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(Continued overleaf)

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In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*)

Physical Adsorption on Heterogeneous Solids

by **M. Jaroniec,** *Institute of Chemistry, Maria-Curie-Sklodowska University, Lublin, Poland, and* **R. Madey,** *Smith Laboratory of Physics, Kent State University, Kent, USA*

(Studies in Physical and Theoretical Chemistry, 59)

This book presents a unified description of physical adsorption on heterogeneous solids from both the gas and liquid phases. All solid adsorbents of great practical importance, such as activated carbons, silica gels, aluminium oxides, zeolites, etc., possess surface and structural heterogeneities, which play a dominant role in the adsorption process especially at low pressures. The assumption of adsorbent heterogeneity in studies of physical adsorption introduces considerable difficulties in the theoretical description of this phenomenon. To overcome this problem, the authors present a relatively simple theory of physical adsorption on heterogeneous solids which provides a quantitative description of this phenomenon and simultaneously permits elaboration of methods for characterizing adsorbent heterogeneity.

The chapter on single-gas adsorption on heterogeneous surfaces is the most extensive one in the book because there is a great deal of scientific literature concerning this problem. Moreoever, the theory of single-gas adsorption provides foundations for deriving equations that apply to adsorption from multicomponent gas and liquid mixtures. Special attention has been paid to the fundamental aspects of physical adsorption on heterogeneous solids, to illustrating similarities and differences between adsorption at the gas-solid and liquid-solid interfaces, and to describing methods for characterizing energetic and structural heterogeneities of solids.

Although many books have been devoted to physical adsorption on solids, none treats gas and liquid adsorption on heterogeneous solids. This book will therefore be useful to researchers in academic institutes and industrial laboratories, whose work is concerned either directly or indirectly with adsorption phenomena at the gas-solid and liquid-solid interfaces as well as with characterization of adsorbents and catalysts.

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REVIEW

APPLICATION OF ENZYME PURIFICATION PROCESSES TO PROTEOLYTIC ENZYMES

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INTRODUCTION

The proportion of proteolytic enzymes in the chain production of industrial enzymes is 60%, that is, *ca.* 40 000 tons per annum. Interest in these enzymes is increasing. In addition to their traditional applications, such as in industrial detergents, new outlets are opening up, *e.g.*, in the production of polypeptides, as they permit new functional or nutritional properties to be introduced and biologically active peptides to be obtained. Further, their use in the field of biosynthesis is now expanding considerably.

Purification methods vary according to the chosen purpose, *e.g.*, preparation of industrial enzymes or standards for the determination of enzymatic activity or for structure analysis.

A decrease in overall activity occurs during the various stages of purification. Hence one must aim to obtain a pure enough enzyme for the intended purpose so that the cost/performance ratio is as small as possible.

As enzymes are more or less unstable, care must be taken in the extraction and purification steps: (i) operation at low temperatures hampers the development of microorganisms, limits the enzyme–substrate reaction and prevents degradation of the thermolabile enzymes; nevertheless, as some proteases are fairly stable at high temperature, this property is used in order to eliminate the thermolabile contaminants; and (ii) addition of antiseptics, antibiotics, reversible inhibitors of enzymatic reactions, such as EDTA, soybean trypsin inhibitor, pepstatin or stabilizing factors such as calcium ions. It is also desirable to avoid denaturing conditions such as extremes of pH, too high ionic strength or addition of surface-active or organic solvents. Foams and films should be avoided whenever possible, as proteins tend to denature on interfaces.

Three main steps can be distinguished in an enzymatic preparation: extraction of endocellular enzymes and solubilization in the medium; obtaining the crude enzyme; and selective purification, and these are treated in the following sections.

1. EXTRACTION OF ENDOCELLULAR ENZYMES AND SOLUBILIZATION IN THE MEDIUM

Before any separation, the enzyme must be solubilized in the medium and separated from insoluble contaminants such as microorganisms, cellular debris or various impurities.

The endocellular enzymes or those obtained from tissue extracts are dissolved after membrane disruption and homogenized in a buffer. The membrane disruption is carried out by various chemical methods, *e.g.*, alkalinization, addition of EDTA or detergents or osmotic shocks, or by physical methods, *e.g.*, sonication, freezing and thawing phases, solid or liquid shear or grinding or agitation with abrasives[1]. In a number of instances, an important part of the enzyme remains adsorbed within the cellular membranes.

Insoluble impurities and cellular waste are then removed by filtration, centrifugation or partition. With conventional methods, because the very small size of the particles which are to be eliminated, there is a risk of plugging, entailing losses or a decrease in activity. Tangential filtration may be a solution.

In addition to the classical methods used to extract enzymes when prepared in suitable amounts, partition techniques are more specially used for expensive enzymes. Hence liquid–liquid extraction is being increasingly used as it permits not only cells and insoluble organelles to be eliminated, but also the enzymatic activity of poorly concentrated media such as fermentation juices or filtrates from cellular cultures[2] to be increased. It is based on the incompatibility in an aqueous solution of two polymers [polyethylene glycol (PEG) and dextran] or of one polymer and an appropriate salt (principally potassium phosphate), which leads to the formation of two non-miscible phases. The two phases contain a high proportion of water (65–90%), which ensures a better enzymatic stability than water–organic solvent mixtures[3].

For each enzyme, one can determine a partition coefficient specific for a given medium, $K = C_T/C_B$, where C_T is the concentration of enzyme in the upper phase and C_B that in the lower phase. For a high degree of purification, the enzyme should have a large (>3) or small ($<1/3$) K value. K is a constant and depends on numerous factors such as the type of polymer being used, molecular weight and molecular weight distribution of the polymers, length of the tie-line (a function of the concentration of the components of the phase system), type and concentration of added salts, pH and temperature[4]. Hydrophobic proteins are usually found in the upper phase and hydrophilic proteins in the lower phase. Enzymes can be extracted into the chosen phase by introducing a specific ligand bound to a polymer concentrated in such a phase[2,5,6]. The output depends on the partition factors and on the relative volumes of the two phases. Enrichment can be carried out in several stages. It is important to know precisely the point of equilibrium and the phase separation method. It is also possible to use three or poly-phase systems; these are advantageous compared with two-phase systems as mixtures of proteins can be resolved between three or more bulk phases. The possibility of binding specific ligands to each of the polymers could lead to a further increase in the selectivity of partitioning[7].

The formation and separation of the phases, recycling of polymers and salts and economic implications have been thoroughly studied by Husted and co-workers[8–11]. The possibilities of continuous output (counter-flow extraction) enables both high yields (50 g proteins/kg medium) and high purities to be obtained. This non-

denaturing technique is fairly recent and apparently seldom used for proteases, but it should be developed more. At present, its industrial application meets with some difficulties regarding the recovery of the various polymers in order to recycle them.

2. OBTAINING THE CRUDE ENZYME

At the beginning of a separation, when large amounts of materials have to be handled, methods of high capacity should be chosen; at this stage resolution is secondary requirement. We can distinguish precipitation techniques and adsorption techniques, and these are consisted below.

2.1. Precipitation techniques

These are likely to be used directly on the starting medium, which has previously been treated to remove insoluble contaminants subjected to a preliminary concentration step.

2.1.1. Precipitation with organic solvents

Organic solvent-induced precipitation (2–5 volumes) is a still in use as a classical method, which nonetheless has some inconveniences, particularly because of the risk of denaturation. In addition, it requires the use of large amounts of solvents, especially for low-molecular-weight enzymes. This makes it necessary to treat large amounts and requires a solvent-recovery apparatus to be installed.

2.1.2. Precipitation with neutral salts

Salting-out of proteins is based on the property of proteins to re-precipitate at a high ionic strength. A protein solution of known concentrations (s) will begin to precipitate at a ionic strength given by the following equation:

$$\frac{T}{2} = \frac{B_1 - \log s}{K_1 s}$$

where T = ionic strength (mol/l), B_1 = constant, $K_1 s$ = (slope of line) salting-out constant and s = solubility of protein (g/l). The value of B_1 is dependent of the salt used and varies with pH, temperature and the nature of protein in solution. $K_1 s$ is independent of pH and temperature but varies with the protein in solution and the salt used[1]. One usually operates at the enzyme pI. One can possibly obtain a fractionation of the medium by using an increasing concentration of salt. The most commonly used salt is ammonium sulphate because of its high solubility, lack of toxicity, cheapness and, in some instances, its stabilizing effect.

In this method, however, the mineral salts are liable to be carried with the precipitate, which requires another step such as dialysis or gel filtration.

Both methods of precipitation have the advantages of being simple, cheap and able to treat large amounts of impure product, but they are hardly applicable to enzymes of low molecular weight because of their high solubility in water.

2.1.3. Precipitation with non-denaturing polymers

It is also possible to utilize non-denaturing polymers such as polyethylene glycol

(PEG). They are used alone or associated with ethanol or ammonium sulphate[12]. The great advantage of this process is the shorter time required for precipitation (0.5–1 h). Other advantages are in aiding crystallization and limiting enzyme degradation. The most commonly used PEG have a nominal average molecular weight (MW) in the range 4000–6000 but polymers with much lower MW, which are cheaper, can also be used. It is advisable to buffer the PEG solution at the required pH in order not to change the pH of the enzyme solution, which might lead to conditions that promote molecular association[12]. The PEG is then removed by ultrafiltration or during the later stages of ion-exchange or affinity chromatography[13]. As PEGs are optically transparent and help to prevent enzyme loss, it is unnecessary to eliminate the last traces[14].

2.1.4. Particular instances

2.1.4.1. Precipitation with metals

The use of bivalent metals such as zinc or copper to precipitate proteins has been employed for many years[15–17]. These metals form relatively stable complexes with histidine and cysteine residues[18–20], which permits the proteins to be precipitated without denaturation by reducing the pH variations and the amounts of solvents required in classical systems. Zaworski and Gill[21] have recently published a method using Zn^{2+} for the rapid concentration of culture media and obtained a porcine urokinase from *Saccharomyces cerevisiae* culture filtrates.

2.1.4.2. Affinity precipitation

The principle consists in complexing the enzyme and precipitating the complex macroligand–molecule. This technique seems very promising as it can be applied on a large scale. Schneider *et al.*[22] produced an acrylamide water-soluble macroligand carrying affinitive molecules for trypsin and benzoic acid that helps its precipitation under certain conditions. As the ligand can easily be recycled, this technique is attractive, it is cheap and it can be applied to large volumes, although it is sometimes difficult to find a suitable ligand. This method is, moreover, fairly recent and has rarely been applied to proteases.

2.2. Adsorption techniques

An alternative to precipitation is selective adsorption of the enzyme on an appropriate support.

2.2.1. Adsorption on an ion-exchange resin

This is the most interesting process, especially when a high-capacity and inexpensive exchanger can be used and/or when batchwise operation is possible. Under precise conditions of pH, this can be a highly selective method.

Whenever the enzymes are fragile or do not withstand high ionic strengths, operating in the reverse mode is possible, *i.e.*, fixing the contaminants on the resin, then concentrating the enzyme.

2.2.2. Adsorption on specific ligands

In this stage, affinity-related chromatography is usually too expensive except for use with a highly active product. It is used for the purification of expensive enzymes.

3. SELECTIVE PROCESSES OF PURIFICATION

The capacity and selectivity of the methods are dependent on the amount of contaminants, the volume to be dealt with and the purity required. On an industrial scale, it is advisable to limit the number of stages. Two types of techniques can be singled out: chromatographic and electrophoretic.

3.1. Chromatographic techniques

3.1.1. Low-pressure liquid chromatography

The choice of the chromatographic support must meet certain requirements, such as specific adsorption, non-denaturation of adsorbed products and perfect reversibility of the adsorption. Other criteria should also be considered, such as the adsorption capacity of the support, the usable flow-rate range, chemical stability and yield–cost ratio[23].

3.1.1.1. Ion-exchange chromatography

This widely used method has a number of advantages: high capacity, good specificity depending on the operating conditions and inexpensive supports. One can distinguish two main type of materials, ion-exchange resins and hydrophilic cellulosic ion exchangers.

Ion-exchange resins are insoluble polymers on which the exchange groups are fixed. Resin have the advantages of being stable over a wide range of pH, they sediment rapidly, they have a high adsorption capacity and high liquid flow-rates can be used. They are commonly used in industrial partitioning (Zeta-prep, LKB), but their high capacity is often disadvantageous for analytical purification as enzymes do not withstand the harsh conditions necessary for their elution.

The chief hydrophilic cellulosic ion exchangers on the market and their properties were given by Segal[24].

The choice of the exchanger depends on the sample properties (charge, pH, stability), the amount to be treated and the level of purity required. The net charge of the molecule determines the type of exchanger. Usually, one uses weak exchangers in the pH range 6–9 and strong exchangers for slightly ionized substances eluted at extreme pH or with a high ionic strength. The nature of the matrix, the height/diameter ratio and the flow-rate are determined according to the required level of purification. On an industrial scale, one usually selects a height/diameter ratio of *ca.* 15–18. The enzyme is then eluted with a gradient of pH or a gradient of ionic strength with or without a pH gradient; stabilizing agents and/or enzymatic reaction inhibitors can be added.

3.1.1.1.1. Chromatofocusing. This is ion-exchange chromatography of a particular kind, developed by Sluyterman and Wijdenes[25–27], which utilizes the elution capacity of a weak cation exchanger. The eluent consists of a mixed buffer of various charged components (polybuffer). At the start, the column is stabilized at the highest value of the chosen pH gradient and the polybuffer is at the lowest pH. A pH gradient is then obtained automatically as elution proceeds, which permits focusing, *i.e.*, proteins will move along the column at or near their p*I* values. Flow-rates even lower than 0.5 ml/min are useful. The gradient slope is controlled by the concentration of

polybuffer being pumped into the column. The two types of column commonly used are Pharmacia Mono P and the Synchrom AX series. This simple, fast technique enables sizeable amounts (0.5–5 mg) of enzymes to be recovered without denaturation. It is particularly suitable for the separation of similar molecules such as isoenzymes.

3.1.1.2. Gel filtration chromatography

This method, also called exclusion or gel permeation chromatography, is well established. The molecules are eluted in order of decreasing molecular weight. The working range is chosen according to the purity desired, the molecular weight range and the concentration of contaminants. A summary of the different gels on the market was published in *Biofutur*[24]. The most commonly used are the Sepharose G series (Pharmacia) and the Bio-Gel P series (Bio-Rad Labs.). For MW above 100 000 daltons, Agarose gels, Sepharose (Pharmacia) and Bio-Gel A series (Bio-Rad Labs.) are currently used.

When the proteases for purification are metallo-enzymes, it is often necessary to maintain the integrity of the metal–enzyme complex during the chromatographic separation, for example to study enzyme activity. In order to avoid loss or substitution of metal ions, it may be necessary to operate on a metal-free chromatographic medium. The mode of preparation of such media and of determination of metal binding capacity were detailed by Martin[28].

3.1.1.3. Hydrophobic interaction chromatography (HIC)

Enzymatic separations on alkyl-agarose (Sephadex C*n*) columns depend on the formation of hydrophobic complexes between the hydrophobic amino acid areas of the enzyme and a ligand (X) covalently linked to an inert matrix on interposing a hydrocarbon chain (an "arm"). It may be possible to modify the intensity of the interaction by modifying the length of the arm. The X groups can be NH_2, COOH, OH, I, $CH=CH_2$, $-C\equiv CH$ or $-CH(CH_3)_2$. The commonest are the NH_2 groups[29]. The association occurs mainly when the medium has a high strength, this technique therefore being especially suitable for samples obtained from a saline precipitation. Nevertheless, as the elution conditions are sometimes drastic, the method is limited to particular cases. Proteases purified by this process include procarboxydase A and B[29], collagenase[29], kininase II[30], renin[31] and hornet chymotrypsin[32]. The selection of the column for a given precipitation, the preparation of column materials and the optimization of separations were studied by Shaltiel[29].

3.1.1.4. Adsorption chromatography on mineral polymers

The most commonly used material is hydroxyapatite. This crystalline calcium phosphate, $Ca_{10}(PO_4)_6(OH)_2$, permits a rapid fractionation of enzymes. The separation process involves the secondary and tertiary structure of the molecule, the neutral and acidic proteins apparently being fixed on the Ca^{2+} sites and the basic proteins on the PO_4^{3-} sites[1].

A great advantage of chromatography on hydroxyapatite is that it can be used with phosphate buffer at a pH close to the physiological pH and be virtually independent of the initial saline concentration of the sample, *i.e.*, it permits elution through a simple increase in phosphate concentration (usually 30–120 m*M*). The resolution depends on the shape, charge and charge/mass ratio. This technique is

particularly suitable for purifying labile enzymes and for the concentration of samples obtained by ion-exchange chromatography that have possibly been chromatographed without previous dialysis. The main inconvenience is the very low flow-rate[33]; different workers have proposed various methods to improve this[34]. Moreover, commercially available supports with shapes suitable for preparative chromatography, such as Bio-Gel HT and HTP (Bio-Rad Labs.) and HA-Ultragel (IBF) help to overcome the difficulties with the technique.

3.1.1.5. Affinity chromatography

Affinity chromatography is an adsorption chromatographic technique in which the molecule to be purified is selectively and reversibly adsorbed on a biospecific ligand. In some instances, the purification was significantly improved by interposing an "arm" which relieves the steric restrictions imposed by the matrix and allows an increased flexibility and availability of the ligand. The main coupling methods and their advantages and inconveniences were outlined by Scopes[35]. After eliminating the contaminants by washing, the enzyme is eluted by pH modification, a salt gradient, specific eluents, distorting eluents (urea) or by reducing the adsorbent polarity.

We can distinguish specific ligands, usable with similar enzymes from different origins, the main proteolytic ligands being listed in Table 1, and group ligands, commonly related to a variety of enzyme. The main ligands for serine proteases can be listed as follows.

The most commonly used aldehydic peptides[36,37] are the arginal derivatives, which show a very strong affinity for trypsin-family enzymes. Enzymes eluted from Argal–Sepharose (4B or 6B) are prekallikrein[38], *Clostridium histolyticum* clostripain[39], human plasminogen activator[40], prothrombin[41], Pronase (mixture of various proteases produced by *Streptomyces griseus*[36]), bovine trypsin[36] and mixtures of trypsin and chymotrypsin[37].

Soybean–trypsin inhibitor[42,43]: the Kunitz soybean inhibitior is frequently used to purify proteases, *e.g.*, trypsin, chymotrypsin[44], crab collagenase or kallikrein[45]. A few proteases can be separated with the aid of the Bowman Birk soybean inhibitor[46].

The benzamidine group has been used for rapid separations of numerous proteases, trypsin[47], thrombin[47], urokinase[48], prekallikrein[49], kallikrein[50,51], collagenase and clostripain[52] or second component of human complement. It is possible to utilize the benzamidine group to detect specifically active forms of some serum proteases such as trypsin, thrombin or human factors IXa and Xa[53].

Phenylboronic acids are used in the separation of trypsin and chymotrypsin[54] and of different subtilisins[55].

A recent technique that makes use of a new isopropyl fluorophosphate gel is of great interest in the separation of serine proteases[56].

The main ligand for the aspartate proteases is the hexapeptide Val–D–Leu–Pro–Phe–Phe–Val–D–Leu[54] and for thiol proteases some peptidyl diazomethyl ketones, which are more selective. They react rapidly at high dilution with thiol proteases but not with other proteases and can be synthesized to satisfy the specificity of individual members of the thiol protease family[57]. However, in the presence of Cu^{2+} ions, certain proteases can be inactivated[57].

As most ligands are inhibitors, research has centred on new inhibitors specific to the enzymes being investigated, such as α_2-macroglobulin, human α_1-proteinase

inhibitor, human α_1-antichymotrypsin, eglin (elastase–cathepsin G inhibitor from leeches), egg white cystatin and potato carboxydase inhibitor (CPI)[58]. Because CPI fixes numerous proteases, it is particularly interesting in the field of affinity chromatography[59]. It is stable at extremes of pH, not denatured by guanidine–6 *M* HCl and can be coupled to Sepharose. It has been used to separate various proteases, such as bovine and porcine carboxypeptidases A and B, carboxypeptidase N and cathepsin[59,60].

3.1.1.5.1. Affinity chromatography on immobilized dyes. Dyes, especially Cibacron Blue F3G-A, appear to be especially effective adsorbents for the purification of numerous proteases, including clotting factors, complement factors and human hexokinase[61]. Other dyes, such as Procion Green H-4G and H-8BN, have also been found to be suitable ligands for a yeast hexokinase[62] and carboxypeptidase G_2, respectively[63]. The mode of action is not well known but there seem to be interactions of the ion-exchange type and/or hydrophobic interactions[61]. This method is advantageous in several respects, being cheap and applicable to coupling, chemical and enzymatic studies, which makes it useful for large-scale purifications, but it lacks specificity. Several techniques for such dye-screening procedures have been published[35,61]. It must be operated under well defined buffer, temperature and pH conditions. The eluents can be non-specific, *i.e.*, molarity, salts, polyols, chaotropic or chelating agents and detergents, or specific, *i.e.*, molarity, substrates and dyes.

3.1.1.5.2. Lectins. When the enzymes are glycoproteins, which is frequently the case for proteases, the use of lectins as ligands is very helpful. The specificities of the main lectins have been reported by Segal[64], the more frequently used being concanavalin A (Con A), which recognizes molecules whose glucosylated part includes D-mannose or D-glucose. Lectins have numerous advantages as they are very selective molecules, available in large amounts. Also, as they do not interact very strongly with sugars ($K = 10^2$–10^4), they can be displaced easily by specific sugars at neutral pH and separated by concentration gradients[65]. Lectins are generally used in the early stages of purification and require additional procedures for complete purification; details of specific methods are not considered. The general method for their use has been reviewed by Lotan and Nicolson[66].

3.1.1.5.3. Immunoaffinity chromatography on antibody columns. Chromatography with antibodies or more recently with monoclonal antibodies has been used to separate some therapeutic proteases such as kallikrein[67], angiotensin I-converting enzyme[68], proteolytic enzymes of the complement system[69–71], blood-clotting enzymes[72–74] and urokinase[75].

3.1.1.5.4. Metal ion affinity chromatography (IMAC chromatography). This technique makes use of the capacity of metals to coordinate molecules containing heteroatoms and to produce a complex with aromatic components. Most often used are the Cu^{2+} and Zn^{2+} (ref. 76). Cu^{2+} has been used to purify an *Aspergillus niger* carboxypeptidase[77,78].

Purification on heparin, classically used for the proteases involved in blood clotting, should also be mentioned.

Numerous experiments have been carried out to try to improve the yield of affinity purifications and to adapt them to processes able to deal rapidly with large volumes. Some newer strategies are outlined below.

3.1.1.5.5. Counter-flow affinity filtration. This method combines the specificity

TABLE 1

AFFINITY CHROMATOGRAPHIC TECHNIQUES APPLIED TO PROTEOLYTIC ENZYMES

DFP = Difluorophosphate.

