. These changes in the elution order can be attributed to adsorption that takes place between the hydrophobic center of the analyte and the adsorption sites on the low-capacity cation-exchange column. Changes in the elution order of organic cations on the low-capacity cation-exchange column can be accomplished by adjusting the amount of added organic modifier and/or by manipulating the mobile phase ionic strength[10].

In IPC, the added UV-absorbing countercation has the dual role of: (1) displacement of an analyte cation from the cation-exchange column and (2) detection of an UV-transparent analyte cation as a dip or trough in the baseline absorbance. When a low-capacity cation-exchange column is used for separating organic analyte cations, the UV-absorbing countercation will be involved in the detection of the organic analyte cation and will compete for the cationic exchange sites. If the UV-transparent analyte is retained predominantly by adsorption, then the UV-absorbing countercations role is just the indirect UV detection of the organic analyte.

Fig. 1 shows the separation and indirect UV detection of several guanidines on a silica-based strong cation-exchange column (I) and on the PRP-X200 column (II). Elution orders were found to be almost reversed when the two columns were compared for this separation. It should be noted that the guanidine separations on the two columns were optimized so that the resulting chromatograms could be directly compared.

It was observed during this study that the elution order of the guanidines on the PRP-X200 column could be changed by adjusting the amount of added acetonitrile or by adjusting the mobile phase ionic strength. If the amount of acetonitrile in the mobile phase was increased, the retention of 1-ethylguanidine (F) was reduced. However, the retention of guanidine (E) does not change. This shows that the adsorption mechanisms plays a more predominant role in the retention of 1-ethylguanidine whereas guanidine is retained exclusively by a cation-exchange mechanism. A combination of adsorption/ion-exchange mechanisms accounts for the retention of the other guanidines.

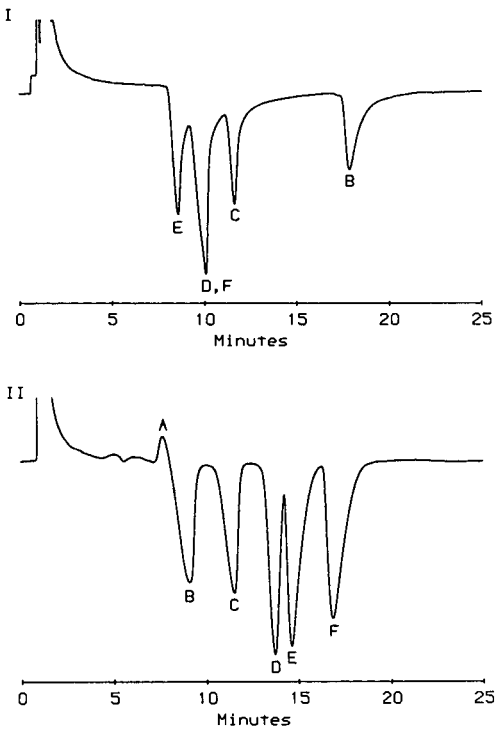

Fig. 1. Chromatogram of (A) system peak, (B) 1,1,3,3-tetramethylguanidine, (C) 1,1-dimethylguanidine, (D) 1-methylguanidine, (E) guanidine, (F) 1-ethylguanidine. Conditions: mobile phase, (I) 0.0025 *M* benzyltrimethylammonium chloride in acetonitrile–water (20:80) (II) 0.0008 *M* benzyltrimethylammonium chloride in acetonitrile–water (20:80); columns, (I) Whatman Partisil 10 SCX (250 × 4.6 mm I.D.), (II) PRP-X200 (150 × 4.1 mm I.D., 10 mm); flow-rate, (I) 2.0 ml/min, (II) 1.0 ml/min; detector, 0.04 a.u.f.s. at 268 nm.

Quantitation was done for the guanidines on the PRP-X200 column. Calibration curves for all of the guanidines were found to be linear over a range of 5 to 800 ppm with a detection limit of 1–3 ppm. Several samples with a known amount of 1,1,3,3-tetramethylguanidine, 1,1-dimethylguanidine, 1-methylguanidine, guanidine and 1-ethylguanidine were prepared and analyzed. Average recoveries of 96.4, 98.5, 98.8, 100.9 and 99.0% with relative standard deviations of 5.6, 1.5, 3.3, 1.9 and 2.4%, respectively, were found for each analyte.