No.	*Enzyme*	*Ligand*	*Eluent*	*Ref.*
1	Acid protease	Pepstatine	Urea	86, 87
2	Amino peptidase	Leu–Gly	NaCl, Zn	88–90
3	Asparaginase	L-Asp	NaCl, Asp	91
4	Carboxypeptidase A	Phenyl propionates, *p*-aminobenzyl succinate	KCl	92 93
5	Carboxypeptidase B	D-Arg Protease inhibitor	NaCl, pH	94
6	Carboxypeptidase N	Aminobenzoylarginine	Guanidoethylmercapto succinate	95
7	Carboxypeptidase	Potato inhibitor	pH	58
8	*Aspergillus niger* carboxy-peptidase	Cu^{2+}–IDA Cu^{2+}–tyrosine	Acetate + EDTA	96
9	Cathepsin	Pepstatin Con-A	NaCl pH	87 97
10	Chymotrypsin	CBZ–(ω-NO_2)–Arg D-Tryptophan methyl ester	Phosphate buffer Acetic acid	37
11	Mammalian collagenase	Heparin, Zn^{2+}	Cacodylate buffer + NaCl Sodium cacodylate buffer, pH	98
12	Human collagenase	Collagen, Arg	NaCl	39,99
13	Porcine collagenase	Zn^{2+}	pH + EDTA	98
14	Dipeptidyl-peptidase	4-Phenylbutyl–Gly–Pro	Ethylene glycol, NaCl	100
15	Elastase	Elastin, $(Ala)_3$	Salt, Ala	99,101,102
16	Tissue factor apoprotein	Con-A	α-Methyl D-glucoside	103
17	Bovine factor VII	Benzamidine	Guanidine, HCl	104
18	Human factor XII (Hageman factor)	Lysine	$(NH_4)_2SO_4$	105
19	Bovine factor XI (plasma thromboplastin antecedent)	Heparin Benzamidine	NaCl + DFP Imidazole, HCl + guanidine, HCl + NaCl	106
20	Bovine factor X (Stuart factor)	Benzamidine	Guanidine, HCl	107
21	Formylmethionine-aminopeptidase	N-Formylbestatine	NaCl	108
22	Human prekallikrein	Heparin Benzamidine Con-A Agmatine	NaCl Guanidine, HCl α-Methyl D-glucoside NaCl	109, 110
23	Porcine kallikrein	Aprotinine	pH	111
24	Human kallikrein	Aprotinine Aminobenzamidine	Phosphate buffer Benzamidine, HCl	109, 110
25	Acid protease	Pepstatine	Urea gradient	87, 112
26	Neutral protease	N-phenylphosphenyl–Phe–Phe	pH	113
27	Pepsin	Poly(L-Lysine) ε-Aminocaproyl–L-Phe–D-Phe–OCH_3	NaCl NaCl	114, 115
28	Plasmin	Lysine	pH + NaCl	116
29	Post-proline hydrolase	CBZ–Pro–D-Ala	CBZ–Pro–Phe	117

TABLE I (*continued*)

No.	*Enzyme*	*Ligand*	*Eluent*	*Ref.*
30	Proteinase	4-Phenylbutylamine	NaCl, urea	118
		Soybean trypsin inhibitor D-Tryptophan methyl ester	$CaCl_2$	119, 120
31	*Aspergillus* proteinase	ε-Aminocaproyl–Phe–D-Phe–OCH_3	NaCl	121
32	Renin	Pepstatine,	pH	122, 123
		haemoglobin octapeptide (D-Leu^6)	pH Sodium acetate	124
33	*S. aureus* staphylocoagulase	Bovine prothrombin	NaSCN	125
34	Staphylokinase	DIP-canine-plasmin	pH	126
35	Subtilisin	*p*-(ω-aminoethyl)phenyl boronic acid	pH + glycerol	127
36	Thrombin	Aminobenzamidine	Benzamidine	128
37	Trypsin	Benzamidine	pH	
		CBZ–(ω-NO_2)–L-Arg	pH + semicarbazide	5
		Soybean trypsin inhibitor	pH + benzamidine	41
38	Tyrosinase	Con-A	α-Methyl D-mannoside	129

of affinity techniques with the efficiency of membrane separation. The substance to be purified, or the impurities if they are not too numerous and of a known nature, is specifically adsorbed on a solid support or an insoluble polymer. The molecular mixture containing the adsorbed substance is separated from the contaminants by filtration. The enzyme is desorbed by a suitable polymer, then the support and polymer are recycled. Such a process has developed for continuous use at the outlet of a bioreactor and has been tested on various biological molecules such as Con A and alcohol dehydrogenase[79,80], but has not, as far as we are aware, been used for proteolytic enzymes.

3.1.1.5.6. Affinity partitioning. The main inconvenience of the previous process is the few affinity sites that are available and the difficult access to such sites. To overcome this disadvantage, some workers have tried to avoid the difficulties by using a water soluble polymeric matrix on which the chosen ligand was coupled. Adamski-Medda *et al.*[81] thus separated trypsin from chymotrypsin by using *p*-aminobenzamidine bound to dextran. Choe *et al.*[82] separated the same enzymes using soybean trypsin inhibitor. Disappointing results ensued, with purification yields of 65% in the former instance and 81% in the latter, and the recovery rate of about 55% shows the difficulties of finding a macroligand specific enough for the enzyme to be purified. Some workers have avoided the difficulty by using synthetic macroligands. Luong *et al.*[83] synthesized an acrylamide polymer upon which *m*-aminobenzamidine groups are coupled. The capacity of such a ligand is close to the theoretical value[81]. By eluting with L-arginine, it is possible to recover 90% of the starting trypsin with a purity of 98%. Such a system can be operated continuously.

A number of other ligand polymers useful for affinity partitioning and methods for their preparation have been summarized elsewhere[84,85]. The amount of protein that can be included is limited by the solubility in the two phases, but 50 g of protein per

kilogram of partition system can often be used[6]. This technical approach is very promising but its industrial application is limited owing to the cost.

Affinity chromatographic techniques that have been applied to proteolytic enzymes are surveyed in Table 1.

3.1.2. High-performance liquid chromatography (HPLC)

The high-resolution HPLC processes are widely used in analysis and research and, because of their speed and efficiency, they are beginning to be used on a preparative scale. In order to limit costs and maximize production in preparative chromatography, the column should be overloaded. Ghodbane and Guiochon[130] described the prediction of the optimum extent of overloading in relation to the required recovery and degree of purity of the final products.

The most commonly used techniques are high-performance size-exclusion chromatography and high-performance ion-exchange chromatography.

3.1.2.1. High-performance size-exclusion chromatography (HPSEC)

HPSEC enables eluted molecules to be separated according to their decreasing molecular weight and size. The breakthrough with this technique was associated with the development of highly resistant columns operating at high back-pressures, *i.e.*, with hydrophilic polymer gels, diol-bonded silica, hydrophilic grafted or coated silica gels and cellulose derivatives[131,132]. HPSEC is applied in four areas: prefractionation, analytical separations, molecular weight determinations and preparative isolation. It is also suitable in the monitoring of the time course of enzymic reactions[132]. It has several advantages: proteins are eluted rapidly with relatively narrow peaks; as an isocratic system generally suffices, it is cheap and makes it possible to isolate easily a few milligrams of enzyme[133]. This compensates for the disadvantages involved, such as limited peak capacity and the fact that it is never entirely quantitative because of parasitic adsorption phenomena between the enzyme and the matrix[134]. The resolution depends on the column (mass loadability, fractionation range), flow-rate and size and shape of the proteins[135]. In order to increase the resolution, several column of decreasing fractionation range can be used. It is strongly recommended to collect only the centre of the peaks and recycle the remainder. The flow-rates vary between 0.5 and 1.5 ml/min in the analytical and between 8 and 10 ml/min in the preparative mode. The commonest commercial columns are those from Toyo Soda (SW and TSK series). Merck (LiChrosorb Diol and LiChrospher), Waters Assoc. (Protein-Pak series), Synchrom (Synchropak) and Lachema (Separon).

The main proteases thus purified are trypsin[133], chymotrypsin[133], pepsin[134], numerous kinases[136] and a gelatinase[135].

3.1.2.2. High-performance ion-exchange chromatography (HPIEC)

In the HPIEC mode, the enzymes are separated not only according to their charge but also their hydrophobicity and molecular weight, The effects of the mobile phase, of ionization and a salt slope play a major role in separations[137–140]. The usual gradient slope is a 3.3% salt gradient accross the column. Mobile phase velocities of 7.2 ml/min per cm^2 cross-sectional area (1 ml/min in a 42–50 mm column) are average[137]. Operating at this velocity with a 3–6%/min gradient slope will give good separations in 15–30 min. Halving the mobile phase flow-rate has been shown to increase the

resolution 1.5-fold when a 1.66%/min gradient slope was used. In this instance, the total separation time would be 60 min. The two variables that have been found to be most useful in controlling retention and selectivity are the pH of the mobile phase and the nature of displacing salt. The influence of the major ions on the retention and resolution of various proteins has been studied by Regnier[137]. The main columns in use are the Mono S and Mono Q (Pharmacia), the Synchropak AX, CM and QX series (Synchrom) and the IEX series (Toyo Soda). In order to separate enzymes with closely similar p*I* values, chromatofocusing (Mono P columns, Pharmacia) is occasionally used[141,142].

3.1.2.3. Reversed-phase high-performance liquid chromatography (RP-HPLC)

This very selective method has the inconvenience of being enzyme denaturing. A number of trials were carried out in order to find supports and elution conditions that permit biological activity to be retained[143–145] by adding ion-pairing agents[146] and stabilizers. Utilization of volatile organic acids (formic, acetic, trifluoroacetic and heptafluoroacetic acids) and of calcium chloride made it possible to recover biological activity after elution by rapid evaporation of the organic solvent under nitrogen[147,148], or by modifying the pH buffer or molarity just after separation[143]. In order to increase the recovery of proteins of MW > 30 000 daltons, some workers advise the use of "end-capped" short linked phases, RP-18 or RP-4, especially in the semi-preparative mode[149–151]. Optimizing the column and separation by sample pretreatment and altering the mobile phase conditions have been extensively studied[152,153]. However, this technique is mainly used for low-molecular-weight enzymes. Because of risks of denaturation, it is advisable to use this technique for checking the purity of a given sample or for purifying small amounts of product for structural investigations.

3.1.2.4. High-performance hydrophobic interaction chromatography (HPHIC)

HPHIC with microparticulate supports, introduced in 1983[154], made it possible to separate proteins rapidly with high resolution and to recover almost quantitatively the injected sample with good preservation of the activity[155]. The supports used contain a silica matrix[156–161] or hydrophylic polymers[162–164]. The hydrophobicity of the support is adjusted so that the proteins can be adsorbed in ionic, anti-chaotropic solutions within the molarity range 1–2 M. The selectivity was shown to change with eluent pH[165]. Gradients often used are ammonium sulphate or potassium phosphate with molarity varying between 1.5–2.5 and 0.05–0 *M* over a 30–60-min period. The flow-rates usually used are 0.5–1 ml/min. Kato[166] reported a detailed study on the various parameters that can influence separation: hydrophobicity of the support ligand, column length, initial salt concentration, type of salt and slope of salt concentration, elution curve, pH, flow-rate, temperature, addition of organic solvents, chaotropic agents or surfactants and sample loading.

3.1.2.5. High-performance affinity chromatography (HPAC)

HPAC combines the high specificity of affinity techniques with the efficiency, sensitivity and speed of HPLC techniques. For proteolytic enzymes, the ligands used may be dyes (Procion Yellow, Cibacron Blue F3G), proteins (soybean trypsin inhibitor, Con-A), antibodies, metals or various other substances. Fassina and

Chaiken[167], Larsson[168] and Olsson[169] have studied the various techniques used for coupling of ligands, to control the determination of bound ligands and to optimize separations. It should be noted that a commercial column (Ultraffinity, Beckman) is available on which the user can couple a larger number of ligands. This technique is very interesting for analytical work but has been little used with proteolytic enzymes.

Proteases separated by this technique include hexokinase (Procion Green MX-5BR)[170], carboxypeptidases (Procion Yellow H-A)[170], various proteolytic enzymes of the complement system C1r, C1s, C2a, etc. (antibodies)[171], chymotrypsin (soybean trypsin inhibitor)[168] and pepsin (L-Phe–D-Phe–OCH_3, methacrylate support)[172].

Examples of HPLC techniques applied to the purification of proteolytic enzymes are summarized in Table 2.

TABLE 2

EXAMPLES OF HPLC TECHNIQUES APPLIED TO THE PURIFICATION OF PROTEOLYTIC ENZYMES

Technique	*Column*[a]	*Ref.*
HPSEC:		
A. melleus semi-alkaline protease	TSK G 3000 SW (Tosoh)	173
Pepsin	TSK G 3000 SW (Tosoh)	174
Leucine aminopeptidase, trypsin, pepsin, α-chymotrypsin	LiChrosorb Diol (Merck)	175
Mastocyta tryptase	TSK G 3000 SW (Tosoh)	176
HPIEC:		
Saccharomyces cerevisiae protease	Mono Q (Pharmacia)	177
Muscular cysteine proteinase	Mono Q (Pharmacia)	178
Entamoeba histolytica proteinase	Mono P (Pharmacia)	179
Human metalloendoprotease	Mono Q (Pharmacia)	180
E. coli membrane protease	Mono P (Pharmacia)	181
Rat thiol proteases	Mono P (Pharmacia)	182
Proteases (T cells)	Mono Q (Pharmacia)	3
Chymopapain	Mono S (Pharmacia)	183
A. niger semi-alkaline protease	Mono Q (Pharmacia)	184
Bovine adrenomedullary granules protease	Mono Q (Pharmacia)	185
HPLAC:		
Trypsin	STI–LiChrospher 500 NH_2 (Merck)	186
A. niger protease–pepsin	ω-Aminocaproyl-L-Phe-D-Phe-OCH_3 Separon H 100[b]	172
RP-HPLC:		
Trypsin	μBondapak C_{18} (Waters Assoc.)	187
Papain	LiChrospher 500-*n*-butyl (Merck)	33
RP-HIC:		
Human metalloendoprotease	TSK-phenyl 5 PW (BioRad Labs.)	180
Procarboxypeptidases A and B	TSK-phenyl 5 PW (BioRad Labs.)	188
Proelastase	TSK-phenyl 5 PW (BioRad Labs.)	188
Renin	TSK-phenyl 5 PW (Toyo Soda)	189

[a] STI = Soybean trypsin inhibitor.

[b] Column packed by workers.

3.2. Electrophoretic techniques

3.2.1. Preparative isoelectric focusing

Isoelectric focusing is a very attractive method for the fractionation and isolation of enzymes. It permits a fairly large amount of product to be purified with excellent resolution and load capacity. The method concentrates and separates molecules different each other in only 10^{-2} pH unit[190]. Four kinds of matrices are used: polyacrylamide gels, agarose, granulated gels (Sephadex G-200, Sephacel, Bio-Gel 60) or rehydratable gels, the choice depending on the amount and to the molecular weight of the enzymes to be separated. Laboratory-scale techniques on polyacrylamide or Sepharose gels enable fractions up to 100–150 mg and large-scale fractionation permits several grams to be purified in one step by using density gradient columns and layers of granulated gels[191,192]. Resolution is influenced by the field strength and the shallowness of the pH gradient. Once focusing is finished, the enzymes must be located in the gel layer and separated at low temperature by rapid, simple and preferably non-destructive methods. Speed is important because keeping the gels without a voltage or at a reduced voltage can lead to broadening of zones by protein diffusion[191]. The recovery depends on a number of factors, such a proper separation including elution from the gel, additional steps that may be necessary for removal of ampholytes and/or concentration of the isolated fraction. Many techniques, such dialysis, electrodialysis, ultrafiltration, salting-out, gel chromatography, ion-exchange or hydrophobic chromatography, two-phases extraction with *n*-pentanol and, more recently, electroosmosis have been suggested for the removal of ampholytes[188]. An alternative is "Trans elution" which consists of the sideways transfer of the macromolecules under the influence of an electric field from the gel slab[189]. The electro-eluted proteins remain in solution and are easily recovered by spin-drying the support. It may be possible to recover 85–92% of the crude sample[190]. The recovery also depends on the load capacity; loads from 0.5 to 10 mg/ml gel increase the recovery of Pronase from 14 to 80%[190].

These techniques are mostly used in research and to separate rapidly analytical samples. The various methodologies have been detailed by various workers[190,192,193].

3.3. Particular instances

3.3.1. Metal-free enzymes

It is sometimes necessary to utilize metal-free enzymes, for instance to study enzyme–metal interactions or to keep an enzyme in its crystalline stage (carboxypeptidase A)[194]. Suitable apoenzymes are prepared by using metal-chelating agents. As proteases are often zinc-activated metalloenzymes, 1,10-phenanthroline is used[195]. Proteases purified in this way include *Acromonas* aminopeptidase[196], angiotensin-converting enzyme[197], *Bacillus subtilis* neutral protease[198], carboxypeptidase A[199,200] and B[201], a bovine aminopeptidase[202], procarboxypeptidase A[202], *Streptomyces griseus* carboxypeptidase[204] and thermolysin[205].

4. ELUATE CONCENTRATION

Following chromatography, the media must be concentrated. The most widespread methods are ultrafiltration and dialysis. Freeze-drying is sometimes used.

4.1. Concentration by ultrafiltration

This is the most common technique. Diffusive ultrafiltration, in which molecules are removed by molecular diffusion under the action of a concentration or activity gradient, is regularly used. To reduce the phenomena of concentration polarization and enzymic adsorption, tangential filtration with a polysulphone or cellulose nitrate membrane is mostly used. The main application have been discussed by Kusiak *et al.*[206]. It is also possible to use diafiltration; such a method of washing with a regulated volume is suitable for eliminating mineral salts and low-molecular-weight substances.

4.2. Concentration by freeze-drying

Although freeze-drying is employed classically for the concentration of enzymes, its use is limited by the fact that unless the salt concentration of solutions is sufficiently reduced, eutectic mixtures may be formed. This may lead to incomplete drying or severe foaming and enzyme denaturation[1]. To avoid denaturation and loss of activity, it is essential to operate under very strict pH conditions, which vary according to the enzymes, and to suppress or limit the final heating during deadsorption.

The "drop dialysis method", developed by Marusyk and Sergeant[207] for dialyse[129] small-volume samples of DNA is interesting. This technique, initially applied to DNA, makes it possible to prepare quickly small samples for electrophoresis or any other analytical method, with high recoveries and a tolerable loss of enzyme activity[208].

5. ENZYME CRYSTALLIZATION

The operating conditions vary according to the enzymes. It is nevertheless necessary to control certain factors, such as pH, temperature and precipitants. There are three distinct phases to be considered: nucleation, post-nucleation and cessation of growth[209]. Crystal formation is possible only at the critical point of supersaturation in an appropriate environment (ionic strength, pH, temperature). Crystals continue to grow as long as the state of supersaturation exists. The optimum protein concentration is *ca.* 10–20 mg/ml. It is essential to add the precipitating agent in small amounts and to follow nucleation[210]. One most often operates at the pH of activity (*i.e.*, pH of maximum enzyme activity), at room temperature or in a cold chamber (4–6°C). Gilliand and Davies[209] published a review that summarizes the chief precipitating agents and the different techniques in use, the most widespread being ammonium sulphate, PEG and 2-methyl-2,4-pentanediol.

6. ENZYME PURIFICATION MONITORING

Enzyme purification is most often carried out by electrophoresis and sometimes HPLC (RP-HPLC, HPSEC or HPIEC). UV detection at 280, 254 or 214 nm provides an excellent means of controlling product purity and quantifying yields. Electrophoretic techniques currently used are analytical electrophoresis, sodium dodecyl sulphate (SDS) electrophoresis and electrofocusing; immunoelectrophoresis is also used. Burton *et al.*[152] explored a new strategy which couples RP-HPLC with SDS electrophoresis.

6.1. Techniques for enzyme separation

6.1.1. Analytical electrophoresis

When it is necessary to separate intact proteins by a non-destructive means for later assessment of biological activity, gels must be prepared under non-denaturing conditions, *i.e.*, polyacrylamide gel electrophoresis (PAGE)[211,212]. As the speed of electrophoretic migration of a protein depends not only on its charge but also on its molecular size and shape, it is possible to separate two enzymes with an identical net charge but of different size by varying the pore size of the support as a function of the acrylamide (range about 2.5–30%, w/v) and the amount of cross-linker used. Gels with less than 2.5% permit proteins with a molecular weight close to 10^6 daltons to be separated and gels with 30% do the same for polypeptides with molecular weights close to 2000 daltons[213]. Separation by charge is achieved by operating between pH 3 and 11 to allow for maximum charge differences between neighbouring protein species[214].

To analyse very complex media or isolate enzymes in very small amounts, it is possible to increase the resolution by two-dimensional operation. Since O'Farrel's original publication[215], numerous modifications have been introduced to improve reproducibility[216–219] and increase the resolution and detection of proteins[220]. The use of a heating apparatus, of denaturing agents such as β-mercaptoethanol[218,221,222], dithioerythritol (DTE) or dithiothreitol (DTT)[216,218] and of zwitterionic detergents[218] has been proposed. Simplification and improvement of the O'Farrel method made it possible to obtain a better separation and to increase the detection sensitivity by reducing the background noise[223].

For analytical purposes, media are often separated by denaturing electrophoresis, the most often used being the SDS techniques of Laemmli[224] or Neville[225]. The advantage of Neville's system is that the lengthy washing required to remove glycine from gels when Tris–glycine is used is avoided. Treatment with denaturing agents. SDS (1–2%), with or without reducing agents, dithiothreitol (40–100 m*M*) or 2-mercaptoethanol (up to 1 *M*) reduces disulphide bonds. The only separative criterion is protein size.

It is possible to prepare gels with an increasing acrylamide concentration gradient (generally 5–20%), which has two advantages, first for separating a larger range of molecular weights and second for increasing the resolution. Electrophoresis can be performed with a single buffer (continuous electrophoresis) or with several buffers (discontinuous electrophoresis). The latter technique permits proteins to be concentrated with extremely small zones prior to their migration. Specific details of the procedure have been described by Blackshear[214]. A single band in SDS electrophoresis is usually accepted as a criterion of purity.

6.1.2. Isoelectric focusing

Isoelectric focusing offers two distinct advantages over the usual methods of electrophoresis: it gives a high resolution, proteins differing in p*I* by 10^{-3} to 10^{-2} being resolved, and it is independent of time so deterioration of the zone definition with time is almost negligible. The resolution is improved by using high field strengths (100–300 V/cm) in thin (0.2–1 mm) gels layers.

Affinity electrophoresis and isotachophoresis can also be used. Kolodzeiskaia[226] recently published a paper in which electrophoretic techniques applied to proteolytic enzymes were reviewed.

6.2. Enzyme localization in gels

6.2.1. Non-specific detection

Such techniques imply an irreversible alteration of the enzyme and their only interest is to permit its observation. Coomassie R_{250}[227] and G_{250} Blue[228] and silver staining[229] are commonly used. The sensitivities are about 50 ng and 1 ng per spot, respectively. Because numerous proteases are glycoproteins, it is of interest to detect them more specifically. Three methods are used[230], the thymol–sulphuric acid method, the periodic acid–Schiff base method and the fluorescein isothiocyanate–labelled lectin method.

6.2.2. Specific detection

It is often relevant to preserve enzymic activity and to determine it. There it is necessary to eliminate the electrophoretic reagents that are likely to interfere in the determination (ampholine, SDS, etc.) and sometimes to renature the enzymes. Different techniques are used, such as removal of SDS by incubation in buffered 25% isopropanol or renaturing of enzyme by using a combination of non-ionic detergent, glycerol and substrate; subsequently several approaches can be chosen[231].

6.2.2.1. Elution of enzymes from the gel

The gel is cut into pieces and the enzymes are eluted with an appropriate buffer and assayed by conventional methods. The measured activity depends on the electrophoretic technique used (more or less denaturing) and of the recovery from the gel. Spectrophotometric assays are widely used to detect proteolytic enzymes. These use fluorochrome[232–236] or chromogenic indicators conjugated to the substrate[237–241]. Soluble substrates[241,244] require the removal of undegraded material (usually trichloroacetic acid precipitation), unless they have been attached to a solid support, such as Sepharose[236,238]. Insoluble substrates such as azocoll[240] can be removed by centrifugation. Proteolytic activity is determined by measuring the amount of indicator released in the supernatant. Alternatively, indicators such as fluorescamine[234] or *o*-phthaldehyde [241] may be added to the supernatant to detect digestion products. These assays can detect trypsin, collagenase or elastase[234,235] and chymotrypsin[233] at concentrations of 50 and 1 mg/ml, respectively. An alternative is to used a radiolabelled substrate such as [^{14}C]collagen[242,243], [^{3}H]elastin[244,245] or [^{125}I]fibrinolectin[246] and casein[247]. All of these assays rely on the release of radiolabelled products into the supernatant, where they are determined by gamma or scintillation counting. An assay using radioiodine–gelatin as substrate was published recently[248]. This assay is able to detect elastase, trypsin and collagenase at concentrations of 1 ng/ml or less.

6.2.2.2. Staining for enzymic activity in situ after electrophoretic detection

Heeb and Gabriel[231] have reported five groups of techniques:

(i) simultaneous capture, where the substrate is converted by the enzyme into a product that couples immediately with a reagent present in the incubation mixture to form an insoluble coloured product;

(ii) post-incubation coupling, in which incubation of substrate with enzyme results in a product, then a reagent is added to the medium to yield coloured material;

(iii) autochromic methods, which make possible direct observation of enzymic activity by changes in the optical properties of either the substrate or reaction product;

(iv) sandwich-type incubation, which uses a matrix containing an auxiliary indicator enzyme or high-molecular-weight substrates; incubation of the separating gel with the matrix permits localization of enzymic activity; and

(v) copolymerization of substrate in the gel, which is used for high-molecular-weight substrates such as gelatin or casein. It is often necessary to prevent the enzyme from acting on the substrate during electrophoresis under enzymic conditions, *e.g.*, inclusion of an inhibitor or chelating agent, use of a less than optimal pH, or other means. Conditions of optimum activity can be restored during the subsequent incubation period in order to detect enzyme activity.

The main techniques used for localizing proteases *in situ* are reported in Table 3.

TABLE 3

LOCALIZATION OF ENZYME ACTIVITY

No.	*Enzyme*	*Substrate*[a]	*Detection*[b]	*Ref.*
1	Acrosin (E.C. 3.4.21.10)	Arg–β-naphthylamine	Coupling with Fast Black	249
2	Aminopeptidase (E.C. 3.4.11.4)	L-Leu–Gly–Gly	Coupling with amino acid oxidase, PMS	231, 250
3	Carboxypeptidase (E.C. 3.4.17)	Carbonaphthoxy–Phe	Coupling with Diazo Blue B	251
4	Cathepsin B (E.C. 3.4.22.1)	Z Arg–Arg–methoxynaphthyl-amide	Coupling with Fast Garnet, GBC/scan	252
		Azocoll	TCA precipitation	252
5	Chymotrypsin (E.C. 3.4.21.1)	Z Tyr–*p*-nitroanilide	Diazotization of *p*-nitroanilide	254
6	Dipeptidase (E.C. 3.4.14)	L-Leu–Leu	Coupling with amino acid oxidase, PMS	231, 250
7	Elastase (E.C. 3.4.31.11)	N-Acetyl-DL-Ala α-naphthyl ester	Coupling with Fast Black	231, 255, 256
		Elastin–orcein	Scan at 550 nm	
			Clear zones	
8	Proteinases (E.C. 3.4)	Casein, azocoll, gelatin	TCA precipitation, scan at 520 nm	257, 258
		Fluorescein–gelatin	Salt precipitation, fluorescence	259–261
		FITC–casein	Clear zones	262, 263
9	*Aspergillus* proteinases (E.C. 3.4)	Azocasein	Clear zones	264
10	Thermolysine (E.C. 3.4.24.4)	Cytochrome *c*	TCA precipitation	253
11	Trypsine (E.C. 3.4.21.4)	Azocoll	TCA precipitation + scan	253, 265, 266
		BAPNA	Diazotization of *p*-nitroanilide	256
		Benzoyl–Arg–Arg *p*-nitroanilide		
12	Subtilisin (E.C. 3.4.21.14)	Benzoxyl–Gly–Gly	Product visible under UV light	267
		Leu–*p*-nitroanilide		
13	Urokinase	Plasminogen, fibrin	Clear zones	268
	Plasminogen activators (E.C. 3.4.31.31)	Plasminogen, gelatin	Amido Black staining	

[a] Z = Carbobenzoxy; FITC = isothiocyanate–fluorescein; BAPNA = benzoylarginine-*p*-nitroanilide.

[b] PMS = Phenazine methosulphate; TCA = trichloroacetic acid; INT = iodophenyl nitrophenyl tetrazolium; Fast Garnet GBC base = 4-amino-2′,3-dimethylazobenzene.

6.2.3. Western blotting

In order to facilitate the detection of an enzyme in a very complex medium (*e.g.*, biological extract, fermentation juice) it may be interesting to try the western blot technique. After the enzyme has been separated by a suitable electrophoretic technique and renaturation, it is transferred on to a membrane, then detected by incubation in the presence of an antibody or a specific ligand and rendered visible. There are seven practical stages of operation:

(i) separation of the crude extract by electrophoresis;

(ii) washing away the gel before transfer and renaturation by removal of detergents:

(iii) transfer (usually electrotransfer); the transfer conditions, *i.e.*, pH, ionic strength, presence of methanol, are determined by the nature of the required protein and the commonly used membranes are made of nylon, nitrocellulose or poly(vinylidene dichloride)[269];

(iv) pre-hybridization, *i.e.*, eliminating non-specific support fixation sites which account for background noise;

(v) detection with a non-specific dye (Coomassie Blue, Amido Black, aurodye), by marking with a radioactive isotope (^{32}P, ^{125}I)[270], a fluorescent marker, an antibody avidin–biotin system[271] or immuno-gold–silver staining[272]; zinc-binding enzymes can be probed with radioactive zinc (^{65}Zn) and detected by autoradiography[273];

(vi) elimination of free ligand by washing; and

(vii) signal spotting through coloration, fluorescence or autoradiography.

The practical aspects of such techniques have been discussed by Durand[274] and Walker[275]. This very sensitive method, the detection threshold of which can be close to 1 ng/mm^2, is very interesting for the detection of therapeutically significant enzymes, *e.g.*, collagenase in some forms of cancer[276]. It may also possible to detect specifically active enzymes by performing the coupling reaction after transfer on to a membrane on which have been previously fixed auxiliary enzymes specific for the reactive product[276].

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8. SUMMARY

A purification scheme has been established and three major steps defined: extraction and solubilization of the enzyme in the medium, obtaining the crude enzyme and selective purification. For each step, the applicable techniques, with their advantages and incoveniences, and the classical parameters in use are discussed. Affinity liquid chromatography and high-performance liquid chromatographic processes have been particularly developed. Techniques for enzyme crystallization and methods for monitoring enzyme purification are considered.

REFERENCES

1 J. Helling and B. W. Phillips, in A. Wiseman (Editor), *Handbook of Enzyme Biotechnology*, Ellis Horwood, Chichester, 1975, p. 59.
2 H. Hustedt, K. H. Kroner, H. Schütte, U. Menge and N. Papamichael, *Bioengineering*, 1 (1986) 12.
3 G. Johansson, *Methods Enzymol.*, 104 (1984) 356.
4 G. Johansson, in H. Welter, D. E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems*, Academic Press, Orlando, 1985, p. 161.
5 T. B. Choe, P. Masse and A. Verdier, *Biotechnol. Lett.*, 8 (1986) 163.
6 S. J. Stocks and D. E. Brooks, *Anal. Biochem.*, 173 (1988) 86.
7 P. A. Albertsson and G. Birkenmeier, *Anal. Biochem.*, 175 (1988) 154.
8 H. Hustedt, K. H. Kroner, U. Menge and M. R. Kula, *Trends Biotechnol.*, 3 (1985) 139.
9 K. H. Kroner, H. Hustedt, S. Granda and M. R. Kula, *Biotechnol. Bioeng.*, 20 (1978) 1967.
10 M. R. Kula, *Ann. N.Y. Acad. Sci.*, 341 (1981) 354.
11 H. Hustedt, B. Börner, K. H. Kroner and N. Papamichael, *Biotechnol. Techn.*, 1 (1987) 49.
12 K. C. Ingham, *Methods Enzymol.*, 104 (1984) 351.
13 T. F. Bussy and K. C. Ingham, *J. Biochem. Biophys. Methods*, 2 (1980) 191.
14 K. C. Ingham and T. F. Bussy, *Chem. Eng. Commun.*, 7 (1980) 315.
15 D. A. Scott, *Biochem. J.*, 28 (1934) 1592.
16 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1922) 384.
17 E. J. Cohn, F. R. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. M. Mittlerman, R. F. Mouton, K. Schmid and E. Uroma, *J. Am. Chem. Soc.*, 72 (1950) 465.
18 P. A. Roche and S. V. Pizzo, *Arch. Biochem. Biophys.*, 256 (1987) 265.
19 V. C. W. Tsang, J. M. Peralta and A. R. Simons, *Methods Enzymol.*, 92 (1983) 377.
20 E. L. Giroux and R. I. Henkin, *Biochim. Biophys. Acta*, 273 (1972) 64.
21 P. G. Zaworski and G. S. Gil, *Anal. Biochem.*, 173 (1988) 440.
22 J. Schneider, J. M. Harris and M. Yalpani, in J. M. Harris and M. Yalpani (Editors), *Partitioning in Aqueous Two-Phase Systems*, Academic Press, Orlando, FL, 1986, p. 126.
23 J. L. Tayot, *Biofutur*, 51 (1986) 69.
24 G. Segal, *Biofutur Technoscope*, No. 12, July (1987) 5.
25 L. A. Æ. Sluyterman and J. Wijdenes, in B. J. Radola and D. Graesslin (Editors), *Proceedings of International Symposium Electrofocusing and Isotachophoresis*, Walter de Gruyter, Berlin, 1977, p. 463.
26 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31.
27 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 429, 441.
28 M. T. Martin, *Methods Enzymol.*, 158 (1988) 15.
29 S. Shaltiel, *Methods Enzymol.*, 104 (1984) 69.
30 T. A. Stewart, J. A. Weare and E. G. Erdös, *Methods Enzymol.*, 80 (1981) 450.
31 T. C. Carilli, L. Cameron Wallace, L. M. Smith, M. A. Wong and J. A. Lewicki, *J. Chromatogr.*, 444 (1988) 203.
32 R. Zwilling and H. Neurath, *Methods Enzymol.*, 80 (1981) 633.
33 G. Bernardi, *Methods Enzymol.*, 22 (1971) 325.
34 H. Hirano, T. Nishimura and T. Iwamura, *Anal. Biochem.*, 150 (1985) 228.
35 R. K. Scopes, *Anal. Biochem.*, 165 (1987) 235.
36 S. I. Ishii and K. I. Kasai, *Methods Enzymol.*, 80 (1981) 842.
37 T. V. Wilkinson, A. H. Patel and R. M. Schultz, *Sep. Sci.*, 11 (1976) 385.
38 C. Kattner and E. Shaw, *Anal. Biochem.*, 80 (1981) 826.
39 I. Emöd and B. Keil, *FEBS Lett.*, 77 (1977) 51.
40 N. Sakuragawa, K. Takahashi and T. Ashizawa, *Acta Med. Biol.*, 25 (1977) 119.
41 A. H. Patel and R. M. Schulz, *Biochim. Biophys. Res. Commun.*, 104 (1982) 181.
42 D. L. R. Hwang, K. T. D. Lin, W. K. Yang and D. E. Foard, *Biochim. Biophys. Acta*, 495 (1977) 369.
43 D. E. Foard and K. D. Lin, *Abstracts, Euroanalysis III*, Abstract 27, August 1978.
44 W. M. Roschlan, *Folia Haematol.*, 101 (1974) 22.
45 H. Fritz, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 893.
46 K. D. Lin, D. L. Hwang and D. E. Foard, *J. Chromatogr.*, 195 (1980) 385.
47 A. Kanamori, N. Seno and I. Matsumoto, *J. Chromatogr.*, 363 (1986) 231.
48 G. Schmer, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 810.

49 L. Holmberg, B. Bladh and B. Åstedt, *Biochim. Biophys. Acta*, 445 (1976) 215.
50 R. L. Heimark and E. W. Davie, *Biochemistry*, 18 (1979) 5743.
51 K. Tasaka, *Pharmacology*, 21 (1980) 43.
52 C. Sampaio, S. C. Wong and W. Shaw, *Arch. Biochem. Biophys.*, 165 (1974) 133.
53 D. M. Monroe, G. B. Sherril and H. R. Roberts, *Anal. Biochem.*, 172 (1988) 427.
54 V. K. Akparov and V. M. Stepanov, *J. Chromatogr.*, 155 (1978) 329.
55 E. Millquist, H. Petersson and M. Ranby, *Anal. Biochem.*, 170 (1988) 289.
56 J. Pohl, M. Zaoral, A. Jindra, Jr. and V. Kostka, *Anal. Biochem.*, 139 (1984) 265.
57 P. Rauber, P. Wikstrom and E. Shaw, *Anal. Biochem.*, 168 (1988) 259.
58 A. J. Barret, *Methods Enzymol.*, 80 (1981) 771.
59 G. M. Hass and C. A. Ryan, *Methods Enzymol.*, 80 (1981) 778.
60 M. T. Everitt and H. Neurath, *FEBS Lett.*, 110 (1980) 292.
61 C. R. Lowe and J. C. Pearson, *Methods Enzymol.*, 104 (1984) 113.
62 Y. D. Clovis, M. J. Goldfinch and C. R. Lowe, *Biochem. J.*, 197 (1981) 203.
63 P. Hughes, C. R. Lowe and R. I. Sherwood, *Biochim. Biophys. Acta*, 700 (1982) 90.
64 G. Segal, *Biofutur Technoscope*, No. 14, October (1987) 18.
65 A. Kamra and M. Gupta, *Anal. Biochem.*, 164 (1987) 405.
66 R. Lotan and G. L. Nicolson, *Biochim. Biophys. Acta*, 559 (1979) 329.
67 R. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
68 J. J. Lanzillo, R. P. Cynkin and B. L. Fanburg, *Anal. Biochem.*, 103 (1980) 400.
69 Y. Mori, *J. Chromatogr.*, 189 (1980) 438.
70 R. A. Wetsel, M. A. Jones and W. P. Kolb, *J. Immunol. Methods*, 35 (1980) 319.
71 R. B. Sim, *Methods Enzymol.*, 80 (1981) 26.
72 J. L. Lane, H. Ekert and A. Vafiadis, *Thromb. Haemostasis*, 42 (1979) 1306.
73 J. A. Katzmann, D. Neshiem, L. S. Hibbard and K. G. Mann, *Proc. Natl. Acad. Sci. U.S.A*, 78 (1981) 162.
74 A. H. Goodall, G. Kemble, D. P. O'Brien and E. Rawlings, *Blood*, 59 (1982) 664.
75 T. C. Wun, L. Ossowski and E. Reich, *J. Biol. Chem.*, 257 (1982) 7262.
76 M. Belew, T. T. Yif, L. A. Andersson and R. Ehrnstrom, *Anal. Biochem.*, 164 (1987) 457.
77 S. Krishnan, M. A. Vijayalakshmi and I. Greahel, *J. Chromatogr.*, 397 (1987) 339.
78 S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 370 (1986) 315.
79 E. Pungor, N. B. Afeyan, N. F. Gordon and C. L. Cooney, *Biotechnology*, 5 (1987) 604.
80 C. Vincent, *Biofutur Technoscope*, No. 20, May (1988) 1.
81 D. Adamski-Medda, Q. T. Nguyen and E. Dellacherie, *J. Membr. Sci.*, 9 (1981) 337.
82 T. P. Choe, P. Masse and A. Verdier, *Biotechnol. Lett.*, 3 (1986) 163.
83 J. H. T. Luong, A. L. Nguyen and K. L. Male, *Biotechnology*, 5 (1987) 564.
84 I. N. Topchieva, *Russ. Chem. Rev. (Engl. Transl.)*, 49 (1980) 260.
85 A. F. Bückmann, M. Morr and G. Johansson, *Makromol. Chem.*, 182 (1981) 1379.
86 E. Hackenthal, R. Hackenthal and U. Hilgengeldt, *Biochim. Biophys. Acta*, 522 (1978) 561.
87 K. Yamamoto, N. Katsuda and K. Kato, *Eur. J. Biochem.*, 92 (1978) 499.
88 S. M. M. Basha, M. N. Horst, F. W. Bazer and P. M. Roberts, *Arch. Biochem. Biophys.*, 185 (1978) 174.
89 C. Kettner, J. Rodriguez-Absi, G. I. Glover and J. M. Prescott, *Arch. Biochem. Biophys.*, 162 (1974) 56.
90 K. H. Rohm, *Hoppe Seyler's Z. Physiol. Chem.*, 363 (1982) 641.
91 T. Tosa, T. Sato, R. Sano, K. Yamamoto, Y. Matuo and I. Chibata, *Biochim. Biophys. Acta*, 334 (1974) 1.
92 G. Oshima and K. Nagasawa, *J. Biochem.*, 81 (1977) 1285.
93 L. B. Cueni, *Anal. Biochem.*, 107 (1980) 341.
94 Y. Narahashi, K. Yoda and S. Honda, *J. Biochem.*, 82 (1977) 615.
95 T. Plummer and M. Y. Hurnitz, *J. Biol. Chem.*, 253 (1978) 3907.
96 S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 329 (1985) 165.
97 R. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
98 J. E. Cawston and G. Murphy, *Methods Enzymol.*, 80 (1981) 711.
99 C. Gillet, Y. Ecckhont and G. Vaes, *FEBS Lett.*, 74 (1977) 126.
100 R. M. Metrione, *Biochim. Biophys. Acta*, 526 (1978) 531.
101 Y. Legrand, G. Pignaud, J. P. Caen, B. Robert and L. Robert, *Biochem. Biophys. Res. Commun.*, 63 (1975) 224.
102 K. Katagiri, T. Takeuchi, K. Taniguchi and M. Sasaki, *Anal. Biochem.*, 86 (1978) 159.

103 F. A. Pitlick and Y. Nemerson, *Methods Enzymol.*, 45 (1976) 37.
104 R. Radcliffe and Y. Nemerson, *Methods Enzymol.*, 45 (1976) 49.
105 J. H. Griffin and C. G. Cochrane, *Methods Enzymol.*, 45 (1976) 56.
106 K. Takahiko, K. Hisao and E. W. Davie, *Methods Enzymol.*, 45 (1976) 65.
107 K. Fujikawa and E. W. Davie, *Methods Enzymol.*, 45 (1976) 89.
108 H. Suda, K. Yamamoto, T. Aoyagi and M. Umezawa, *Biochim. Biophys. Acta*, 616 (1980) 60.
109 R. W. Colman and A. Bagdasarian, *Methods Enzymol.*, 45 (1976) 303.
110 B. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
111 F. Fiedler, E. Fink, H. Tschesche and H. Futz, *Methods Enzymol.*, 80 (1981) 493.
112 E. Hackenthal, *Biochim. Biophys. Acta*, 522 (1978) 563.
113 B. Holmquist, *Biochemistry*, 16 (1977) 4591.
114 P. F. Fox, J. R. Whitaker and P. A. O'Leary, *Biochem. J.*, 161 (1977) 389.
115 J. Turková, K. Bláha, J. Horáček, J. Vajtner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
116 G. E. Sieffring and K. C. Robbins, *Methods Enzymol.*, 45 (1976) 257.
117 T. Yoshimoto, M. Fischl, R. C. Orlowski and R. Walter, *J. Biol. Chem.*, 253 (1978) 3708.
118 G. Feinstein and A. Janoff, *Biochim. Biophys. Acta*, 403 (1975) 477.
119 E. J. B. Fodor, H. Ako and K. A. Walsh, *Biochemistry*, 14 (1975) 4923.
120 D. Y. Twumasi and I. E. Liener, *J. Biol. Chem.*, 252 (1977) 1917.
121 U. Kašhe, K. Buchholz and B. Galunsky, *J. Chromatogr.*, 216 (1981) 169.
122 H. Yokosawa, T. Inagami and E. Haas, *Biochem. Biophys. Res. Commun.*, 83 (1978) 306.
123 T. Matoba, K. Murakami and T. Inagami, *Biochim. Biophys. Acta*, 526 (1978) 560.
124 H. J. Chou, J. H. Sharper and R. I. Gregerman, *Biochim. Biophys. Acta*, 524 (1978) 123.
125 T. Morita, H. Igarashi and S. Iwanaga, *Methods Enzymol.*, 80 (1981) 311.
126 K. W. Jackson, N. Esmon and J. Tang, *Methods Enzymol.*, 80 (1981) 387.
127 V. K. Akparov and V. M. Stepanov, *J. Chromatogr.*, 155 (1978) 329.
128 W. Holleman and L. J. Weiss, *J. Biol. Chem.*, 251 (1978) 1691.
129 K. Nishioka, *Eur. J. Biochem.*, 85 (1978) 137.
130 S. Ghodbane and G. Guiochon, *J. Chromatogr.*, 444 (1988) 275.
131 T. A. Anderson, *J. Chromatogr.*, 326 (1985) 33.
132 X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55.
133 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 133 (1983) 251.
134 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Biochem.*, 52 (1980) 177.
135 K. Unger, *Methods Enzymol.*, 104 (1984) 154.
136 K. H. Rohm, *Hoppe-Seyler's Z. Physiol. Chem.*, 363 (1982) 641.
137 F. E. Regnier, *Methods Enzymol.*, 104 (1984) 170.
138 M. L. Heinitz, L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 173.
139 J. S. Richey, *Methods Enzymol.*, 104 (1984) 223.
140 M. T. W. Hearn, A. N. Hodder and M. I. Aguilar, *J. Chromatogr.*, 443 (1988) 97.
141 E. Kopetzki and K. D. Entian, *Anal. Biochem.*, 121 (1982) 181.
142 J. H. L. Franssen, T. Janssen-Claessen and G. Van Diesjen, *Biochim. Biophys. Acta*, 747 (1983) 186.
143 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190.
144 R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 81.
145 D. P. Lee, *J. Chromatogr.*, 443 (1988) 143.
146 M. J. O'Hare, M. W. Capp, E. C. Nice, N. H. C. Cooke and B. G. Archer, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, p. 161.
147 K. Titani, J. Sasagawa, K. Resing and K. A. Walsh, *Anal. Biochem.*, 123 (1982) 6552.
148 M. W. Berchtold, K. J. Wilson and C. W. Heiznann, *Biochemistry*, 21 (1982) 6552.
149 M. Rubinstein, *Anal. Biochem.*, 98 (1979) 1.
150 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
151 M. T. W. Hearn and B. Greco, *J. Chromatogr.*, 266 (1983) 75.
152 W. G. Burton, K. D. Nugent, T. K. Slattery, B. R. Summers and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 363.
153 K. D. Nugent, W. G. Burton, T. K. Slattery, B. F. Johnson and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
154 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.