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CHROM. 21 206

Note

Determination of 2,6- and 4,6-dinitrocresols by high-performance liquid chromatography on a β-cyclodextrin bonded column

A. M. TRIPATHI, J. G. MHALAS and N. V. RAMA RAO*
Research & Development Division, Hindustan Organic Chemicals Limited, Rasayani 410207, Maharashtra (India)
(First received September 9th, 1988; revised manuscript received December 21st, 1988)

2,6-Dinitro-*p*-cresol (2,6-DNPC) and 4,6-dinitro-*o*-cresol (4,6-DNOC) are formed as side reaction products in the manufacture of nitrotoluene isomers, the extent of formation of 2,6-DNPC being larger. Their build-up above certain limits, especially of 2,6-DNPC, can cause undesirable thermal run-away reactions. Further, the nitrocresols are toxic compounds and are listed as priority pollutants[1,2] by the U.S. Environmental Protection Agency. Also, 4,6-DNOC finds extensive use as an herbicide. These dinitrocresols enter the effluent stream also. Hence, it is of utmost importance to monitor their formation during the manufacture of nitrotoluene isomers.

A literature survey has not revealed any high-performance liquid chromatographic (HPLC) method for the determination of 2,6-DNPC, though some HPLC methods[3–5] have been reported for the analysis of 4,6-DNOC as an herbicide among other nitro-containing herbicides like dinoseb, dinoterb and dinobuton. However, the simultaneous determination of 2,6-DNPC and 4,6-DNOC by HPLC has not so far been reported. Therefore, it was thought worthwhile to develop an efficient HPLC method for the analysis of these two isomers.

A β-cyclodextrin bonded phase column, which is effective for the separation of positional isomers[6,7], has been employed for the separation of the two isomers and the results are presented herein.

EXPERIMENTAL

Instrumental

A Perkin-Elmer chromatograph Series 10, equipped with a Rheodyne sample injector, a Perkin-Elmer Lambda 3-B variable wavelength UV–VIS spectrophotometric detector and an LCI-100 computing integrator were used for chromatographic work. The β-cyclodextrin bonded column was obtained from Advanced Separation Technologies (Whippany, NJ, U.S.A.). Its dimensions were 250 mm × 4.6 mm I.D. and the particle size was 5 μm.

0021-9673/89/$03.50

Reagents and chemicals

All solvents used were of HPLC grade. 2,6-Dinitrophenol and 4,6-DNOC were obtained from Fluka (Buchs, Switzerland). 2,6-DNPC was isolated from the caustic washings of a nitrotoluene plant and its purity established by NMR and gas chromatographic (GC) methods.

Preparation of standard solutions

Stock solution of 2,6-dinitro-*p*-cresol (2,6-DNPC), 4,6-dinitro-*o*-cresol (4,6-DNOC), *p*-nitrotoleune (p-NT) and 2,6-dinitrophenol (2,6-DNP) (1 mg/ml) were prepared in methanol. Various amounts of 2,6-DNPC, 4,6-DNOC and p-NT were taken in 25-ml volumetric flasks and 5 ml of 2,6-DNP (internal standard) were added. The volumes were made up to 25 ml with mobile phase and 6.0 μl from this solution were injected for HPLC.

Chromatographic conditions

A 6.0-μl volume of each standard sample was injected. Flow-rate: 1 ml/min. Detection: UV at 254 nm. Mobile phase: methanol–acetonitrile–acetic acid (20:78.5:1.5, v/v).

RESULTS AND DISCUSSION

The β-cyclodextrin bonded phase column is packed with silica material covalently bonded with β-cyclodextrin molecules by means of a non-nitrogen-containing

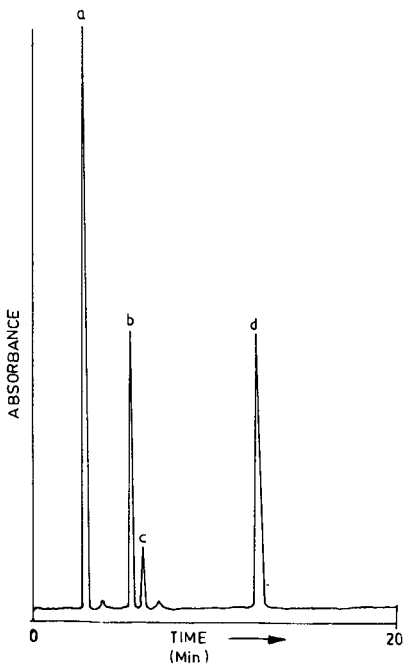

Fig. 1. HPLC separation of dinitrocresol isomers in a plant sample. Peaks: a = nitrotoluenes; b = 2,6-dinitro-*p*-cresol; c = 4,6-dinitro-*o*-cresol; d = 2,6-dinitrophenol. Experimental conditions as in the text.