155 R. J. Linhardt, *Appl. Biochem. Biotechnol.*, 11 (1985) 49.
156 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
157 J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, *J. Chromatogr.*, 317 (1984) 141.
158 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1984) 519.
159 J. P. Chang, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
160 L. A. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 73.
161 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
162 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
163 S. Hjertén, K. Yao, Z.-Q. Liu, D. Yang and B.-L. Wu, *J. Chromatogr.*, 354 (1986) 203.
164 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 360 (1986) 260.
165 M. L. Heinitz, L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 173.
166 Y. Kato, *Adv. Chromatogr.*, 26 (1987) 97.
167 G. Fassina and I. M. Chaiken, *Adv. Chromatogr.*, 27 (1987) 247.
168 P. O. Larsson, *Methods Enzymol.*, 104 (1984) 212.
169 P. O. Olsson, *Biol. Chem. Hoppe-Seyler*, 368 (1987) 767.
170 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 216 (1981) 175.
171 S. P. Colowick and O. Kaplan, *Methods Enzymol.*, 80, Sect. I (1981) 3.
172 J. Turková, K. Bláha, J. Horáček, J. Vajčner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
173 S. Sasaki, S. Kobayashi, S. Kiryu and M. Sugiura, *Chem. Pharm. Bull.*, 33 (1985) 746.
174 S. Kobayashi, H. Endo, M. Sasaki, S. Kiryu and M. Sugiura, *Chem. Pharm. Bull.*, 33 (1985) 697.
175 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Biochem.*, 52 (1980) 177.
176 G. H. Caughey, N. F. Viro, J. Ramachandran, S. C. Lazarus, D. B. Borson and J. A. Nadel, *Arch. Biochem. Biophys.*, 258 (1987) 555.
177 K. Mizuno and H. Matsuo, *Nature (London)*, 309 (1984) 558.
178 B. Dahlmann, L. Kuehn and H. Reinauer, *FEBS. Lett.*, 160 (1983) 243.
179 W. E. Keene, M. G. Petitt, S. Allen and J. H. Mekerrow, *J. Exp. Med.*, 163 (1986) 536.
180 J. L. Seltzer, M. L. Eschbach and A. Z. Eisen, *J. Chromatogr.*, 326 (1985) 147.
181 P. B. Wolfe, P. Silver and W. Wickner, *J. Biol. Chem.*, 257 (1982) 7898.
182 K. Tanaka, N. Ikegaki and A. Ichihara, *J. Biol. Chem.*, 259 (1984) 5937.
183 D. J. Buttle and A. J. Barret, *Biochem. J.*, 223 (1984) 81.
184 C. Barthomeuf, H. Pourrat and A. Pourrat, *Biotechnol. Tech.*, 2 (1988) 29.
185 G. E. Maret and J. L. Fauchere, *Anal. Biochem.*, 172 (1988) 248.
186a W. Machleidt, J. Otto and E. Wachter, *Methods Enzymol.*, 47 (1977) 210.
186b A. Kanamori, N. Seno and I. Matsumoto, *Chem. Pharm. Bull.*, 35 (1987) 3783.
187 K. Titani, J. Sasagawa, K. Resing and K. A. Walsh, *Anal. Biochem.*, 123 (1982) 408.
188 P. J. Padkield and R. M. Case, *Anal. Biochem.*, 104 (1984) 416.
189 C. T. Carilli, L. C. Wallace, L. H. Smith, H. A. Wong and J. A. Lewicki, *J. Chromatogr.*, 444 (1988) 203.
190 M. Heeb and O. Gabriel, *Methods Enzymol.*, 104 (1984) 416.
191 B. J. Radola, in J. G. Radola (Editor), *Isoelectric Focusing*, Butterworth, London, 1975, p. 145.
192 B. J. Radola, *Methods Enzymol.*, 104 (1984) 256.
193 *Isoelectric Focusing, Principles and Methods*, Pharmacia Fine Chemicals, Uppsala, 1981.
194 F. Wagner, *Methods Enzymol.*, 158 (1988) 21.
195 E. A. Lance, C. W. Rhodes, III, and R. Nakon, *Anal. Biochem.*, 33 (1983) 492.
196 J. M. Prescott, F. W. Wagner, B. Holmquist and B. L. Vallee, *Biochim. Biophys. Res. Commun.*, 114 (1983) 646.
197 P. Buenning and J. F. Riordan, *Bioinorg. Chem.*, 24 (1985) 183.
198 J. D. McConn, *J. Biol. Chem.*, 239 (1964) 3706.
199 J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, 235 (1960) 390.
200 D. W. Auld and B. Holmquist, *Biochemistry*, 13 (1974) 4355.
201 E. Winsterberger, H. Neurath, T. L. Coombs and B. L. Vallee, *Biochemistry*, 4 (1965) 1526.
202 H. Hanson and M. Frohne, *Methods Enzymol.*, 44 (1980) 504.
203 W. D. Behnke and B. L. Vallee, *Proc. Natl. Acad. Sci. USA*, 69 (1972) 2442.
204 K. Breddam, T. J. Bazzone, B. Holmquist and B. L. Vallee, *Biochemistry*, 18 (1979) 1563.
205 B. Holmquist and B. L. Vallee, *J. Biol. Chem.*, 249 (1974) 4601.
206 J. W. Kusiak, J. M. Quirk and R. O. Brady, *J. Biol. Chem.*, 253 (1978) 184.
207 R. Marusyk and A. Sergeant, *Anal. Biochem.*, 105 (1980) 403.

208 H. Görish, *Anal. Biochem.*, 173 (1988) 393.
209 G. L. Gilliand and D. R. Davies, *Methods Enzymol.*, 104 (1984) 370.
210 K. M. Gernet, R. Smith and D. C. Carter, *Anal. Biochem.*, 168 (1988) 141.
211 L. Orstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
212 B. J. Davies, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
213 C. Vincent, *Biofutur Technoscope*, No. 9, February (1987) 1.
214 P. J. Blackshear, *Methods Enzymol.*, 104 (1984) 237.
215 P. H. O'Farrel, *J. Biol. Chem.*, 250 (1975) 4007.
216 N. G. Anderson and L. Anderson, *Clin. Chem.*, 28 (1982) 739.
217 R. P. Tracy and D. S. Young, in J. E. Celis and R. Bravo (Editors), *Two Dimensional Gel Electrophoresis*, Academic Press, New York, 1984, p. 193.
218 P. Blangarin and J. J. Madjar, in M. M. Galteau and G. Siest (Editors), *Recent Progress in 2-Dimensional Gel Electrophoresis*, University of Nancy, Nancy, 1986, p. 25.
219 D. F. Hochstrasser, V. Angsburger, M. Funk, R. Appel, C. Pellegrini and A. F. Muller, *Electrophoresis*, 7 (1986) 505.
220 J. J. Madjar, M. Arpin and J. P. Reboud, *Anal. Biochem.*, 83 (1977) 304.
221 P. G. Righetti, G. Tudor and E. Gianazza, *J. Biochem. Biophys. Methods*, 6 (1982) 219.
223 D. F. Hochstrasser, M. G. Harrington, A. C. Hochstrasser, M. J. Miller and C. R. Merril, *Anal. Biochem.*, 173 (1988) 424.
224 V. K. Laemmli, *Nature (London)*, 277 (1970) 680.
225 D. M. Neville, *J. Biol. Chem.*, 246 (1971) 6328.
226 M. V. Kolodzeiskaia, *Lab. Delo*, 7 (1986) 426.
227 A. H. Reisner, *Methods Enzymol.*, 104 (1984) 439.
228 O. Vesterberg, L. Hansen and A. Sjosten, *Biochim. Biophys. Acta*, 491 (1977) 160.
229 C. R. Merril, D. Goldman and M. L. Keuren, *Methods Enzymol.*, 104 (1984) 441.
230 J. E. Gander, *Methods Enzymol.*, 104 (1984) 447.
231 M. J. Heeb and O. Gabriel, *Methods Enzymol.*, 104 (1984) 416.
232 H. Maeda, *Anal. Biochem.*, 92 (1979) 222.
233 H. Khalfan, R. Abuknesha and D. Robinson, *Biochem. J.*, 209 (1983) 265.
234 C. H. Evans and J. D. Ridella, *Anal. Biochem.*, 142 (1984) 411.
235 S. S. Twining, *Anal. Biochem.*, 143 (1984) 30.
236 U. Tisljar and H. W. Denker, *Anal. Biochem.*, 152 (1986) 39.
237 H. Nagase and J. F. Woessner, *Anal. Biochem.*, 107 (1980) 385.
238 A. T. Andrews, *FEBS Lett.*, 141 (1982) 207.
239 M. D. Taylor and A. T. Andrews, *FEBS Lett.*, 157 (1983) 200.
240 R. Chavira, T. J. Burnett and J. H. Hageman, *Anal. Biochem.*, 136 (1984) 446.
241 F. C. Church, D. H. Porter, G. L. Catignani and M. E. Swaisgood, *Anal. Biochem.*, 146 (1985) 343.
242 M. T. Gislow and B. C. McBride, *Anal. Biochem.*, 68 (1975) 70.
243 J. T. Dingle, A. M. J. Blow, A. J. Barrett and P. E. N. Martin, *Biochem. J.*, 167 (1977) 775.
244 M. J. Banda and Z. Werb, *Biochem. J.*, 193 (1981) 589.
245 H. S. Jensen, *Biochem. J.*, 218 (1984) 645.
246 J. C. Unkless, T. L. Ossowski, J. P. Quigley, D. B. Rifkin and E. Reich, *J. Exp. Med.*, 137 (1973) 85.
247 E. D. Sevier, *Anal. Biochem.*, 74 (1976) 592.
248 B. D. Robertson, G. E. Kwan-Lim and R. M. Maizels, *Anal. Biochem.*, 172 (1988) 284.
249 D. L. Garner, *Anal. Biochem.*, 67 (1975) 688.
250 M. Suguira, Y. Ito, K. Hirano and S. Sawaki, *Anal. Biochem.*, 81 (1977) 481.
251 M. Pacaud and J. Vriel, *Eur. J. Biochem.*, 23 (1971) 435.
252 J. S. Mort and H. Leduc, *Anal. Biochem.*, 119 (1982) 148.
253 K. R. Lynn and N. A. Clevette-Radford, *Anal. Biochem.*, 117 (1981) 280.
254 B. G. Ohlsson, B. R. Weström and B. W. Karlsson, *Anal. Biochem.*, 152 (1986) 239.
255 J. Dijhof and C. Poort, *Anal. Biochem.*, 83 (1977) 315.
256 G. Feinein and A. Janoff, *Anal. Biochem.*, 71 (1976) 358.
257 P. E. Burdett, A. E. Kipps and P. H. Whitehead, *Anal. Biochem.*, 72 (1976) 315.
258 D. Every, *Anal. Biochem.*, 116 (1981) 519.
259 P. J. Kelleher and R. L. Juliano, *Anal. Biochem.*, 136 (1984) 470.
260 M. Hofelmann, R. Kittsteiner-Eberle and P. Schreier, *Anal. Biochem.*, 128 (1983) 217.
261 P. Sinha, *Histochemistry*, 81 (1984) 217.

262 S. A. Lacks and S. S. Sprinchorn, *J. Biol. Chem.*, 255 (1980) 7467.
263 S. S. Twining, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 42 (1983) 1951.
264 H. Ansari and L. Stevens, *Soc. Gen. Microbiol. Q.*, 8 (1981) 277.
265 A. Gerther, Y. Tencer and G. Tinman, *Anal. Biochem.*, 54 (1973) 270.
266 C. W. Ward, *Anal. Biochem.*, 74 (1976) 242.
267 K. A. Killick and L. W. Wang, *Anal. Biochem.*, 106 (1980) 367.
268 C. Heussen and E. B. Doxdle, *Anal. Biochem.*, 102 (1980) 196.
269 J. Thelu, *Anal. Biochem.*, 172 (1988) 124.
270 J. G. Linner, S. A. Livesey, D. S. Harrison and A. L. Steiner, *J. Histochem.*, 34 (1986) 1123.
271 M. Wilchek and E. Bayer, *Anal. Biochem.*, 171 (1988) 1.
272 K. Yamaguchi and H. Asakawa, *Anal. Biochem.*, 172 (1988) 104.
273 A. Mazen, G. Gradwohl and G. de Murcia, *Anal. Biochem.*, 172 (1988) 39.
274 B. Durand, *Biofutur Technoscope*, No. 19, April (1983) 3.
275 D. Walker, *Biotechnol. Lab.*, December (1988) 8.
276 J. Sock and R. Rohringer, *Anal. Biochem.*, 171 (1988) 310.

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CHROM. 21 461

FACTORIAL OPTIMIZATION FOR FLOWS IN THE HALL ELECTROLYTIC CONDUCTIVITY DETECTOR

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SUMMARY

Statistical optimization of flows is carried out for a Hall electrolytic conductivity detector (HECD). Yates' method was used to quickly estimate main factors affecting HECD response to organochlorine. Also, more detailed evaluation of variations of HECD response for three flow variables was performed via central composite design factorial experiments. Corresponding three-factor, three-level factorial experiments provided data for least-squares regression analyses. The resulting fitted-polynomial yielded an appropriate mathematical model of the HECD response surface for the corresponding three dimensions in flow-space.

Optimum values for three detector flows are thereby selected from the response surface to provide maximum HECD sensitivity, *i.e.*, response to organochlorine in the halogen mode. Supplemental data then allow for flow optimization with respect to other criteria, *e.g.*, signal-to-noise ratios and resolution of eluates.

INTRODUCTION

Hall electrolytic conductivity detector (HECD) sensitivity can be influenced by many variables. Selection of HECD reactor temperature affects detector response, and depends upon eluites to be detected[1]. Also, flow-rates for carrier gas, reaction gas and conductivity solvent are important parameters affecting HECD response[2–4].

Carrier gas, often helium which contains gas-phase eluates, is introduced to the high-temperature HECD reactor from the chromatographic column. Hydrogen, the reaction gas, to produce $HCl_{(g)}$ for the halogen mode, is added to the carrier gas prior to exposure to the reactor's nickel catalyst. Reactor effluents containing carrier gas, hydrogen, $HCl_{(g)}$ and other reactor products mix with buffered *n*-propanol in the gas–liquid contactor; the gas–liquid separator then isolates the flowing liquid phase from undissolved gases for electrometric measurements of dissolved Cl^- in the analytical cell. The two gas flows and the flow of the liquid through these mixing,

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separation and measurement regions of the differential conductivity cell significantly affect HECD response.

In this work factorial experiments were conducted to obtain mathematical models representing HECD response surfaces which estimate effects of carrier gas flow, reaction gas flow and conductivity solvent flow upon HECD sensitivity[5]. These three flow variables, or factors[6], were deliberately varied in a controlled fashion and corresponding HECD response factors were determined. A second-order multiple regression model was used to estimate the relationship between the three factors and the response surface[7]. This regression model and Yates' method of analysis[6] were employed to evaluate the main effects for each factor, *i.e.*, variations of the average response factor as a single flow variable changes. The interaction effects were also calculated for each combination of pairs of flows[6]. Optimized flow settings were then estimated for achieving maximum detector sensitivity for the HECD system. Results of this study are reported herein for both packed and capillary gas chromatographic (GC) systems with a HECD.

Other variables may also affect HECD sensitivity such as conductivity cell temperature, reactor catalyst integrity, eluate peak shape, background conductance and solvent pH. These variables were not evaluated in this study because previous work has shown these variables to change slowly with time or cause only minor changes in HECD response factors[7,8].

EXPERIMENTAL

Reagents

1-Chlorooctane (>99%) (1COA) was purchased from Aldrich. Resi-analyzed grade *n*-hexane and propanol were purchased from J. T. Baker. Dilutions of 1-chlorooctane in hexane were used as injected aliquots for these studies.

Instrumentation

A Tracor Model 560 gas chromatograph equipped with a Tracor Model 700A HECD was used for these studies. Packed-column separations were done with a 2 m × 2 mm I.D. glass column, packed with 3% OV-17 on 100–120 mesh Supelcoport. For capillary column separations the HECD was interfaced with a J&W Scientific 30 m × 0.32 mm I.D. fused-silica capillary column with 0.25-μm DB-5 bonded phase.

The HECD reactor assembly was adapted for capillary systems for some of this work. A 4 in. × 1/4 in. O.D. × 1 mm I.D. glass insert was placed into the HECD column interface and anchored with a 1/4-in. graphite ferrule. A 1/4 in. O.D. to 1/16 in. O.D. stainless-steel Swagelok reducing union, modified for make-up gas introduction, was attached to the other end of the glass insert. The capillary column was passed through the reducing union into the flow channel of the glass insert and located such that the termination of the capillary column was about 1 mm from the entrance to the nickel catalyst reaction tube. The capillary column was anchored by a 0.8 mm to 1/16 in. O.D. graphite ferrule at the reduction union.

An SGE OCI-3 on-column injector and syringe were used to deliver sample injection volumes onto the capillary column. Hydrogen carrier gas flow was set with the precision flow-control valve provided with the on-column injector. Hydrogen

make-up gas for capillary separations was introduced through the modified Swagelok reducing union and the reaction gas inlet. The HECD conductivity solvent, *n*-propanol, was delivered by the metering pump provided with the HECD.

The HECD was used in the halogen mode for these studies. A nickel-catalyst reactor temperature of 950°C was used, as recommended for chlorinated hydrocarbon analyses[1]. A Hewlett-Packard Model 3390A integrator was used to measure HECD peak areas and retention times.

Procedures

Gas flow-rates were measured with a soap-bubble meter and a stopwatch at the differential conductance cell exit. Solvent flows were measured with a 10-ml graduated cylinder at the same cell outlet. The following procedure was used to set and monitor the three flows: (a) solvent flow was turned off, (b) reactor gas flow was turned off, (c) vent valve was closed, (d) carrier gas flow was adjusted and measured, (e) reaction gas flow was adjusted and the carrier gas plus reaction gas flow was measured, (f) solvent flow was adjusted and measured.

A volume of 1 μl of a 1-chlorooctane solution (59 nmol Cl/ml) in *n*-hexane was repeatedly injected and separated at 65°C for each set of flow conditions evaluated. Peak areas for 1-chlorooctane from chromatograms for each set of flow conditions were used according to the central composite factorial experiment described below. The baseline noise was always small relative to peak areas for these experiments.

Factorial experimental design

Factorial experiments allow the main effects and interaction effects within a multivariate system to be evaluated. This approach is efficient since it requires fewer experimental trials than normally required by corresponding single-factor experiments[5]. Fractional factorial experiments further reduce the number of required trials by neglecting selected high-order interactions[5] between variables.

In this study a three-level, three-factor experiment was implemented to evaluate effects of three flow factors upon HECD response. The experiment can be pictorially represented by a cube with the center placed at the origin, or base-point[9], of a Cartesian coordinate system (see Fig. 1). The *X*, *Y*, and *Z* axes represent the three evaluated flow factors, k_1, k_2 and k_3. Each position in the cube defines a combination of the three flows. Each position in the cube can also be related to a point, *i.e.*, an estimated response, on a calculated response surface; this relationship can be established by fitting the factorial data to a polynomial function with multiple linear regression[5]. For the experiments described herein the response surface mathematically

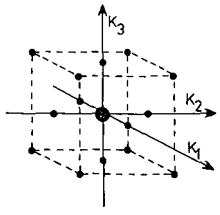

Fig. 1. A three factor, $2^k + 2k + 1$ factorial experimental space. Dots represent the 15 positions at which measurements are required for a central composite factorial experiment.

models the relationship between the three flow factors and the HECD response to 1-chlorooctane.

The number of trials required for a complete factorial experiment is Q^k where k factors are evaluated at Q different values, *i.e.*, Q levels[6]. Thus a complete, or 3^k, factorial design for three factors requires HECD response measurements for at least the 27 flow combinations represented by 27 specified cube positions[6,10]. The data obtained from a 3^k design may then be modeled by a second-order polynomial with parameters for main effects, two-way interactions, three-way interactions and an error term[10].

For this work a fractional factorial experiment was selected instead of a complete factorial experiment. The central composite factorial design used, called a $2^k + 2k + 1$ design, requires only 15 experiments, corresponding to 15 HECD flow combinations represented in Fig. 1[10]. Central composite data were modeled by a second-order polynomial which includes parameters for each main effect, second order two-way interaction effects and an error term[11], and neglects three-way interactions:

$$y = B_0 + B_1X_1 + B_2X_1^2 + B_3X_2 + B_4X_2^2 + B_5X_3 + B_6X_3^2 + \\ + B_7X_1X_2 + B_8X_1X_3 + B_9X_2X_3 + e \quad (1)$$

Similar to the 3^k design, y estimates the response surface for factors x_1, x_2 and x_3. The central composite design was preferred for fitting factorial data by multiple regression since fewer trials are required[5,9–13]: the central composite design uses a minimum of $(Q-1)^k + (Q-1)k + 1$ experiments compared to the Q^k experiments required for a complete factorial experiment[10].

Yates' method

Yates' method was also used to evaluate flow-response relations for the HECD. It is also a two-level evaluation which does not consider second-order relationships which may exist between the individual flow factors and the HECD response. Yates' method consists of simple calculations which allow a prompt evaluation of factorial experimental data[6].

RESULTS AND DISCUSSION

Multivariate evaluation of the effects of reaction gas, carrier gas, and conductivity solvent flow-rates on the HECD response by a central composite factorial experiment

Effects of the helium carrier gas, hydrogen reaction gas, and *n*-propanol conductivity solvent flows on HECD sensitivity in the halogen mode were evaluated with a three-level, three-factor, factorial experiment. The three factors were reaction gas flow, k_1, carrier gas flow, k_2, and conductivity solvent flow, k_3. The measured response was the peak area for 1-chlorooctane obtained from packed-column HECD chromatograms, using the halogen mode and 1-μl injections.

The central composite design was used for evaluating the HECD response surface for the three flows. A corresponding second-order polynomial was found by multiple regression and modeled the factorial data. Selection of the central composite design was justified based on limited single-factor flow data in the literature, *i.e.*,

hydrogen flows in the nitrogen mode[2]; hydrogen flow and conductivity solvent flow in the nitrogen mode[4]; and conductivity solvent flow in the sulfur mode[3]. Those studies indicate that HECD response generally increases as hydrogen flow increases and decreases as conductivity solvent flow increases in a second-order fashion without major inflection points. Our experience with the HECD in the halogen mode suggested the same trends. High-order interaction effects were therefore not evaluated, hence reducing the number of required experiments from 27 to 15.

Reasonable flow ranges for the HECD system were used as boundaries for the experimental space, *i.e.*, the cube (hydrogen reaction gas low flow = 12.7 ml/min, base point = 30.0 ml/min, and high flow = 51.2 ml/min; helium carrier gas low flow = 12.3 ml/min, base point = 35.8 ml/min, and high flow = 55.8 ml/min; solvent low flow = 0.3 ml/min, base point = 1.4 ml/min, and high flow = 2.7 ml/min). Packed-column carrier gas flow conditions are limited since analyte elution is severely retarded at very low flows, *e.g.*, less than 10 ml/min, but analytes may be lost during the venting procedure at very high flows, *e.g.*, greater than 40 ml/min. Conductivity solvent flow is limited by the useful range of the delivery pump since at low flows, less than 0.2 ml/min, the system becomes erratic, and the upper flow limit is about 4 ml/min. Reaction-gas flow was also restricted since the gas-to-liquid flow ratio in the gas–liquid contactor and separator must be approximately 100:1, according to previous studies[7].

For the three-factor, three-level, second-order, central composite design the system was evaluated at points corresponding to the eight vertices, the centers of the six faces, and the base-point for the experimental cube-space[5]. Replicate measurements were obtained for the base point to establish expected uncertainties. Accordingly, appropriate chromatograms were developed; eight with the flow conditions set as defined by the vertices of the cube, six as defined by the centers of the faces of the cube, and five replicate chromatograms for the base-point. The experimental sequence was not completely randomized due to practical limitations in precisely resetting flows.

The data-analysis computer program (available upon request from the authors) was used to mathematically model the factorial data with the best-fit second-order polynomial using multiple linear regression. The expected values for 1-chlorooctane peak area responses, as modeled by the regression polynomial function, and regression residuals were calculated. Regression parameters for eqn. 1 resulting from modeling factorial data are shown in Table I. Table I also includes confidence intervals based on the Student's *t*-statistic for each of the ten regression parameters. The 90% confidence intervals were calculated from the diagonal elements of the inverted regression matrix and parameter standard deviations as estimated from the corresponding sum of squared residual values[6].

The regression parameters indicate that solvent flow has the largest effect among the three flow factors on the HECD response; the first- and second-order regression parameters for the main-effect of solvent flow on the HECD response are much larger than for any other effect. The hydrogen and helium flow factors yield smaller main-effect parameters than solvent flow, with the first and second main-effect parameters being similar for the two gas flow factors. This indicates that the total gas flow through the HECD is more important for HECD response evaluation than the individual flow-rates of hydrogen or helium within the flow boundaries evaluated in this experiment. The 90% confidence intervals for hydrogen and helium flows indicate that the second-order parameters, as well as the interaction-effect terms, are all relatively small when compared to other parameters.

TABLE I

MULTIPLE REGRESSION PARAMETERS FOR THE ICOA PEAK AREA RESPONSE SURFACE TO HYDROGEN, HELIUM AND SOLVENT FLOW RATES

90% Confidence interval estimates for the individual regression parameters are included[6].

Regression parameter	*Parameter value*	*Estimated 90% confidence interval for parameters*	*Parameter description*		
			Effect type	*Factors*	*Parameter order*
β_0	$2.7 \cdot 10^2$	$1.0 \cdot 10^2$	–	–	Intercept
β_1	5.6	5.1	Main	Hydrogen	First
β_2	$-2.9 \cdot 10^{-2}$	$7.0 \cdot 10^{-2}$		Flow	Second
β_3	7.3	4.3	Main	Helium	First
β_4	$-2.9 \cdot 10^{-2}$	$6.0 \cdot 10^{-2}$		Flow	Second
β_5	$-3.8 \cdot 10^2$	$0.7 \cdot 10^2$	Main	Solvent	First
β_6	97.0	18.0		Flow	Second
β_7	$-4.7 \cdot 10^{-2}$	$4.4 \cdot 10^{-2}$	Interaction	Hydrogen and helium	Second
β_8	−0.76	0.80		Hydrogen and solvent	Second
β_9	1.4	0.7		Helium and solvent	Second

The polynomial model was evaluated for effects of each flow on the HECD response while the remaining two flows were held constant at base-point values. If solvent flow is increased while carrier and reaction gas flows remain constant, then the HECD response is reduced (see Fig. 2). For example, a conductivity solvent flow of 0.2 ml/min yields about ten times the HECD response found with a flow of 2.5 ml/min.

HECD response increases as either reaction gas or carrier gas flows are increased. Increasing the carrier gas flow from 10 ml/min to 50 ml/min produces a 120% increase in the HECD response if the other two factors are held constant at base-point values (see Fig. 3). Similarly, hydrogen flow increase over the same flow ranges yields a 45% increase in HECD response (Fig. 4); however, since the helium flow at the base-point is larger than the hydrogen flow at the base-point, different total gas flow-rates are compared. Evaluation of the hydrogen flows from 10 ml/min to 50

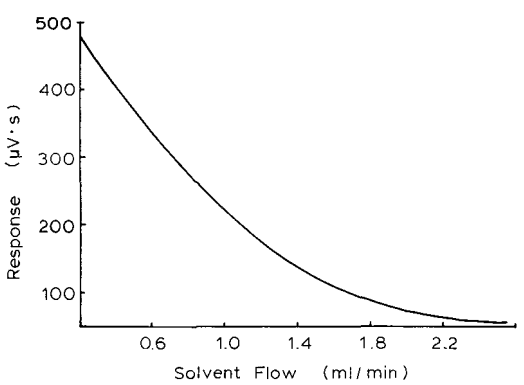

Fig. 2. Effect of HECD solvent flow on ICOA peak area, hydrogen flow = 28.9 ml/min, helium flow = 36.1 ml/min.

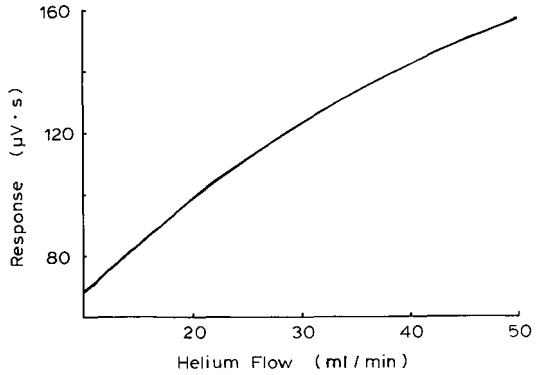

Fig. 3. Effect of HECD helium carrier gas flow on 1COA peak area, hydrogen flow = 28.9 ml/min, solvent flow = 1.4 ml/min.

ml/min, with the helium flow constant at 28.9 ml/min and solvent flow constant at 1.4 ml/min, shows an HECD response increase of 65% (Fig. 5).

Within the 90% confidence intervals for the parameters in eqn. 1 (see Table I), increases of either helium and hydrogen flow causes similar enhancement of HECD response (see Figs. 3 and 5). The increase in the HECD response as total gas flow increases may reflect enhanced mixing dynamics within the gas–liquid contactor and gas–liquid separator. However, a specified total gas flow-rate yields approximately the same HECD response even if the ratio of hydrogen to helium flow changes. This indicates that hydrogen gas is probably only needed in small amounts as a reagent, contrary to conventional assumptions.

Evaluation of the main effects and interaction effects of reaction gas, carrier gas and conductivity solvent flows on the HECD response by Yates' method

To supplement the central composite factorial calculation above, Yates' method for analysis of factorial experiment data was applied to the evaluation of effects of the

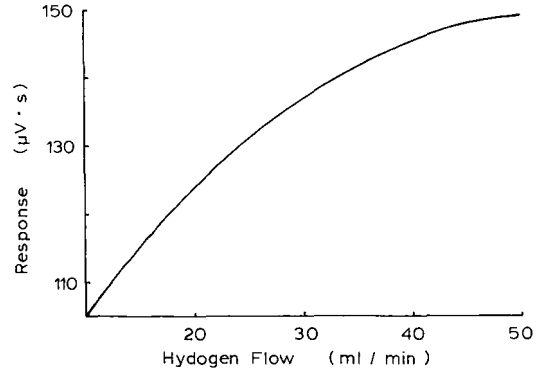

Fig. 4. Effect of HECD hydrogen flow on 1COA peak area, helium flow = 36.1 ml/min, solvent flow = 1.4 ml/min.

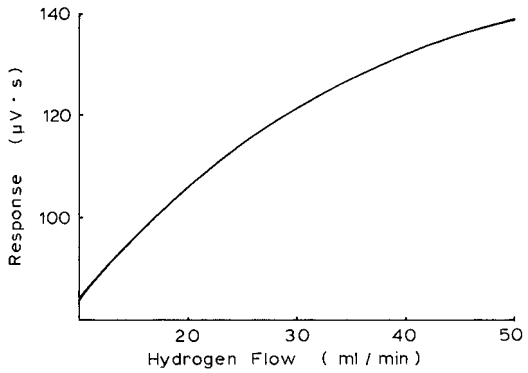

Fig. 5. Effect of HECD hydrogen flow on 1COA peak area, helium flow = 28.9 ml/min, solvent flow = 1.4 ml/min.

three flow factors on HECD response[6]. Yates' method utilizes simple calculations to quickly estimate the main and interaction effects, however, the method is restricted to two-level experiments. Thus, only the eight chromatograms, corresponding to the eight vertices of the experimental cube in Fig. 1 were used. Resulting estimates of Yates' effects suggest trends similar to those suggested by the multiple regression analyses described above.

For calculations according to Yates' method, only three effects are significantly different from zero at the 90% confidence level based upon the Student's *t*-statistic: the main effects of solvent and helium flows and the interaction effect between solvent and helium flows[6]. The estimate of the main effect of solvent flow on the HECD response is much larger than the main effect for hydrogen or helium flows, consistent with the dependence found via the multiple regression evaluation above. However, results of Yates' method indicate that the interaction effect between helium and solvent flows is much more significant than estimated by the multiple regression evaluation. This second-order relationship between solvent flow and the HECD response is also evident from the multiple regression parameters discussed above and from previous work[3].

Flow conditions for optimum HECD response for GC separations

Flow conditions were selected, based upon results described above, to provide a maximum HECD response to organochlorine for the HECD under practical operating conditions. (Flows for maximum HECD response were hydrogen = 50 ml/min, helium = 50 ml/min and solvent = 0.2 ml/min; practical optimum flows were hydrogen = 50 ml/min, helium = 20 ml/min and solvent = 0.5 ml/min) However, criteria other than HECD response also must be considered for optimal GC measurements; signal-to-noise evaluations and resolving power are also important. For example, a solvent flow of 0.5 ml/min provides an enhanced HECD response over higher flows but does not introduce significant pump-related noise into the system. Similarly, a solvent flow of 0.2 ml/min is too low, as it increases HECD response but includes substantially increased noise, thus jeopardizing measurements of analytes and degrading the limit of detection.

Experimental results described above suggest that the HECD response mainly changes with total gas flow. However, carrier gas flow also affects resolution and

retention times in addition to the HECD sensitivity. Thus, carrier gas flow may also be optimized for resolution, and reaction gas flow may be increased to raise the total gas flow if the HECD response needs to be further enhanced. An optimum helium carrier gas flow of 20 ml/min was thereby selected for subsequent packed-column separations. Under those conditions hydrogen reaction gas flow of 50 ml/min provides the most sensitive HECD response according to the experimental results described above.

HECD responses were calculated, based upon the response surface, as single factors were changed while the other flow factors remain constant at the selected optimum flow-rates. The effect of solvent, helium and hydrogen flows on the HECD response near the optimization point are consistent with the discussion above but shows greater dependences than shown in Figs. 2–5 above.

Evaluation of flow factors for capillary separations using hydrogen carrier gas and hydrogen make-up gas eliminated the need for addition of reaction-gas. The major dependence of the HECD upon the main factors of total gas flow and solvent flow, discussed above, therefore allowed for easy adaptation to capillary GC. Because high resolution was provided at very low capillary carrier gas flows, *e.g.*, 1 ml/min, hydrogen as make-up gas was varied to provide maximum HECD response without degrading eluate resolution; this was approximately 60 ml/min. Similarly, HECD solvent flow was selected to provide sufficiently low noise, *e.g.*, at approximately 0.5 ml *n*-propanol/min.

These statistical optimizations for maximized sensitivities allow for efficient assessments of appropriate HECD flows via few experiments. Other parameters such as signal-to-noise ratios or resolution might also be considered in other evalutions which use criteria other than enhanced responses. The optimum flows reported here are consistent with typical flows recommended for the HECD based upon extensive empirical efforts[2–4,7,8]. However the results herein also contradict some conventional assumptions: (a) only small amounts of hydrogen are required for effective reductions of organochlorine to HCl, and (b) total gas flow is typically much more important than the ratio of hydrogen-to-carrier gas flows.

REFERENCES

1 T. L. Ramus and L. C. Thomas, *J. Chromatogr.*, 328 (1985) 342.
2 M. Galoux, J.-C. Van Damme, A. Bernes and J. Potvin, *J. Chromatogr.*, 177 (1979) 245.
3 S. Gluck, *J. Chromatogr. Sci.*, 20 (1982) 103.
4 B. P. Wilson and W. P. Cochrane, *J. Chromatogr.*, 106 (1975) 174.
5 W. G. Cochran and G. M. Cox, *Experimental Designs*, Wiley, New York, 2nd ed., 1957, p. 347.
6 M. G. Natrella, *Experimental Statistics, National Bureau of Standards Handbook 91*, U.S. Government Printing Office, Washington, DC, 1966, Ch. 2, 5, 6.
7 *Operations Manual, 700A Hall Electrolytic Conductivity Detector*, Tracor Instruments, Austin, TX, 1983.
8 R. J. Anderson, *The Hall Book*, Tracor Instruments, Austin, TX, 1983.
9 D. L. Massert, A. Dijkstra and L. Kaufman, *Evaluation and Optimization of Laboratory Methods and Analytical Procedures*, Elsevier, 1978, p. 292.
10 W. Mendenhall, *Introduction to Linear Models and the Design and Analysis of Experiments*, Wadsworth Publishing Co., Belmont, CA, 1968, p. 287.
11 I. B. Rubin, T. J. Mitchell and G. Goldstein, *Anal. Chem.*, 43 (1971) 717.
12 C. R. Hicks, *Fundamental Concepts in the Design of Experiments*, Holt, Rinehart and Winston, New York, 3rd ed., 1982, p. 204.
13 P. W. M. John, *Statistical Design and Analysis of Experiments*, MacMillan, New York, 1971, p. 204.

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SOME FACTORS AFFECTING THE PRECISION IN THE DETERMINATION OF RETENTION INDICES ON POLAR CAPILLARY COLUMNS FOR GAS CHROMATOGRAPHY

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SUMMARY

Three series of homologues were evaluated for use as standards for the determination of retention indices on capillary columns coated with a silicone with 60% cyanopropyl substitution. The homologous series tested were *n*-alkanes according to Kováts, 2-ketones and fatty acid methyl esters. Using a highly polar stationary phase, *n*-alkanes were adsorbed at the gas–liquid interface. The presence of such an additional retention mechanism resulted in *n*-alkane retention times being highly dependent on the stationary phase area to volume ratio. The retention of the polar homologues was, however, relatively insensitive in this respect, and the reproducibility of the retention index determinations was thereby highly improved.

INTRODUCTION

It is well documented that gas chromatographic (GC) retention data can be of great value in qualitative analysis. Such data are influenced by several factors, and in order to be useful in qualitative analysis they should be measured under standardized conditions and reported in a uniform manner. A relatively high insensitivity towards experimental parameters may be achieved when retention is measured under isothermal conditions as relative retention. A retention index system fulfilling these requirements was defined by Kováts[1–3] by reference to two standard *n*-alkanes, the peaks of which bracket the band of the compound of interest. The retention index is given by

$$I(x) = 100z + 100\frac{\log t'_{R(x)} - \log t'_{R(P_z)}}{\log t'_{R(P_{z+1})} - \log t'_{R(P_z)}} \quad (1)$$

where $I(x)$ is the retention index of compound x, $t'_{R(x)}$ is the adjusted retention time of compound x and P_z and P_{z+1} are the two *n*-alkanes which are eluted just before and just after the peak of compound x. The adjusted retention time is $t'_R = t_R - t_m$, where t_R is the retention time and t_m is the retention time of a non-retained substance. The

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retention index scale elaborated by Kováts is founded on the relationship between log t'_R and the carbon number, z, of the members of the homologous series of n-alkanes:

$$\log t'_R = az + b \tag{2}$$

where a and b are constants. Eqn. 2 is based on the assumption that each methylene group makes a constant contribution to the retention, this not being valid, however, for the lower homologues. Wainwright and Haken[4] showed non-linearity for methane and ethane; some workers consider that the equation shows linearity from n-pentane[5,6] onwards and others from n-heptane onwards[7]. Rohrschneider[8] and Golovnya and Grigoryeva[9] indicated linearity from n-nonane. A further complication may be a possible oscillation in the chromatographic properties of successive members of the homologous series. In order to avoid such problems, Kováts first defined the retention index on the basis of even-carbon n-alkanes. This was experimentally found to be unnecessary, and Kováts redefined the retention index and proposed an increment of 100 between n-alkanes[3]. The influence of odd or even carbon numbers on the retention of n-alkanes was later discussed by Vigdergauz and co-workers[10–12].

It is well known that several compounds can have the same retention index on a given column, so retention data cannot be taken as a positive identification. Nevertheless, in combinations of GC and other analytical methods such as mass spectrometry and Fourier transform infrared spectrometry, retention data can be of considerable help. The utility of the retention data in this connection is, of course, dependent on their reliability.

The Kováts retention index system is very widely used, as demonstrated in recent reviews[7,13]. It can be applied to all possible combinations of solutes and stationary phases; the reliability is, however, not equally acceptable in all situations.

The reproducibility of retention indices between different laboratories is generally considered to be within one index unit for non-polar columns and within a few index units for polar columns. It is the opinion of the present authors that the precision obtained on polar columns could be improved. To achieve this, the sources of error should be identified and minimized.

Accurate values of the retention index are thought to result, in an ideal case, when retention is due solely to dissolution of the sample constituents in the pure stationary phase, thus reflecting only the distribution constants between the stationary and mobile phases. Other retention mechanisms, such as adsorption at the support surface and at the gas–liquid interface, should thus be suppressed. Capillary deactivation methods have been much improved in recent years. Now, typical polar solutes can be successfully chromatographed when present in at least nanogram amounts. Adsorption at the gas–liquid interface is, however, almost inevitable for the combination of n-alkanes and polar stationary phases[14]. The sample capacity for n-alkanes here becomes so low that, in practice, column overloading cannot be avoided, thus leading to retention times that are dependent on sample amounts in the range normally encountered in GC with flame ionization detection (FID). The retention of the reference substances, n-alkanes, thus becomes ill-defined and thereby also the retention indices. The use of more polar standards has been suggested for improving the precision[3,15–17], *e.g.*, 2-alkanones[16,18], n-aldehydes[19] and methyl esters of saturated fatty acids (equivalent chain length)[20,21] have been investigated. The

function of the polar standards in isothermal analysis is primarily to provide a reference system based on dissolution in the stationary phase. In temperature-programmed analysis, the relative insensitivity of the retention index towards small fluctuations in experimental conditions is lost[13]. The distribution coefficients of different compounds are differently affected by changes in temperature. This sensitivity towards fluctuations in experimental conditions can be partly compensated, however, by the use of reference compounds having structures similar to those of the sample components, provided, of course, that the sample is homogeneous. The reasons for the use of alternative standards are therefore different in isothermal and temperature-programmed analysis.

A method for the calculation of invariant retention indices was described by Berezkin[22,23]:

$$I_i = I_0 + a_i\,(1/V_L) \tag{3}$$

where I_0 is the limiting value of the retention index determined only by dissolution of a compound i in the stationary phase, a_i is a constant characterizing adsorption of this compound in the given system (the assumption being that the retention of the standard solute is due only to dissolution in the stationary phase) and V_L is the stationary phase volume in the column. This equation was suggested to be applied if the retention index was measured on packed columns. A similar equation, intended for use when retention indices were measured on capillary columns, was also described by Berezkin[23,24]:

$$I_i = I_0 + a_{ij}\,(1/k'_{st}) \tag{4}$$

where k'_{st} is the capacity ratio for a standard compound, being unaffected by adsorption effects. In our experience, it is possible to obtain linearity of eqn. 3, even though the standards are adsorbed on the gas–liquid interface, if the solute to be indexed is unaffected by adsorption. The constant will then be a_{st}, representing the adsorption on the gas–liquid interface of the standard compounds. It is worth noting that the constants a_i and a_{ij} in eqns. 3 and 4 includes the stationary phase surface area, the assumption for the validity of eqns. 3 and 4, thus being that with changes in the amount of stationary phase in the column, the gas–liquid interfacial area remains more or less unchanged[25]. This assumption is, however, not valid in this work, where columns of different dimensions and film thicknesses have been used. Therefore, a modified version of eqn. 3 has been applied:

$$I_i = I_0 + a_{st}\,(A_L/V_L) \tag{5}$$

where A_L is the gas–liquid interfacial area, the assumption being, in this instance, that the film thickness corresponds to the concentration of the coating solution and that the adsorption of the solute is negligible. For capillary columns used in GC, d_f is much smaller than r, and A_L/V_L is then approaching $1/d_f$.

In this work, indexing of retention data obtained on capillary columns coated with a polar stationary phase was compared for three different systems, *n*-alkanes, 2-ketones and methyl esters of saturated fatty acids. Further, the influence on the retention index of the method used for measurement of the gas hold-up time was

studied. The possible existence of an oscillation of the chromatographic properties of odd- and even-carbon *n*-alkanes was also examined.

EXPERIMENTAL

Columns

Five fused-silica capillary columns were coated with a silicone gum stationary phase (60-CN) having *ca.* 60% cyanopropyl, 37% methyl and 3% vinyl substitution. The synthesis of this stationary phase has been described previously[26]. One column was coated with a poly(dimethylsiloxane)–(5–6% diphenyl)–(0.1–0.3% methylvinylsiloxane) copolymer gum, PS264 (Fluka, Buchs, Switzerland) and used as a non-polar reference column.

Reagents

For the silylation of the polar columns a biscyanopropylcyclosiloxane mixture[26,27] was used, and the non-polar column was silylated by reaction with decamethylcyclopentasiloxane[28].

Column preparation

Polar columns. Fused-silica capillary tubing (20 m × 0.32 mm, 26.5 m × 0.22 mm and 26.5 m × 0.25 mm I.D.) (Chrompack, Middelburg, The Netherlands) was used as the column material. Prior to silylation, the capillaries were pretreated according to a previously described method[26], including leaching with 20% (v/v) hydrochloric acid, rinsing with hydrochloric acid (pH 3) and methanol and dehydration of the leached fused silica. Immediately after the dehydration step, the capillaries were deactivated with the biscyanopropylcyclosiloxane mixture [2% (w/v) in dichloromethane] and silylated at 395°C in an inert atmosphere[26,27]. The silylation was followed by rinsing with at least 10 ml of dichloromethane and further drying with nitrogen for several hours. Before coating, the stationary phase was dissolved in dichloromethane (analytical-reagent grade, freshly distilled) at concentrations giving film thicknesses of 2.0, 1.0, 0.16 and 0.20 μm. All the columns were coated by the static method at room temperature. When the solvent evaporation was complete, the columns were flushed with dry nitrogen overnight in order to remove residues of dichloromethane.

Non-polar column. Fused-silica capillary tubing (18 m × 0.32 mm I.D.) from Quartz et Silice (Paris, France) was used as the column material. The capillary was first flushed with nitrogen and then deactivated by reaction with decamethylcyclopentasiloxane at 370°C for 2 h[28], followed by rinsing with dichloromethane and drying with nitrogen. PS264 was dissolved in dichloromethane at a concentration giving a film thickness of 0.5 μm. The coating procedure was the same as for the polar columns. The column dimensions are given in Table I.

Cross-linking

The 60-CN stationary phase was immobilized by the use of azo-*tert.*-butane (ATB) (Ventron, Karlsruhe, F.R.G.). Columns coated with film thicknesses of 1 and 2 μm were flushed with ATB-saturated nitrogen for 1 h, first from one end and then from the other, while the columns coated with film thicknesses of 0.16 and 0.20 μm

TABLE I
COLUMN DIMENSIONS

Column No.	*Length (m)*	*I.D. (mm)*	d_f *(μm)*	*Stationary phase*
1	20.0	0.32	2.0	60-CN
2	20.0	0.32	1.0	60-CN
3	26.5	0.22	0.16	60-CN
4	26.5	0.25	0.20	60-CN
5	26.5	0.25	0.20	60-CN
6	18.0	0.32	0.5	PS264

were flushed for 30 min, also from each end. After flame sealing, the columns were cured at 220°C for 1 h after programming at a rate of 4°C/min. The non-polar stationary phase was not immobilized.

Testing conditions

In order to evaluate the stationary phase properties, and especially the retention indices, the polar columns were tested three times according to the following procedure: (a) after a short conditioning at 220°C for 30 min, programming rate 5°C/min; (b) after immobilization with ATB and conditioning as in (a), and (c) after rinsing with 10 ml of dichloromethane and conditioning as in (a).

Test mixtures

Three test mixtures were used: (1) *n*-alkane and 2-methylnaphthalene; C_{13}–C_{20} *n*-alkanes were used for the polar columns and C_{10}–C_{16} *n*-alkanes for the column coated with PS264; (2) 2-ketones (C_{10}, C_{11}, C_{12} and C_{15}) and 2-methylnaphthalene; (3) Saturated fatty acid methyl esters (FAME) (C_{10}, C_{11}, C_{12} and C_{14}) and 2-methylnaphthalene.

In standard 2, the ketone standard (C_{10}) was 2-decanone, so that all carbon atoms were included when this was set to have retention index value of 1000 i.u. In standard 3, the FAME standard (C_{10}) was methyl decylate, and therefore the methyl ester carbon was not included when this was set to have a retention index value of 1000 i.u.

The concentration of the *n*-alkanes in the standard intended for the polar columns was *ca.* 20 ng/μl and all other components were in the concentration range 100–200 ng/μl.

Apparatus

All chromatographic measurements were performed on a Carlo Erba (Milan, Italy) Mega gas chromatograph equipped with a flame ionization detector. Hydrogen was used as the carrier gas and the gas velocity was 40–50 cm/s at the oven temperature for the run. The injector temperature was held at 220°C, the detector temperature was 250°C and the injections were performed at column temperatures of 115, 125 and 135°C. A splitting ratio of 1:100 was used for all columns and temperatures and the injection volume was 1 or 2 μl.

A laboratory data system, ELDS881 (Chromatography Data System, Kungs-

hög, Stenhamra, Sweden) was connected to the gas chromatograph for signal registration and treatment. The sampling frequency was set to give 40–400 data points over a chromatographed peak.

Column dead-time measurement

The column dead time was measured either by injection of methane or by the mathematical method described by Guardino *et al.*[29].