TABLE I

RETENTION DATA

Compound	*Retention time (min)*	*Capacity factor, k'*
Nitrotoluenes	2.97	0.32
2,6-Dinitrocresol	5.47	1.44
4,6-Dinitrocresol	6.10	1.72
2,6-Dinitrophenol (internal standard)	12.28	4.48

spacer 6–10 atoms in length. The macrocyclic molecule β-cyclodextrin contains seven glucopyranose units arranged in the shape of hollow truncated cone of which the interior cavity is relatively hydrophobic comprising essentially the methylene and glucoside linkages. The exterior faces are hydrophilic because of many hydroxyl groups. Separation of compounds on such columns essentially takes place either by inclusion complexation phenomena or by strong polar interactions.

In the present case, the effective separation of the two isomers of dinitrocresols along with the internal standard 2,6-dinitrophenol has been achieved in the normal phase mode using a methanol and acetonitrile mixture containing acetic acid. To achieve better resolution, sharp peaks and rapid elution of these strongly interacting compounds, it is necessary to add acetic acid to the mobile phase. Without such addition, the components are strongly retained with practically no separation. This suggests a normal phase behaviour[8]. The effect of acetic acid has been studied systematically and it is found that 1.5% (v/v) affords very sharp peaks with good resolutions. A typical chromatogram illustrating the separation of the isomeric dinitrocresols from a plant sample is shown in Fig. 1. It is not clear whether the inclusion process is significant in this mode. A normal adsorption column containing HS-Silica and with the same solvent system was tested but did not yield any separation.

The retention data for the two isomers are given in Table I. The method of separation has been evaluated for quantitation of these isomers. A statistical evaluation of the method is given in Table II. The method has been found to be quite

TABLE II

STATISTICAL EVALUATION OF THE METHOD FOR DETERMINATION OF DINITROCRESOL ISOMERS

S.D. = Standard deviation; C.V. = coefficient of variation.

Isomer	*Amount taken (μg/ml)*	*Amount found*[a] *(μg/ml)*	*% Error*	*S.D.*	*C.V.*
2,6-DNPC	40	39.69	0.78	0.13	0.33
	80	79.37	0.78	0.26	0.33
	120	120.41	0.34	1.05	0.88
4,6-DNOC	40	39.19	2.03	0.47	1.21
	80	77.86	2.67	0.91	1.17
	160	155.25	2.97	1.42	0.41

[a] Based on five measurements.

effective with an accuracy of ±3% for 4,6-DNOC and ±0.8% for 2,6-DNPC.

Efforts have been made to separate the components in reversed-phase mode on a β-cyclodextrin column using a water–methanol mixture as the mobile phase. The components were not eluted with this mobile phase. Further, increasing the water content in the mobile phase leads to increased retention of the components, suggesting a strong inclusion process. The resolution of the peaks was also not improved and elution takes much longer when compared to the normal phase separation.

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CHROM. 21 251

Letter to the Editor

Chromatographic determination of cholesteryl esters synthesized *in vitro*

Sir,

Earlier experiments in this laboratory showed that a cholesteryl ester-synthesizing system present in the cytosol of rat liver is significantly stimulated in rats bearing experimental tumours[1]. Evidence was provided recently[2] that the blood serum of tumour-bearing animals as well as of patients with cancer enhances the production of cholesteryl esters in normal rat liver. For further studies, a method suitable for serial separations of radioactive cholesteryl esters from labelled cholesterol was required.

Thin-layer chromatography (TLC) gives an excellent separation of both these lipid classes but the procedure is rather complicated and time-consuming[3]. The method now described was developed for a simple and rapid separation of labelled cholesteryl esters from radioactive cholesterol (which is used as the starting material for the subcellular synthesis of esters). This procedure shows a good reproducibility and is useful for serial analyses.

[1a,2a(n)-^{3}H]Cholesterol (50 Ci/mmol) was a product of Amersham (Amersham, U.K.). Cholesteryl 14-methylhexadecanoate and [^{3}H]cholesteryl 14-methylhexadecanoate (50 Ci/mmol) were synthesized as described by Helmich and Hradec[4]. Separations of cholesteryl esters from cholesterol were performed on thin layers of silica gel G (Merck, Darmstadt, F.R.G.) developed with *n*-hexane–diethyl ether (95:2, v/v). Mixtures for the cell-free synthesis of cholesteryl esters contained rat liver cytosol and [^{3}H]cholesterol as described in detail elsewhere[2].

The novel procedure for the determination of newly formed radioactive cholesteryl esters included two steps. (1) Portions (100 μl) of the cytosol after the incubation were applied to strips of Whatman 31ET (2 cm × 2 cm) filter-paper and dried under an IR lamp. The dried filters were placed in 3 ml of ethanol–diethyl ether (1:2, v/v) in glass tubes, the tubes were stoppered and left for 60 min at room temperature with occasional shaking. The filters were then removed and washed with *ca.* 1 ml of the extraction mixture. The pooled extracts were evaporated to dryness at 70°C under a stream of nitrogen. (2) Residues were dissolved in 100 μl of chloroform and 75-μl portions were applied to microcolumns of silica gel (Silica-cart) obtained from Tessek (Prague, Czechoslovakia). Plastic syringes attached to the microcolumns were filled with 5.0 ml of *n*-heptane–diethyl ether (90:10, v/v) and the columns were eluted using a moderate pressure. The eluate was collected directly in scintillation vials containing a toluene-based scintillation mixture and the radioactivity (dpm) was assayed using a Beckman 5801 liquid scintillation system.