RESULTS AND DISCUSSION

Determination of column dead time, t_m

The retention indices were established using eqn. 2, by iteration of t_m, to obtain the the best fit to a straight line for carbon number *versus* log t'_R according to Guardino *et al.*[29]. However, as discussed by Smith and co-workers[30,31] and Ettre[32], this value of the mathematical dead time may not be the real column dead time, but a function of it. The different homologous series used here thus resulted in different t_m values. For example, on column 2 (Table I) at 125°C, the t_m value obtained by injection of methane was 41.4 s, the mathematical dead time obtained with *n*-alkane homologues was 40.8 s and with 2-ketone and the FAME homologues 34.2 and 36.6 s, respectively. In this work, the retention indices were calculated using the mathematical dead time obtained with the homologous series used for indexing. For the calculation of k' values, the dead time obtained by injection of methane was used.

Column efficiency

Polar columns are known to be less efficient than non-polar columns, especially when the stationary phase film is thicker. This is partly due to a lower diffusion coefficient in the polar stationary phases, but also to the difficulty in preparing columns with an even stationary phase film. In Table II the column efficiencies are represented by the height equivalent to a theoretical plate (HETP) values for *n*-heptadecane, *n*-eicosane, 2-pentadecanone, methyl myristate and 2-methylnaphthalene at 125°C. As expected, the HETP values for the *n*-alkanes are significantly higher than those for the other solutes. This is mainly due to the poor solubility of these non-polar compounds in the polar stationary phase, the sample capacity being low in this instance. As has been shown in an earlier evaluation of the 60-CN phase[33], an overloading of *n*-alkanes on this stationary phase will lead to concentration-dependent retention indices. One way of avoiding overloading is, of course, to inject smaller amounts of *n*-alkanes on to the column, but detectability may then become a problem; the consequence of a low signal-to-noise ratio will be ill-defined retention times and hence possible uncertainty in the retention index determination. Table II also shows that the ATB treatment has a significant effect on the column efficiency. All the test solutes are affected here, but the *n*-alkanes give by far the greatest decrease in efficiency. This decrease is even more pronounced with increasing stationary phase film thickness. In Fig. 1, the chromatograms obtained before ATB treatment of the columns, using a test mixture containing *n*-alkanes, are shown for (a) column 2 at 135°C, (b) column 3 at 125°C and (c) column 5 at 125°C. The corresponding chromatograms obtained after ATB treatment are shown in Fig. 2a, b and c.

TABLE II
COLUMN EFFICIENCIES AT 125°C

Column No.[a]	*Treatment*[b]	*HETP (mm)*				
		n-Heptadecane	*n-Eicosane*	*2-Pentadecanone*	*Methylmyristate*	*2-Methylnaphthalene*
1	a	3.39	1.35	0.78	0.96	0.80
2	a	1.71	1.15	0.54	0.67	0.55
3	a	1.27	1.14	0.42	0.50	0.29
4	a	0.48	0.58	0.28	0.32	0.27
5	a	0.45	0.34	0.28	0.29	0.27
6	a	–	–	0.36	0.38	0.31
1	b	5.14	2.78	0.94	1.21	0.95
2	b	4.96	3.26	0.96	1.25	0.81
3	b	1.69	2.00	0.55	0.68	0.36
4	b	3.27	7.71	0.80	1.11	0.30
5	b	1.04	1.79	0.39	0.44	0.28

[a] See Table I.

[b] a = Before ATB treatment, after conditioning at 220°C for 30 min; b = after ATB treatment and additional conditioning at 220°C for 30 min.

Comparison between retention index systems

Three retention index systems were evaluated for use on polar columns. The retention indices were determined by use of the equation $\log t'_R = aI + b$, where the constants a and b are dependent on the stationary phase and on the nature of the chemical group bound to the alkyl chain[34], and a should deviate only slightly for all

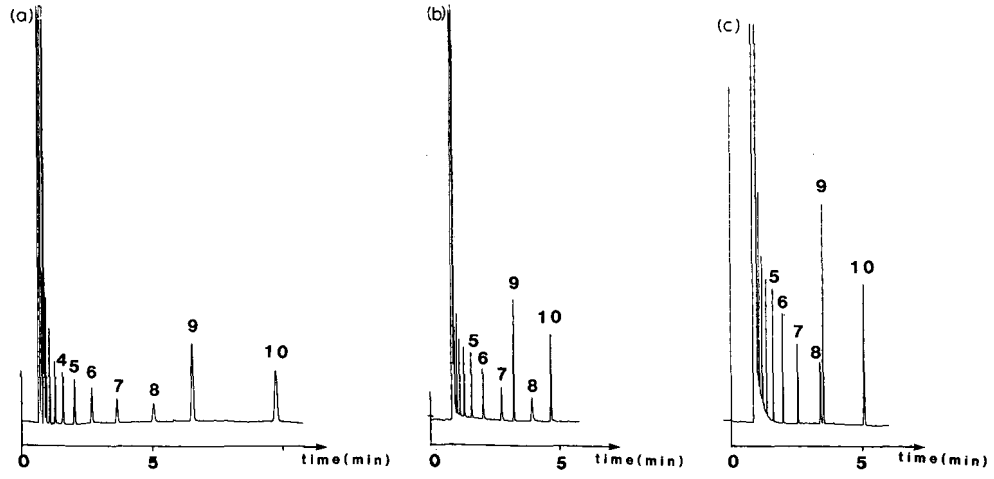

Fig. 1. Gas chromatograms (FID) of a mixture containing *n*-alkanes, 2-methylnaphthalene and biphenyl on three capillary columns coated with 60-CN stationary phase. Conditions: split injections at 135 or 125°C after the columns had been conditioned at 220°C for 30 min. (a) Column 2 at 135°C; (b) column 3 at 125°C; (c) column 5 at 125°C. Peaks: 1 = *n*-tridecane; 2 = *n*-tetradecane; 3 = *n*-pentadecane; 4 = *n*-hexadecane; 5 = *n*-heptadecane; 6 = *n*-octadecane; 7 = *n*-nonadecane; 8 = *n*-eicosane; 9 = 2-methylnaphthalene; 10 = biphenyl.

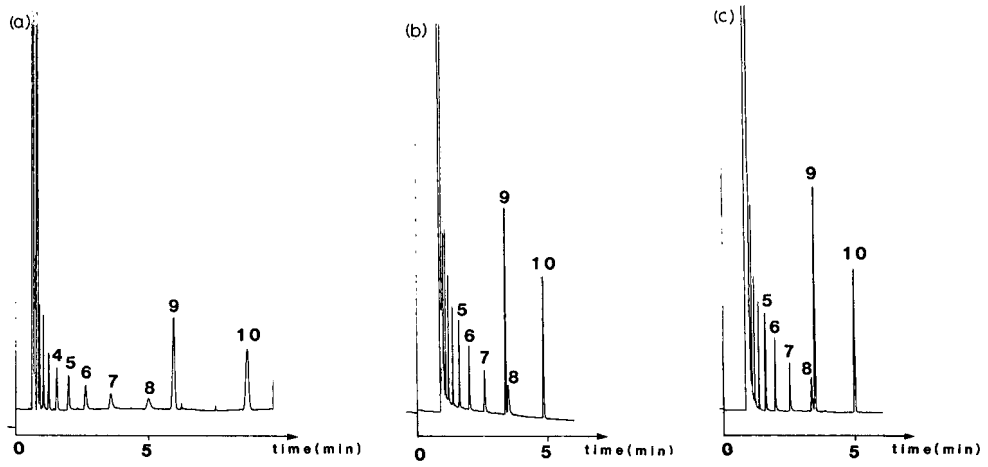

Fig. 2. Gas chromatograms (FID) of a mixture containing *n*-alkanes, 2-methylnaphthalene and biphenyl on three capillary columns coated with 60-CN stationary phase. Conditions: split injections at 135 or 125°C after ATB treatment and conditioning at 220°C for 30 min. (a) Column 2 at 135°C; (b) column 3 at 125°C; (c) column 5 at 125°C. Peaks as in Fig. 1.

series. However, this seems to be true for the non-polar column and for column 1, but for the other columns the a values for the *n*-alkane homologous series deviate considerably from the a values for the 2-ketone and FAME series (Table III). The greatest deviation was observed for column 3, where the adsorption effects for the

TABLE III

CONSTANTS a AND b IN LOG $t'_R = aI+b$, AT 125°C (BEFORE ATB TREATMENT)

Column No.	*Standard*	t_m *(min)*		$a \cdot 10^2$	b
		Mathematical	*Methane*		
1	*n*-Alkanes	0.675	0.690	0.172	−2.334
2	*n*-Alkanes	0.706	0.688	0.178	−2.713
3	*n*-Alkanes	0.873	0.855	0.202	−3.554
4	*n*-Alkanes	0.947	0.917	0.182	−3.221
5	*n*-Alkanes	0.932	0.907	0.182	−3.231
6	*n*-Alkanes	0.523	0.535	0.243	−2.656
1	2-Ketones	0.584	0.688	0.173	−1.076
2	2-Ketones	0.657	0.685	0.174	−1.364
3	2-Ketones	0.840	0.852	0.176	−1.927
4	2-Ketones	0.933	0.920	0.176	−1.873
5	2-Ketones	0.916	0.907	0.175	−1.877
6	2-Ketones	0.506	0.537	0.243	−2.179
1	FAME	0.605	0.688	0.173	−1.051
2	FAME	0.659	0.685	0.173	−1.339
3	FAME	0.841	0.830	0.177	−1.921
4	FAME	0.935	0.922	0.175	−1.847
5	FAME	0.917	0.906	0.175	−1.853
6	FAME	0.469	0.537	0.242	−1.847

TABLE IV

I VALUES OF THE DIFFERENT STANDARDS IN THE DIFFERENT SYSTEMS, CALCULATED FROM LOG $t'_R = aI + b$, AT 125°C (BEFORE ATB TREATMENT)

Column No.	*I in the n-alkane system*[a]						*I in the 2-ketone system*[a]			*I in the FAME system*[a]		
	Ket_{10}	Ket_{11}	Ket_{12}	$FAME_{10}$	$FAME_{11}$	$FAME_{12}$	$FAME_{10}$	$FAME_{11}$	$FAME_{12}$	Ket_{10}	Ket_{11}	Ket_{12}
1	1734.5	1836.9	1939.2	1749.4	1851.3	1952.8	1017.0	1116.6	1216.5	986.8	1087.3	1188.1
2	1728.6	1824.5	1926.4	1740.3	1839.0	1937.5	1009.9	1109.4	1209.0	989.4	1089.9	1190.2
3	1665.0	1755.9	1845.8	1676.5	1767.6	1857.7	1011.4	1112.0	1212.8	989.5	1089.2	1189.1
4	1701.5	1799.4	1897.0	1715.2	1812.8	1910.0	1012.1	1111.5	1211.1	988.3	1088.8	1189.5
5	1702.5	1800.5	1898.1	1716.3	1814.0	1911.3	1016.3	1116.2	1216.3	988.1	1088.4	1188.9
6	1195.2	1296.0	1396.7	1325.6	1426.1	1526.4	1130.1	1230.2	1330.4	871.2	970.1	1069.9

[a] Ket_{10}, Ket_{11}, Ket_{12} = 2-decanone, 2-undecanone, 2-dodecanone; $FAME_{10}$, $FAME_{11}$, $FAME_{12}$ = methyl decylate, methyl undecylate, methyl dodecylate.

n-alkanes were pronounced (Fig. 1b). An effect of this deviation is that the increase between two consecutive members in the homologous series is no longer 100 i.u. In Table IV, the retention indices of 2-decanone, 2-undecanone, 2-dodecanone, methyl decylate, methyl undecylate and methyl dodecylate are given. The values were calculated for the *n*-alkane standard system and for the 2-ketone and FAME systems, respectively. As expected, on column 3 the difference between two consecutive homologues was only *ca* 90 i.u. when the *n*-alkanes were used as standards, whereas a value of 100 i.u. was found with the other two systems. As pointed out by Kováts[3], "a homologous series is an ideal secondary reference series when the indices of its consecutive members increases by 100...". Obviously, this rule fails in systems where interfacial adsorption contributes seriously to the retention[35].

Influence of odd/even n-alkanes

It has been suggested that plots of eqn. 2 would show an oscillating shape owing to differences in the molecular structure of odd- and even-carbon homologues[10–12,36]. In this work, looking at fine details, no such oscillation could be detected (Table V).

Influence of film thicknesses and column dimensions on retention indices

The peculiar behaviour of *n*-alkanes on the polar stationary phase can also be considered in terms of changes in retention indices with column dimensions and film thicknesses. Table VI shows the column capacity factors observed after a short column conditioning, and the ratio of gas–liquid surface area to stationary phase volume in the columns. In Table VII the corresponding retention indices are listed. These data are also presented in Fig. 3 as a plot of I *vs.* A_L/V_L according to eqn. 5. In the *n*-alkane reference system (Fig. 3a), a large effect was observed on the retention index for 2-methylnaphthalene when the area to volume ratio increased. The difference was more than 100 i.u. on comparing columns 1 and 3. The value of a_{st} in eqn. 5 was $-14.1 \cdot 10^{-3}$. However, the same trend was observed when 2-ketones and FAME were used as standards (Fig. 3b and c), but to a far smaller extent, the values of a_{st} being $-1.15 \cdot 10^{-3}$ and $-1.90 \cdot 10^{-3}$, respectively, indicating that adsorption represents a significant contribution to the retention for the *n*-alkanes on this highly polar stationary phase.

TABLE V

EFFECT OF THE LINEARITY AND THE RETENTION INDEX OF 2-METHYLNAPHTHALENE AT 115°C, USING ODD- OR EVEN-CARBON *n*-ALKANES (BEFORE ATB TREATMENT)

Column No.	*Carbons*	t_m *(min)*		I	r^2
		Methane	*Mathematical*		
1	13–20	0.678	0.667	2018.6	0.9999980
1	Odd: 13–19		0.666	2018.4	0.9999997
1	Even: 14–20		0.685	2018.5	0.9999994
3	13–20	0.860	0.899	1914.0	0.9999800
3	Odd: 13–19		0.896	1914.0	0.9999928
3	Even: 14–20		0.908	1913.6	0.9999915

TABLE VI

COLUMN CAPACITY FACTORS AT 125°C AND AREA TO VOLUME RATIO (BEFORE ATB TREATMENT)

t_m values used for the calculations were measured by methane injections.

Column No.	*k' values*				$(A_L/V_L) \cdot 10^{-3}$ (mm^{-1})
	n-Eicosane	*2-Pentadecanone*	*Methyl myristate*	*2-Methyl naphthalene*	
1	18.9	48.4	34.2	23.4	0.50
2	10.2	25.4	17.8	12.2	1.00
3	3.6	6.0	4.4	2.8	6.29
4	2.8	6.3	4.4	2.9	5.00
5	2.8	6.3	4.4	2.9	5.00
6	–	54.6	64.2	6.2	2.04

Influence of temperature on retention indices

In Table VIII, the changes in retention indices for 2-methylnaphthalene in the three reference systems are given. The temperature difference was 10°C (125–135°C). On the polar columns, the *n*-alkane reference system was the most sensitive, d*I*/10°C being 36–33 i.u., whereas in the 2-ketone system it was only *ca.* 15 i.u. The retention

TABLE VII

RETENTION INDICES OF 2-METHYLNAPHTHALENE AT 125°C IN THE THREE DIFFERENT STANDARD SYSTEMS (AVERAGE OF TWO OR THREE MEASUREMENTS)

Column No.	*Treatment*[a]	*n-Alkane*		*2-Ketone*		*FAME*	
		I	*S.D.*	*I*	*S.D.*	*I*	*S.D.*
1	a	2053.4	0.62	1317.4	0.13	1307.4	0.13
2	a	2042.4	0.20	1316.9	0.10	1306.2	0.28
3	a	1946.9	0.12	1311.7	0.09	1296.7	0.01
4	a	2010.0	0.06	1311.1	0.05	1298.0	0.05
5	a	2010.6	0.08	1311.6	0.08	1298.6	0.04
6	a	1305.2	0.03	1111.8	0.07	982.6	0.02
1	b	2050.2	0.79	1320.6	0.29	1308.9	0.06
2	b	2014.7	0.09	1312.0	0.30	1300.1	0.04
3	b	1990.7	0.46	1319.5	0.01	1306.9	0.03
4	b	2019.4	1.10	1319.1	0.03	1307.5	0.09
5	b	2012.8	0.48	1313.6	0.05	1299.7	0.02
1	c	2011.1	0.53	1316.9	–	1307.0	–
2	c	1951.7	1.74	1302.6	0.81	1288.8	0.65
3	c	1986.1	0.16	1306.6	0.02	1290.1	0.03
4	c	1999.4	0.51	1309.9	0.09	1295.2	0.04
5	c	1996.6	0.22	1308.0	0.02	1293.1	0.01

[a] a = Before ATB-treatment, after conditioning at 220°C for 30 min; b = after ATB treatment and additional conditioning at 220°C for 30 min; c = after extraction with dichloromethane and additional conditioning at 220°C for 30 min.

indices on the non-polar column (column 6) showed the smallest dependence on temperature and all three reference systems were changed to the same extent. A linear relationship between I and temperature was obtained in the temperature range examined, *i.e.*, 115–135°C.

Influence of immobilization with ATB

Treatment with ATB did not give reproducible results (Table VII). When using 2-ketones or FAME for indexing, the treatment resulted in an increase in retention

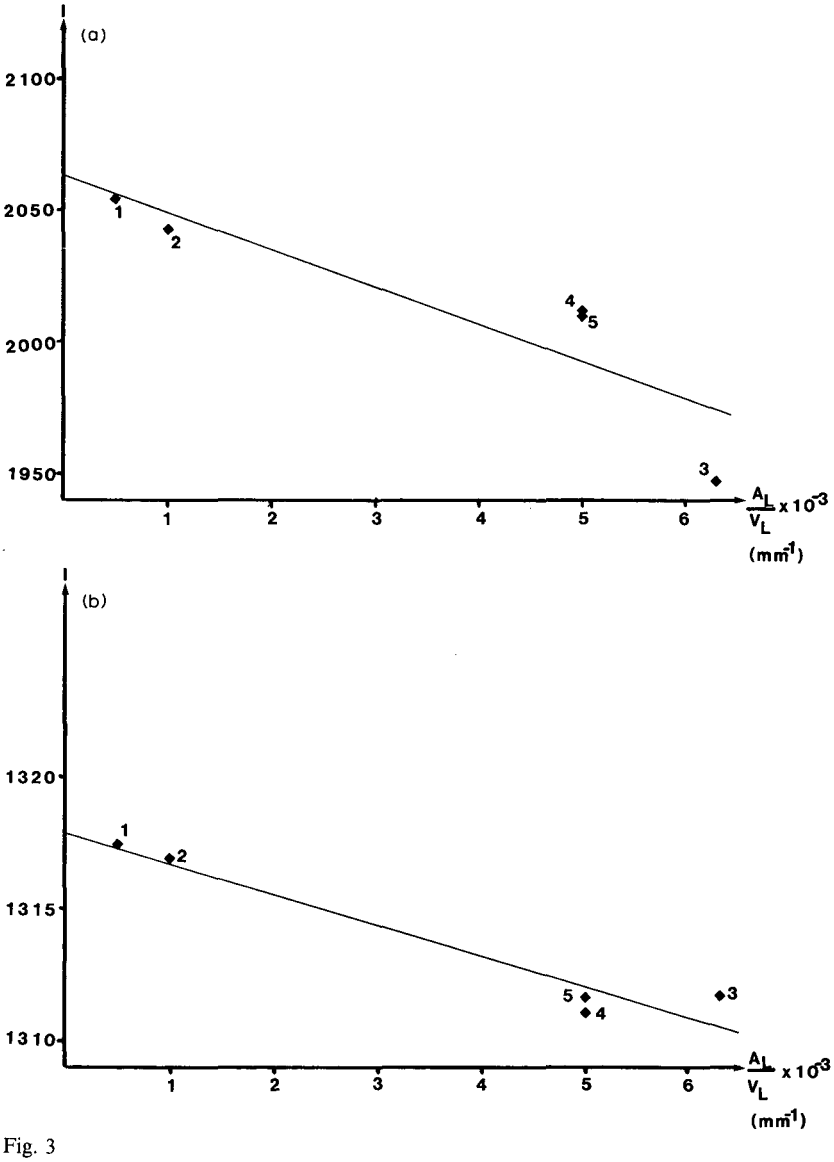

Fig. 3

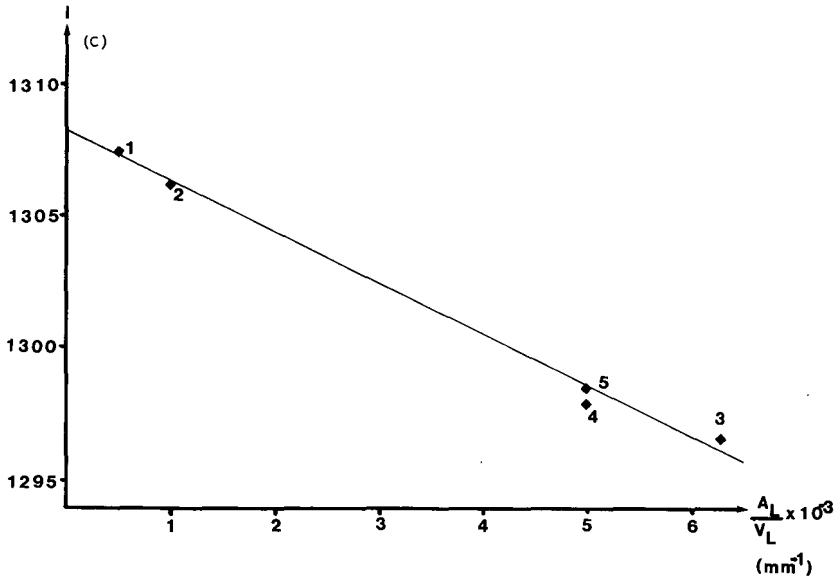

Fig. 3. Dependence of the retention index of 2-methylnaphthalene on the stationary phase area to volume ratio (A_L/V_L). Conditions: split injections at 125°C after the columns had been conditioned at 220°C for 30 min. (a) *n*-Alkane standard, $I = 2063 - 14.1 \cdot 10^{-3} (A_L/V_L)$, $r = 0.890$; (b) 2-ketone standard, $I = 1318 - 1.15 \cdot 10^{-3} (A_L/V_L)$, $r = 0.968$; (c) FAME standard, $I = 1309 - 1.90 \cdot 10^{-3} (A_L/V_L)$, $r = 0.996$. Numbers correspond to column numbers in Table I.

indices for 2-methylnaphthalene for all columns except for column 2. The same result was obtained when *n*-alkanes were used as standards; however, column 1 gave a slightly lower retention index in this system.

Influence of column rinsing with dichloromethane

The influence of extraction of the ATB-treated columns is demonstrated in Table IX. For the thick-film columns (columns 1 and 2), the degree of immobilization was approximately 70%. Column 3 was almost completely immobilized, and columns

TABLE VIII

TEMPERATURE DEPENDENCE OF THE RETENTION INDEX FOR 2-METHYLNAPHTHALENE IN THE THREE DIFFERENT STANDARD SYSTEMS (BEFORE ATB TREATMENT)

Column No.	*dI/10°C (125–135°C)*		
	n-Alkane	*2-Ketone*	*FAME*
1	36.2	15.6	22.4
2	34.5	15.2	21.7
3	33.1	14.9	21.1
4	33.5	14.9	21.2
5	33.8	15.0	21.2
6	6.9	6.2	7.0

TABLE IX

PERCENTAGE DECREASE IN COLUMN CAPACITY FACTORS

Column No.	*Temperature (°C)*	*Decrease in k' values after extraction with CH_2Cl_2 (%)*			
		n-Eicosane	*2-Pentadecanone*	*Methyl myristate*	*2-Methylnaphthalene*
1	125	17.3	30.8	32.0	30.3
1	135	19.4	42.0	31.1	29.5
2	125	1.0	28.2	27.5	33.2
2	135	–	27.9	28.1	32.2
3	125	20.7	0.5	0.2	4.6
3	135	17.8	0.5	1.1	3.6
4	125	9.5	11.7	11.1	12.6
4	135	8.8	11.2	11.0	12.1
5	125	–	17.7	17.7	19.7
5	135	17.2	20.7	20.3	21.9

4 and 5 were immobilized to *ca.* 80–90%. Solvent rinsing resulted in a similar decrease in *k'* values for 2-pentanone, methyl myristate and 2-methylnaphthalene, whereas the decrease for *n*-eicosane showed a significant deviation. Comparison of the retention indices before and after extraction in Table VII shows that the result of column rinsing is a lowering of column polarity, for all three reference systems. As reported earlier[26,27], there may be an over-representation of polar or moderately polar fragments in the mixed cyclic compounds that are removed from the column on rinsing.

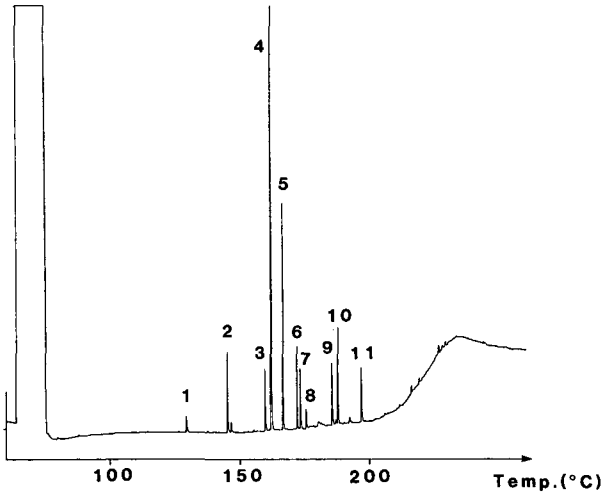

Fig. 4. Gas chromatogram (FID) of a fatty acid methyl ester mixture (rapeseed oil, Supelco) on a fused-silica capillary column coated with 60-CN stationary phase (column 4, Table I). The chromatogram was obtained after ATB treatment, rinsing with dichloromethane and conditioning at 220°C for 10 h. Conditions: splitless injection at 70°C and, after 2 min, programming at 5°C/min to 220°C. Peaks: 1 = 14:0; 2 = 16:0; 3 = 18:0; 4 = 18:1; 5 = 18:2; 6 = 18:3; 7 = 20:0; 8 = 20:1; 9 = 22:0; 10 = 22:1; 11 = 24:0.

Applications

The suitability of the 60-CN stationary phase for the separation of FAME is demonstrated in Fig. 4 for a rapeseed oil sample on column 4.

CONCLUSIONS

It may be considered that the greatest advantage of the Kováts retention index system is that it permits a comparison between results obtained in different laboratories. This universal role is hindered by the use of different systems for data presentation, including other retention index systems[7]. It is well known that the reproducibility of retention index determinations is much lower on polar than on non-polar columns. It seems that the explanation mainly involves the use of *n*-alkanes as reference substances. For the *n*-alkanes, a large proportion of the retention on polar columns is due to adsorption at the gas–liquid interface. Retention based on such a mechanism is highly dependent on the experimental conditions, such as amount injected, stationary phase surface area to volume ratio and small alterations in stationary phase properties. In this work, it has been shown that, when using polar columns, a relatively high insensitivity to experimental conditions in isothermal analysis can be obtained when utilizing 2-ketones or fatty acid methyl esters as standards. The merit of these, in this context, is that they interact with the stationary phase primarily by dissolution. The reproducibility of retention index determinations is thereby much improved. It seems that the universality of the retention index must be sacrificed for the improvement of reproducibility in cases where adsorption may contribute significantly to the retention.

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REFERENCES

1 E. sz. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
2 A. Wehrli and E. sz. Kováts, *Helv. Chim. Acta*, 42 (1959) 2709.
3 E. sz. Kováts, *Adv. Chromatogr.*, 1 (1965) 229.
4 M. S. Wainwright and J. K. Haken, *J. Chromatogr.*, 256 (1983) 193.
5 M. S. Wainwright, J. K. Haken and D. Srisukh, *J. Chromatogr.*, 188 (1980) 246.
6 L. Soják, J. Krupčik and J. Rijks, *Chromatographia*, 7 (1974) 26.
7 M. V. Budahegyi, E. R. Lombosi, T. S. Lombosi, S. Y. Mészáros, Sz. Nyiredy, G. Tarján, I. Timár and J. M. Takáçs, *J. Chromatogr.*, 271 (1983) 213.
8 L. Rohrschneider, *Chromatographia*, 2 (1969) 437.
9 R. V. Golovnya and D. N. Grigoryeva, *Chromatographia*, 17 (1983) 613.
10 M. S. Vigdergauz and V. I. Semkin, *Russ. J. Phys. Chem.*, 46 (1972) 546.
11 M. S. Vigdergauz and V. I. Seomkin, *J. Chromatogr.*, 158 (1978) 57.
12 M. S. Vigdergauz and E. I. Petrova, *Chromatographia*, 25 (1988) 589.
13 L. G. Blomberg, *Adv. Chromatogr.*, 26 (1987) 229.
14 R. L. Martin, *Anal. Chem.*, 33 (1961) 347.
15 A. Gröbler, *J. Chromatogr. Sci.*, 10 (1972) 128.
16 R. G. Ackman, *J. Chromatogr. Sci.*, 10 (1972) 535.
17 S. J. Hawkes, *J. Chromatogr. Sci.*, 10 (1972) 536.

18 J. Raymer, D. Wiesler and M. Novotny, *J. Chromatogr.*, 325 (1985) 13.
19 U. Heldt and H. J. K. Köser, *J. Chromatogr.*, 192 (1980) 107.
20 T. K. Miwa, K. L. Mikolajczak, F. R. Earle and I. A. Wolff, *Anal. Chem.*, 32 (1960) 1739.
21 J. K. Haken, *Adv. Chromatogr.*, 14 (1976) 367.
22 V. G. Berezkin, *J. Chromatogr.*, 91 (1974) 559.
23 V. G. Berezkin, *Adv. Chromatogr.*, 27 (1987) 1.
24 V. G. Berezkin, *J. Chromatogr.*, 98 (1974) 477.
25 J. A. Rijks, L. A. Leiten, C. A. Cramers and V. G. Berezkin, *J. Anal. Chem. U.S.S.R.*, 29 (1974) 736.
26 K. Markides, L. Blomberg, S. Hoffmann, J. Buijten and T. Wännman, *J. Chromatogr.*, 302 (1984) 319.
27 K. Markides, *Thesis*, University of Stockholm, 1984.
28 J. Buijten, L. Blomberg, S. Hoffmann, K. Markides and T. Wännman, *J. Chromatogr.*, 301 (1984) 265.
29 X. Guardino, J. Albaigés, G. Firpo, R. Rodríguez-Viñals and M. Gassiot, *J. Chromatogr.*, 118 (1976) 13.
30 R. J. Smith, J. K. Haken and M. S. Wainwright, *J. Chromatogr.*, 334 (1985) 95.
31 R. J. Smith, J. K. Haken, M. S. Wainwright and B. G. Madden, *J. Chromatogr.*, 328 (1985) 11.
32 L. S. Ettre, *Chromatographia*, 13 (1980) 73.
33 A. Bemgård and L. Blomberg, *J. Chromatogr.*, 395 (1987) 125.
34 G. Guiochon and C. L. Guillemin, *Quantitative Gas Chromatography (Journal of Chromatography Library Series*, Vol. 42), Elsevier, Amsterdam,1988.
35 D. F. Fritz, A. Sahil and E. sz. Kováts, *J. Chromatogr.*, 186 (1979) 63.
36 R. E. Kaiser and A. J. Rackstraw, *Computer Chromatography*, Vol. 1, Hüthig, Heidelberg, 1983, p. 26.

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RETENTION CHARACTERISTICS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BASIC DRUGS AND PLASMA EXTRACTS ON AN ALUMINA COLUMN

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SUMMARY

The retention characteristics of 35 drugs on an alumina column using buffered aqueous methanolic mobile phases have been investigated. It has been shown that the effect of pH on retention depends on the pK_a of the drug and the type of buffer ion used; that there is an inverse relation between ionic strength and solute retention; and that a decrease in the amount of methanol in the mobile phase causes the drugs to be held longer on the column. The chromatographic system was also coupled to an on-line column-switching assembly to facilitate extraction of the drugs from plasma, and results are presented which indicate that the alumina column lends itself well to this convenient method of plasma clean-up.

INTRODUCTION

The use of bare (unmodified) silica and alumina as stationary phases in liquid chromatography had declined in popularity with the advent of chemically modified hydrophobic silicaceous supports. In particular, the use of alumina became even less widespread, as chemical modification of its surface in a manner analogous to the reaction of silica with alkylsilanes, proved not to be a practicable proposition[1,2]. Although some workers have reacted alumina with aminosilane, cyanosilane, and pyridylsilane[3], as well as octadecylsilane[4], Laurent *et al.*[1] found that most chemical reagents which had been used successfully in the chemical modification of silica were unreactive on alumina. They were, however, able to produce a non-polar material by reacting hexamethyldisilane (HMDS) with alumina, but subsequently proved it to be strongly adsorbed rather than covalently bound, and subject to erosion by the kind of polar solvents employed in liquid chromatography.

Among the problems commonly associated with reversed-phase chromatography are poor efficiencies and peak tailing for many organic bases. These effects result from ionic interaction between the charged bases and unreacted silanol moieties

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on the silica surface. Methods which have been used to overcome these problems include ion-suppression chromatography[5], or the use of organic ammonium compounds at high pH[6,7]. More recently, attention has been focussed on the exploitation of the amine–silanol interaction, since Jane[8] first reported that basic compounds could be efficiently separated on bare silica using the kind of aqueous–organic eluents more commonly associated with reversed-phase chromatography. The mechanisms involved in these types of polar interactions are complex and multifunctional, but have been shown to consist at least in part of ion-exchange reactions[9–11], with hydrophobic interactions involving siloxane bridges contributing to some extent[5,10]. This mode of separation demands that eluents of high pH be used where a large proportion of silanol moieties are ionised, and thus available to partake in ion-exchange reactions with oppositely-charged protonated bases[9]. A limitation with the use of high pH eluents is the tendency of silica to dissolve in alkaline solutions. This problem may be partially overcome by the incorporation of a pre-column between the pump and injector in order to saturate the mobile phase with silica, and by the use of mobile phases containing a high proportion of organic component.

Recently, Laurent *et al.*[1,2] have re-appraised the application of alumina as a solid phase support in column liquid chromatography. They showed that alumina can be used in an ion-exchange mode in a manner similar to unmodified silica, with the added advantage that it is stable over a wide pH range (2–12)[1]. Furthermore, due to the amphoteric nature of alumina, it behaves (like silica) as a cation exchanger in neutral or basic solutions, and (unlike silica) as an anion exchanger in acidic solutions, thus permitting the chromatographic separation of both anions and cations.

The objective of the current study was to examine the retention characteristics of a number of basic drugs on an alumina column under varying eluent conditions, and to investigate the possibility of using a column switching assembly in order to facilitate on-line clean-up of plasma samples followed by chromatography on the alumina column.

EXPERIMENTAL

Reagents and solvents

The drugs used were received as a gift from the Institute of Clinical Pharmacology (Dublin, Ireland). Potassium dihydrogen phosphate (analytical grade) was obtained from May and Baker (Dagenham, U.K.) and potassium hydroxide (AnalaR grade) from BDH Chemicals (Poole, U.K.). Orthophosphoric acid and dipotassium hydrogenphosphate (all analytical grade) were supplied by Riedel de Haen (Seelze, Hannover, F.R.G.). HPLC-grade methanol was purchased from Labscan Analytical Sciences (Dublin, Ireland). Deionised water was obtained by passing distilled water through a Milli-Q water purification system. Dried human plasma was obtained from the Blood Transfusion Board (Dublin, Ireland) and dissolved in Milli-Q water. This plasma was then used within seven days of reconstitution.

Drug solutions

Stock solutions equivalent to 1 mg ml^{-1} of the drugs in methanol were prepared,

and working standards were made up to the required concentration, which varied between 1 and 10 $\mu g\ ml^{-1}$ according to the detector response of the drug.

Plasma solutions

Aliquots of reconstituted drug-free plasma were spiked with drug solutions to produce the desired concentrations. These plasma solutions were then diluted (1:1) with water prior to injection into the high-performance liquid chromatographic (HPLC) system.

Instrumentation and operating conditions

The drugs were separated on a Techsphere alumina 5 μm column (150 mm × 4.6 mm I.D.), supplied by HPLC Technology (Macclesfield, U.K.). Stock solutions of phosphate buffer were prepared by mixing solutions of potassium dihydrogenphosphate and dipotassium hydrogenphosphate to produce solutions of varying pH in the range 5–8.5. Phosphoric acid was added to potassium dihydrogenphosphate to give a solution of pH 3, and potassium hydroxide was added to dipotassium hydrogenphosphate to generate solutions of pH 9 and 11. All component solutions were 1 *M*, and the mixtures were diluted with water to give the desired ionic strength. Mobile phases were made by mixing the aqueous component with methanol to produce solutions containing 30–90% organic phase. The pH of the buffer solutions and the aqueous–organic mixtures were measured at 20 ± 1°C using a standard pH glass electrode. The mobile phase was passed through a 0.45-μm filter under vacuum, and delivered by a Waters (Milford, MA, U.S.A.) Model 501 HPLC pump at a flow-rate of 1.0 ml min^{-1}. The drugs were detected by UV absorption at 254 nm using a Shimadzu SPD-6A variable-wavelength detector with a detector setting of 0.04 a.u.f.s. The resulting chromatograms were recorded with a Linseis (Selb, F.R.G.) recorder at a chart speed of 200 mm h^{-1}.

For direct injection, 20-μl aliquots of the drug solutions in mobile phase were introduced into the chromatographic system. For the purposes of column switching, a second Waters Model 501 HPLC pump and the concentration column were connected to the analytical assembly via a Rheodyne (Cotati, CA, U.S.A.) 7000 six-port switching valve. The instrument arrangement used is shown in Fig. 1.

The 10 × 1.5 mm I.D. concentration columns were dry-packed with Corasil (Waters Assoc.) RP-18 (37–50 μm) packing material. The second pump eluent was Milli-Q water filtered through a 0.45-μm filter and degassed under vacuum. When a 500-μl plasma sample is introduced via the injector port, it is swept onto the concentration column by water from pump A. The drugs are selectively retained on this column while the plasma components are eluted to waste. Meanwhile, the mobile phase eluent is being passed by pump B through the analytical column, and upon switching the valve the drugs are swept from the concentration column onto the analytical column where they are separated.

RESULTS AND DISCUSSION

Effect of pH

The retention of the 35 drugs on the alumina column was investigated as a function of pH over the range pH 3 to pH 11. The mobile phase contained

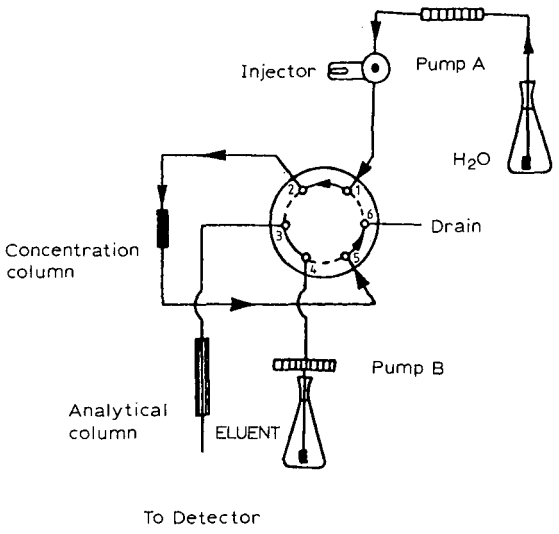

Fig. 1. Column switching assembly incorporating a six-port switching valve.

methanol–0.04 *M* potassium phosphate buffer (80:20) adjusted to the required pH. Additions were not made to compensate for variations in ionic strength from one pH to the next as it was assumed that that the changes in cation concentration would be small in relation to the changes in solute and column ionisation arising from pH variation.

The results of this study are presented in Table I. From this it can be seen that the drugs may be roughly divided into three groups; namely (A), those drugs with retention times which increase between pH 3 and pH 5, followed by a decline in retention at higher pH; (B) those drugs with exhibit decreased retention as the pH is increased; and (C), drugs whose retention times are largely unaffected by pH. These observations may be explained by considering the pK_a of the drugs, the pH of the mobile phase, and the amphoteric nature of the alumina packing. Whether the alumina surface is positively or negatively charged depends on the pH of the surrounding medium. In acidic solutions it is positively charged and behaves as an anion exchanger, whereas in basic solutions the surface is negatively charged and behaves as a cation exchanger. The pH at which the alumina surface bears no charge and is neutral is known as the zero point charge (ZPC). The ZPC is known to be related to the method of production of the alumina, and to depend heavily on the nature of ions present in the surrounding solvent. Laurent *et al.*[12] have shown that in the presence of phosphate buffer, the ZPC of alumina is reduced to 6.5 from the value of 9.2 originally quoted by Parks[13].

Group A. Although these compounds are fully ionised at low pH, interaction with the alumina packing is minimal as the latter will also bear a positive charge, thus repelling the like-charged protonated bases. As the pH is increased to 5, there is an observed increase in retention consistent with the reduced positive charge on the alumina, but as the pH is further increased to 7 and above there is a general decrease in retention. These results may be explained in terms of reduced protonation of the

TABLE I

EFFECT OF pH ON RETENTION

Mobile phase: 0.04 *M* phosphate buffer, pH 7–methanol (20:80).

Drug	pK_a	*Retention time (min)*				
		pH 3	*pH 5*	*pH 7*	*pH 9*	*pH 11*
Group A						
Amitriptyline	9.5	7.5	9.6	6.0	5.1	4.2
Atenolol	9.6	3.9	5.4	2.7	2.1	2.0
Acetopromazine	NA[a]	6.0	7.6	3.6	3.6	3.0
Chlorpheniramine	8.9	8.7	13.8	7.2	6.6	5.1
Chlorpromazine	9.3	6.2	7.5	3.6	3.0	2.7
Desipramine	10.2	5.4	7.5	7.8	6.6	5.4
Imipramine	9.6	8.1	9.6	6.0	5.1	3.9
Mefloquine	NA	7.5	10.8	5.1	4.5	3.6
Nortriptyline	9.7	5.4	8.7	7.5	6.3	5.4
Phentoloxamine	9.1	6.2	7.8	7.2	3.6	3.0
Propranolol	9.5	3.6	4.5	3.5	3.0	2.7
Pindolol	9.5	3.3	3.6	2.4	2.1	2.1
Promethazine	9.1	7.4	10.5	5.1	3.3	3.0
Protriptyline	10.1	4.8	6.6	6.9	6.3	6.0
Quinine	8.5	4.5	8.2	3.0	2.4	2.1
Tripelennamine	9.0	4.5	5.4	2.7	2.7	2.1
Tyramine	10.2	4.2	9.9	6.6	3.6	3.0
Group B						
Diltiazem	NA	4.2	3.3	1.8[b]	1.8[b]	1.8[b]
Dextromethorphan	8.3	4.8	4.5	3.0	2.7	2.4
Fluphenazine	8.1	6.1	6.0	2.4	2.4	2.4
Lidocaine	7.9	3.0	2.7	2.4	2.1	2.1
Mepivacaine	7.7	3.0	2.7	2.1	1.8[b]	1.8[b]
Perphenazine	7.8	6.9	5.1	3.3	3.0	2.7
Phentermine	10.1	5.4	4.5	3.3	3.0	2.4
Phenylpropanolamine	9.5	7.2	4.8	4.8	2.7	2.4
Trimethoprim	7.2	4.5	2.7	1.8[b]	1.8[b]	1.8[b]
Verapamil	NA	3.0	3.0	2.4	2.4	2.4
Group C						
Caffeine	14.0	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Chlorthalidone	9.4	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Chloramphenicol	NA	2.1	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Frusemide	3.6	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Nitrazepam	3.2	2.1	2.0	1.8[b]	1.8[b]	1.8[b]
Sulfamerazine	7.1	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Sulfamethoxazole	5.6	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
N-Acetylsulfamethoxazole	NA	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]
Theophylline	8.6	2.0	1.8[b]	1.8[b]	1.8[b]	1.8[b]

[a] NA = Not available in standard reference texts.

[b] Drug unretained under these conditions.

analytes at higher pH, and possibly a shift upwards of the ZPC of alumina, as the changing buffer composition causes a reduction in the negative charge of the column[14]. Furthermore, Laurent *et al.*[2] have demonstrated an increase in the apparent

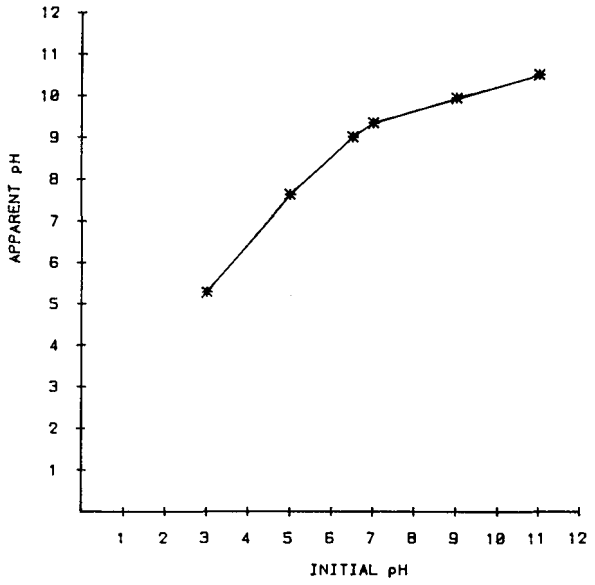

Fig. 2. Effect of the addition of 80% methanol on apparent pH. Mobile phase: 0.04 *M* potassium phosphate buffer–methanol (20:80).

pH in the presence of organic solvents, and the resulting decrease in retention is exaggerated by a concomitant decrease in solute pK_a. This is illustrated in Fig. 2, where it can be seen that there is a substantial difference between the initial and apparent pH values of solutions of initial pH 3–7. This becomes less pronounced for initial pH values of 8–10, until for a solution of initial pH of 11, the addition of methanol has the effect of producing a lower apparent pH value.

Group B. The drugs in this category exhibit reduced retention times with increasing pH. As they generally have lower pK_a values than those in group A, it is to be expected that these compounds would be less ionised than the more basic amines at any given pH, and that there would be a more pronounced reduction in their retention times under conditions of increasing eluent pH.

Group C. As expected, solutes which do not ionise, such as caffeine, are unretained by the column in this chromatographic system where the principal mechanisms of solute–column interaction are known to be ionic in nature. Likewise, compounds with low pK_a values, *e.g.* nitrazepam (pK_a = 3.2), are similarly unretained as they would not be sufficiently ionised to interact with the column at those pH values where alumina behaves as a cation exchanger. The acidic drug frusemide was also investigated, and was found not to be retained under any pH conditions. Frusemide has a pK_a of 3.6[15], so that at pH 3, when the apparent pH was 5.28, the drug should have been ionised. However, this pH is high enough for the column to behave more as a cation- than an anion-exchanger; hence negatively charged solutes would be repelled by the like-charged alumina surface, and therefore be unretained by the column.

Effect of ionic strength

The effect of lowering the ionic strength from 0.04 *M* to 0.02 *M* of the potassium

phosphate buffer was investigated. The results of this study are presented in Table II, where it can be seen that drug retention increases as the ionic strength is reduced. These observations agree with other workers[1,14] who also found an increase in retention on alumina as the ionic strength was reduced. These findings are consistent with the ion-exchange theory of retention which explains enhanced solute interaction in terms of decreased competition by the reduced number of competing cations for the charged sites on the column packing.