Analyses of lipids require mostly extraction by conventional methods[5]. These procedures are time-consuming and losses of material may occur during the extraction of small samples. Mazière *et al.*[6] overcame this difficulty by drying suspensions of

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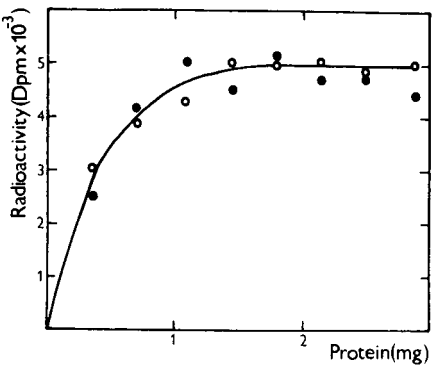

Fig. 1. Comparison of liquid–liquid extraction of samples with the extraction of dried samples on filters. Increasing quantities of rat liver cytosol were incubated with [^{3}H]cholesterol as described[2] and mixtures were extracted by the method of Folch *et al.*[5] (●) or as described (○). Extracts were then separated on microcolumns of silica gel.

TABLE I

RECOVERY OF CHOLESTERYL ESTERS AFTER CHROMATOGRAPHY ON MICRO-COLUMNS OF SILICA GEL

All samples were chromatographed in duplicates on microcolumns of silica gel as described. Quantitation of cholesteryl esters was performed as described by Burke *et al.*[8].

Ester	*Input (mg)*	*A_{550} units*		*Recovery (%)*
		Before chromatography	*After chromatography*	
14-Methylhexadecanoate	0.5	0.057, 0.061	0.060, 0.055	97.5
	1.0	0.130, 0.125	0.118, 0.321	98.0
	2.0	0.243, 0.251	0.210, 0.217	86.4
Stearate	0.5	0.067, 0.055	0.055, 0.058	92.6
	1.0	0.123, 0.117	0.114, 0.098	88.3
	2.0	0.235, 0.239	0.206, 0.222	90.3

cultured cells directly on thin layers used for the subsequent separation by the usual TLC and reported recoveries comparable with usual extraction methods. For our purposes, only the separation of two lipid classes was required and extraction of the material dried on filter-papers was found preferable. Recoveries obtained for cholesteryl esters using this technique were comparable with those obtained by the usual extraction procedure of Folch *et al.*[5] (Fig. 1).

Microcolumns of silica gel were found very useful for a rapid and efficient separation of radioactive cholesteryl esters from labelled cholesterol. Similar micro-columns were used for the separation of polar from non-polar lipids[7]. Recoveries of radioactive cholesteryl esters obtained after chromatography on silica microcolumns (Table I) were in good agreement with those after TLC.

Department of Molecular Biology, Research Institute of Tuberculosis and Respiratory Diseases, 180 71 Prague (Czechoslovakia)

DANA REJŠKOVÁ
JAN HRADEC*

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Author Index

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PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	461 462 463/1	463/2 464/1	464/2 465/1 465/2	466 467/1 467/2	468 469 470/1 470/2	471 472/1 472/2 473/1	The publication schedule for further issues will be published later.					
Bibliography Section		486/1		486/2		486/3		486/4				
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2	490/1 490/2	491/1	491/2	492/1	492/2 493			

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Ketoprofen	1.46	OJ
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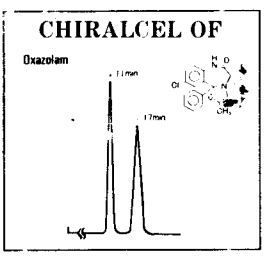

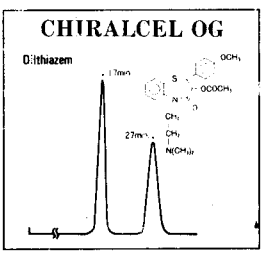

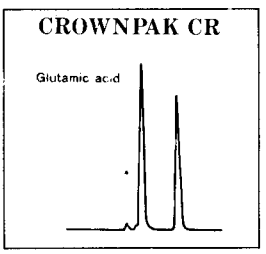

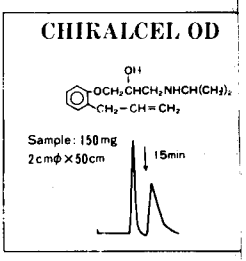

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