Effect of methanol content

The effect of varying the percentage methanol concentration from 90% to 30% (in a mobile phase where the aqueous component was 0.02 *M* phosphate buffer, pH 7) on the retention of 9 of the drugs is shown in Table III. From this it can be seen that as the methanol content is reduced, there is a corresponding increase in drug retention; so much so, that when the methanol content is 50% or below most of the drugs would appear to be fully retained, not having eluted after 60 min or more. These observations differ from those made by Laurent *et al.*[2], and Lingeman *et al.*[14], who found a point of maximum retention *vs.* percentage methanol concentration which is solute-dependent, but was about 40% methanol for most compounds they studied. Laurent *et al.*[2] attributed low solute retention at high methanol contents to a decrease in solute ionisation, and at low methanol contents to reduced solvation of the competing ions.

TABLE II

EFFECT OF IONIC STRENGTH ON RETENTION

Mobile phase: phosphate buffer pH 7–methanol (20:80).

Drug	*Retention time (min)*		*Drug*	*Retention time (min)*	
	0.02 M	*0.04 M*		*0.02 M*	*0.04 M*
Amitriptyline	18.3	6.0	Phentermine	6.3	3.3
Atenolol	3.6	2.7	Phenylpropanolamine	4.8	4.8
Acetopromazine	6.3	3.6	Propranolol	5.1	3.0
Caffeine	1.8[a]	1.8[a]	Pindolol	3.0	2.4
Chlorpheniramine	11.1	7.2	Promethazine	6.3	5.1
Chlorthalidone	1.8[a]	1.8[a]	Protriptyline	10.5	6.9
Chlorpromazine	4.5	3.6	Nitrazepam	1.8[a]	1.8[a]
Chloramphenicol	1.8[a]	1.8[a]	Quinine	3.0	3.0
Diltiazem	2.1	1.8[a]	Trimethoprim	1.8[a]	1.8[a]
Desipramine	11.7	7.8	Tripelennamine	3.3	2.7
Dextromethorphan	3.9	3.0	Tyramine	9.0	6.6
Fluphenazine	2.7	1.8[a]	Verapamil	2.4	2.1
Frusemide	1.8[a]	1.8[a]	Sulphamerazine	1.8[a]	1.8[a]
Imipramine	8.4	6.0	Sulphamethoxazole	1.8[a]	1.8[a]
Lidocaine	2.1	2.1	N-Acetylsulfamethoxazole	1.8[a]	1.8[a]
Mefloquine	7.4	5.1	Theophylline	1.8[a]	1.8[a]
Mepivacaine	2.4	1.8[a]			
Nortriptyline	11.4	7.5			
Perphenazine	3.3	3.0			
Phentoloxamine	4.5	7.2			

[a] Drug unretained under these conditions.

TABLE III

EFFECT OF METHANOL CONTENT ON RETENTION

Mobile phase: 0.02 *M* potassium phosphate buffer, pH 7–methanol.

Drug	*Retention time (min) at methanol content (%)*				
	30	*50*	*70*	*80*	*90*
Amitriptyline	–[a]	–	17.1	6.0	3.3
Chlorpromazine	–	–	9.3	3.6	3.0
Dextorphan	–	–	4.8	3.7	2.7
Nitrazepam	9.6	3.6	2.1	1.8	1.5
Perphenazine	–	–	6.9	3.0	2.4
Pindolol	6.9	3.9	3.0	2.4	2.4
Quinine	–	–	3.9	3.0	2.4
Tripelennamine	–	–	5.4	2.7	2.4
Sulfamethoxazole	1.8[b]	1.8[b]	1.8[b]	1.8[b]	1.8[b]

[a] Not eluted after 60 min.
[b] Drug unretained under these conditions.

At any given pH these two mechanisms will be operating in opposition to one another, as evidenced by the appearance of a retention maximum, with reduced solute ionisation predominating at high methanol contents, and reduced competing ion solvation predominating at low methanol contents. However, as Laurent *et al.*[2] point out, the enhanced solvation of large competing ions (such as tetramethylammonium hydroxide) is the opposite to what happens when the smaller lithium ion is transferred from aqueous to organic media; in this case the smaller ion is less solvated in methanol. It is quite possible that in the present study where the competing ion is the relatively small potassium ion, the degree of buffer ion solvation is reduced as the methanol content is increased, an effect which would act in concert with reduced solute ionisation to minimise the drugs interaction with the stationary phase.

Plasma experiments

Having previously carried out on-line solid–liquid extraction of drugs from plasma, followed by chromatography on a bare silica column[16], it was decided to attempt similar experiments on the alumina column. The instrument arrangement and column switching operation have been previously described in the experimental section. Plasma clean-up and drug extraction were performed on a 10 × 1.5 mm I.D. concentration column, dry-packed with Corasil C_{18} 25–40 μm packing material. In earlier work on the silica column, the C_{18} packing was compared with C_8 material in terms of drug recovery and plasma clean-up. It was found that a C_{18} concentration column retained less of the endogenous plasma components than the C_8 packing because the former is more hydrophobic and has less affinity for the relatively polar plasma constituents.

The objective of this study was to compare drug-free plasma profiles when the pH of the aqueous component was increased from pH 5 to pH 8, and when the ionic strength was increased from 0.02 *M* to 0.04 *M*. The mobile phases studied, along with the resultant chromatograms are presented in Fig. 3, where it can be seen that a slightly

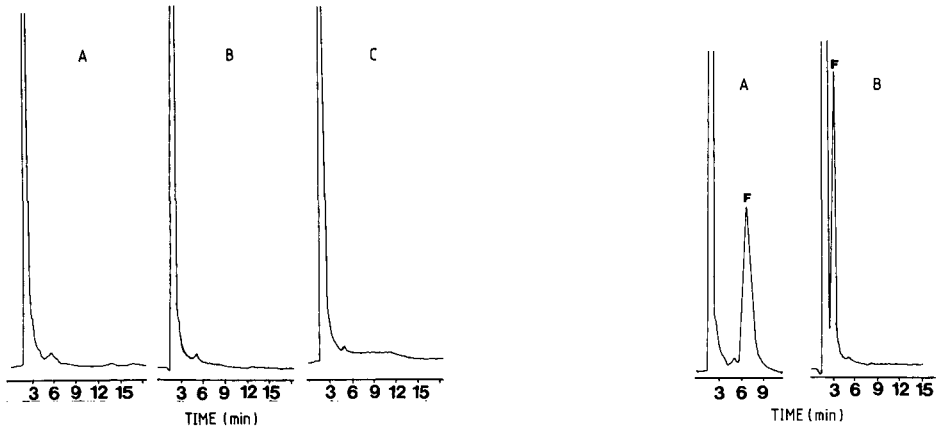

Fig. 3. Blank plasma chromatograms. Mobile phases: (A) 0.02 *M* buffer, pH 5–methanol (20:80); (B) 0.02 *M* buffer, pH 8–methanol (20:80); (C) 0.04 *M* buffer, pH 8–methanol (20:80).

Fig. 4. Spiked plasma chromatograms. Plasma spiked with fluphenazine (F) 200 ng ml^{-1}. (a) Mobile phase: 0.02 *M* phosphate buffer, pH 5–methanol (20:80); (b) mobile phase: 0.02 *M* phosphate buffer, pH 8–methanol (20:80).

better blank plasma chromatogram is obtained at the higher eluent pH with little difference observed when the ionic strength is doubled. The effect of pH on the retention of plasma constituents is not as manifest as its effect on the individual drug species. To illustrate this point, a test compound, *i.e.* fluphenazine, was extracted on-line from spiked plasma prior to chromatography. As may be seen from the chromatograms presented in Fig. 4, there is a pronounced shift in the retention time of the drug relative to that of the plasma constituents as the pH is increased from pH 5 to pH 8. These results demonstrate that it is possible to manipulate the mobile phase to produce the most appropriate retention time for the compounds of interest with little danger of introducing unacceptable interfering peaks from the plasma matrix. Hence this type of chromatography would seem to offer an advantage over conventional bonded-phase techniques, in that the plasma constituents are not sufficiently ionised to be retained by the alumina column, and hence are rapidly eluted. An important example is caffeine which can interfere with drug analysis in reversed-phase chromatography: because it does not ionise, it will not interact with alumina by the principal mechanism of retention, *i.e.* ion exchange, and as a result would elute too early to interfere with any of the peaks of interest.

Using a mobile phase containing methanol–0.04 *M* phosphate buffer, pH 5 (80:20), five replicate analyses of plasma containing 200 ng ml^{-1} fluphenazine were made. The drug eluted with a mean retention time of 7.0 min (standard deviation 0.1 min). The mean drug peak height was 65.4 mm ($\pm$ 2.3 mm) using a detector sensitivity setting of 0.04 a.u.f.s. The coefficient of variation was 3.5%, and 73.1% of the drug was recovered from plasma when compared with authentic aqueous standards injected in the same concentrations as spiked plasma. These results indicate that it should be possible to develop a fully validated bioanalytical method incorporating on-line solid-phase extraction for any drug which can be separated using this chromatographic system.

CONCLUSION

A preliminary study of the behaviour of drug compounds and plasma extracts on an alumina column has been carried out. Results would indicate that alumina presents an attractive alternative to bonded-phase separations of basic drugs which can present difficulties associated with the presence of unreacted silanol moieties on the silica surface. Alumina has an advantage over silica in that it is more stable at high pH, and thus in the separation of bases, the cationic character of the column may be maximised without fear of column degeneration. The retention of ionic compounds is strongly influenced by pH, ionic strength, and the percentage methanol in the mobile phase. Any or all of these parameters may be manipulated to produce the desired retention for the compound or compounds of interest. Chromatography on alumina would appear to be well suited to the analysis of drugs in plasma samples as it lends itself to the rapid and convenient technique of solid-phase extraction by column switching.

REFERENCES

1 C. J. C. M. Laurent, H. A. H. Billiet and L. de Galan, *Chromatographia*, 17 (1983) 253.
2 C. J. C. M. Laurent, H. A. H. Billiet and L. de Galan, *Chromatographia*, 17 (1983) 394.
3 J. H. Knox and A. Pryde, *J. Chromatogr.*, 112 (1975) 171.
4 Y. Hirata and M. Novotny, *Anal. Chem.*, 51 (1979) 1807.
5 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.*, 54 (1982) 442.
6 A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. J. Stampfli and R. W. Frei, *J. Chromatogr.*, 149 (1978) 199.
7 J. C. Kraak and P. Bijster, *J. Chromatogr.*, 143 (1977) 499.
8 I. Jane, *J. Chromatogr.*, 111 (1975) 227.
9 R. W. Schmid and C. Wolf, *Chromatographia*, 24 (1987) 713.
10 R. J. Flanagan and I. Jane, *J. Chromatogr.*, 323 (1985) 173.
11 G. B. Cox and R. W. Stout, *J. Chromatogr.*, 384 (1987) 315.
12 C. J. C. M. Laurent, H. A. H. Billiet, L. de Galan, F. A. Buytenhuys and F. P. B. van der Maeden, *J. Chromatogr.*, 287 (1984) 45.
13 G. A. Parks, *Chem. Rev.*, 65 (1965) 177.
14 H. Lingeman, H. A. Van Munster, J. H. Beynen, W. J. M. Underberg and A. Hulshoff, *J. Chromatogr.*, 352 (1986) 261.
15 R. S. Rapaka, *J. Chromatogr.*, 342 (1985) 293.
16 M. Kelly, M. R. Smyth and D. Dadgar, manuscript in preparation.

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LIQUID CHROMATOGRAPHIC SEPARATION OF ACIDIC PHOSPHOSERINE PEPTIDES ON MACROPOROUS COPOLY(STYRENE–DIVINYLBENZENE) USING AMINES TO REGULATE RETENTION

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SUMMARY

Using phosphoserine peptides as model substances, we have studied the separation of acidic peptides as substituted ammonium ion-pairs on a macroporous copoly(styrene–divinylbenzene) (PLRP-S) chromatographic column. Various hydrophobic amines were examined, of which long-chain N-methyl- and N,N-dimethyl-*n*-alkylamines proved to be the best with respect to selectivity and peak symmetry. Excellent separations were obtained in ammonium bicarbonate buffer (pH 7.5) with ethanol as organic modifier under isocratic conditions using the corresponding heptyl- to nonylamines.

INTRODUCTION

In recent years, reversed-phase high-performance liquid chromatography (HPLC), owing to its inherent sensitivity, resolution, speed, and general convenience, has become an invaluable tool in the analysis of natural as well as synthetic peptides[1–3]. Among refinements of this technique the application of various ionic or ionizable additives as counter-ions should be emphasized in this context. Additives to have been applied include carboxylates, perfluorinated carboxylates, sulphonates, sulphates and picrate, with a vast number of organic cationic analytes, including drugs and peptides, whereas mainly quaternary ammonium ions have been used for anionic substances[4–6].

Earlier it was shown that for the analysis of basic hydrophilic peptides, such as the arginine-containing serine peptides, prepared as substrates for cAMP-dependent protein kinase[7], hexanesulphonate could be applied as the counter-ion[8]. The peptides were separated on a C_{18} column in phosphate buffer (pH 3–4.5) with ethanol as modifier. Later it was demonstrated that the corresponding phosphoserine peptides could be resolved under the same conditions[9].

In a parallel fashion, attempts were made to resolve mixtures of acidic phosphopeptides on C_{18} microsilica columns. The idea was to apply amines of varying hydrophobicity to regulate the retention but, even under essentially neutral conditions (pH below 7.5), the columns had limited stability and lost their resolving capacity.

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With the advent of new stationary phases such as copoly(styrene–divinylbenzene), which are stable at a higher pH, new attempts were undertaken in this direction.

EXPERIMENTAL

Materials and solvents

To regulate the retention, amines and a quaternary ammonium salt were used. Hexylamine, octylamine, N-ethyldiisopropylamine, N,N-dimethylcyclohexylamine and tributylamine were obtained from Fluka (Buchs, Switzerland), N-methyloctylamine (MOA) and trimethylnonylammonium bromide from Eastman Kodak (Rochester, NY, U.S.A.), N,N-dimethylheptylamine, N,N-dimethylnonylamine and N-methylnonylamine from Pfaltz & Bauer (Waterbury, CT, U.S.A.) and N,N-dimethyloctylamine (DMOA) from ICN Biomedicals (Plainview, NY, U.S.A.). The quaternary ammonium bromide was converted into the corresponding hydroxide by means of an anion exchanger before use.

Isocratic mobile phases were used, consisting of binary aqueous solvents with ethanol or acetonitrile as organic modifier. The acetonitrile used was LiChrosolv from Merck-Schuchardt (Darmstadt, F.R.G.). The ethanol was of spectroscopic quality. The buffers were prepared from ammonium hydrogencarbonate, generally to an ionic strength of 0.1 *M*, and neutralized by carbon dioxide to the required pH. The mobile phases also contained the mentioned amines to serve as counter-ions.

Synthesis of phosphoserine peptides

The phosphopeptide Arg–Ala–Ser(P)–Val–Ala (**1**) was prepared and purified as already described[10]. Employing the earlier established experimental procedures[10], the tetrapeptide Ala–Ser(P)–Val–Ala (**2**) and the tripeptide Ser(P)–Val–Ala (**3**) were obtained in similar yields, as outlined previously[11]. The protected peptide intermediates were thoroughly characterized by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and amino acid analyses, and their high purity was confirmed with thin-layer chromatography (TLC). The final free phosphopeptides were examined with TLC, HPLC, and amino acid analyses, and exhibited satisfactory purity.

Instrumentation

Our liquid chromatographic system consisted of a Model 6000 A solvent-delivery device and a Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The detector head was carefully isolated in order to reduce any influence on the baseline stability at high sensitivity due to variations in the ambient temperature. The flow-cell had a volume of 8 μl. The detection wavelength was 210 nm. A laboratory-made block-heater was used and thermostatted at 30.0 $\pm$ 0.1°C. A Model 7125 sample-loading injector was obtained from Rheodyne (Cotati, CA, U.S.A.).

The separation column (150 $\times$ 4.6 mm I.D.) was obtained from Polymer Labs. (Shropshire, U.K.). It contained a polymeric material, macroporous copoly(styrene–divinylbenzene), PLRP-S, 100 Å, 5 μm.

Procedure

The column was carefully conditioned by pumping the mobile phase through the

system at a flow-rate of 1 ml/min. for 6 h, when the retention times had generally stabilized. In the studies on the effect of the amine concentration, the experiments were conducted in the order from lower to higher concentrations, because otherwise the results would be poorly reproducible. Before changing from one amine to another, the column was carefully washed with 0.1 *M* phosphate buffer (pH 3.0) and distilled water.

Standard chromatographic conditions

Support, PLRP-S, 5 μm; column, 150 × 4.6 mm I.D.; mobile phase, 0.1 *M* ammonium hydrogencarbonate (pH 7.50)–modifier (93:7); flow-rate, 1.0 ml/min; amine, as indicated (otherwise 0.010 *M*); temperature, 30.0 ± 0.1°C; detection wavelength, 210 nm. The three phosphopeptides are indicated in the figures as follows: (■) Arg–Ala–Ser(P)–Val–Ala (**1**); (●) Ala–Ser(P)–Val–Ala (**2**); and (×) Ser(P)–Val–Ala (**3**).

RESULTS AND DISCUSSION

Although various chromatographic systems containing trifluoroacetic acid (TFA) have been successfully used for the separation of many peptides on reversed-phase C_{18} microsilica columns[12], preliminary experiments with peptides **2** and **3** indicated that even in the absence of an organic modifier, poor retention was obtained (capacity factor, $k' < 2$). This led us to consider the possibilities for chromatographing our peptides as anions with cationic counter-ions (amines and quaternary ammonium salts), but these experiments were hampered by the poor stability of this column material. Under the conditions used, at pH 7.0 the resolving power of our columns already deteriorated at an unacceptable rate. In this work we therefore exclusively used copoly(styrene–divinylbenzene) as the support, because it is stable over a much wider pH range and various improved types of this material have been used successfully in recent years for the separation of peptides[13,14]. In the presence of 0.1% TFA, peptides 1–3 passed through the column without any retardation, even in the absence of an organic modifier.

With this new type of column, a number of hydrophobic (C_6–C_{12}) amines were screened for their usefulness in the separation of acidic hydrophilic peptides, using three phosphopeptides as model substances. A preliminary study of several different types of amine was made at this stage (Table I), but this list is by no means complete. Nevertheless, as a result of this screening, two apparently good candidates for further study appeared. With the majority of the amines tested, however, neither satisfactory selectivity nor the required peak symmetry could be obtained. This was the case with the two primary amines studied, hexylamine and octylamine. On the other hand, with N-methyloctylamine the results were dramatically improved, and N,N-dimethyl-octylamine proved equally useful. Three other tertiary amines tested in this context did not seem promising. An attempt has been made to summarize the findings in qualitative terms in Table I.

At this stage it was decided to study the influence of N,N-dimethyloctylamine and N-methyloctylamine on the separation of the three model peptides mentioned above in more detail. In this context the effect of pH as well as of ionic strength on k' also had to be investigated, and this was done with the former of these two amines. These results are presented in Fig. 1. Considering the pK_a values of the various groups

TABLE I

SCREENING OF AMINES FOR THEIR USEFULNESS IN THE SEPARATION OF THREE ACIDIC HYDROPHILIC PHOSPHOSERINE PEPTIDES

The code used to grade retardation, selectivity, and peak symmetry: – – (poor); – (less satisfactory); + (satisfactory); + + (good to excellent). For further details, see Standard chromatographic conditions, at the end of the Experimental section.

Amine	*Retardation*	*Selectivity*	*Symmetry*
Hexylamine	+	– –	–
Octylamine	+	– –	+
N-Methyloctylamine	+ +	+ +	+ +
N-Ethyldiisopropylamine	–	– –	– –
N,N-Dimethylcyclohexylamine	–	– –	– –
Tributylamine	–	+	– –
N,N-Dimethyloctylamine	+ +	+ +	+ +
Trimethylnonylammonium (hydroxide)	+ +	– –	+ +

involved[15], the pH 7–8 range was studied. As can be seen from Fig. 1a, k' has a maximum at *ca.* pH 7.5 for the model peptides, but appears to be quite high throughout the pH interval. (It should be noted that this applies to the resolution as well.) On the basis of this result, we chose pH 7.5 as a standard value in subsequent experiments. Correspondingly, in these experiments the ionic strength was kept at 0.1 *M*. As can be seen from Fig. 1b, k' is only modestly influenced by a change above this value, whereas the retention is satisfactory and the resolution quite high.

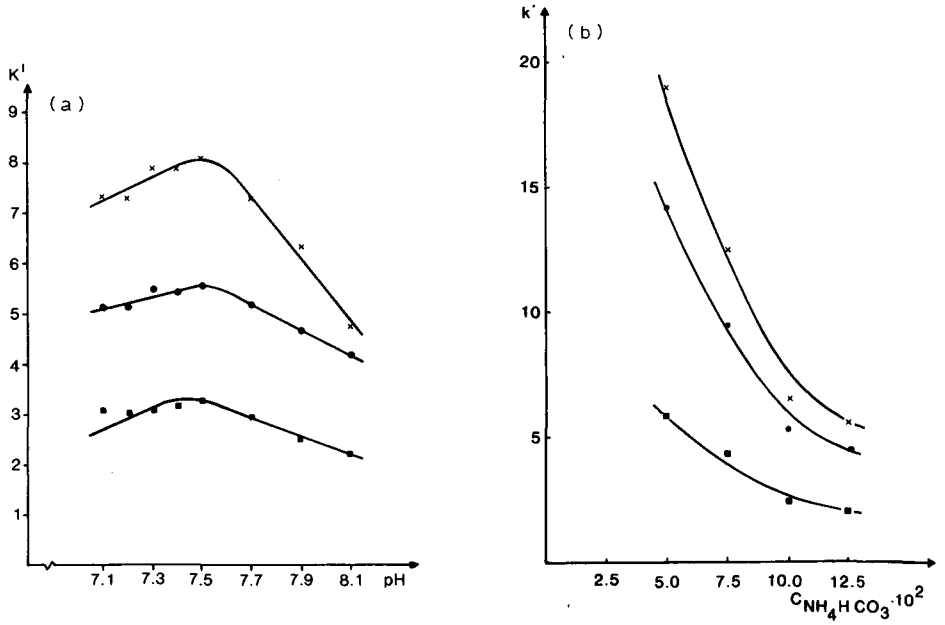

Fig. 1. The influence of (a) pH and (b) ionic strength on k' for peptides **1–3** in the presence of N,N-dimethyloctylamine and NH_4HCO_3 (in the latter, the pH used was 7.60). For further details, see Standard chromatographic conditions, at the end of the Experimental section.

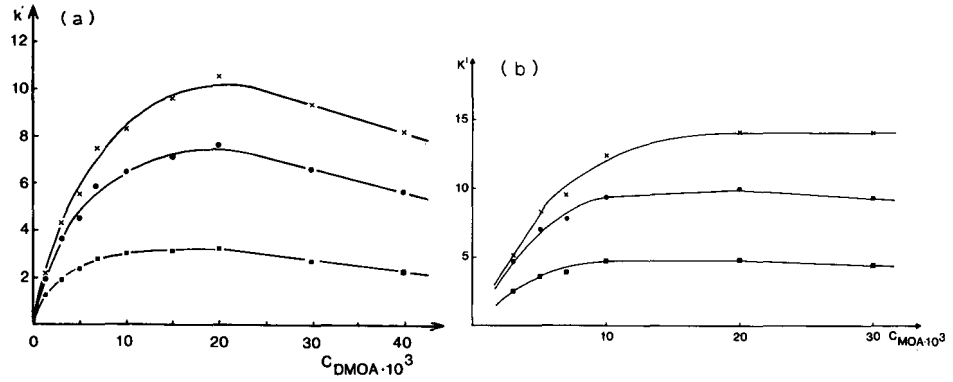

Fig. 2. Effect of the concentrations of (a) N,N-dimethyloctylamine and (b) N-methyloctylamine on k' for peptides **1–3**. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

The effect of the concentration of N,N-dimethyloctylamine and N-methyloctylamine on k' is presented in Fig. 2. For both amines, k' for all the model peptides first increases sharply on the addition of amine, reaching its half-maximum value at *ca.* 4 mM, to exhibit more or less pronounced maxima at *ca.* 20 mM. The resolving capacity is satisfactory (for α values at $C_{amine} = 0.010$ M, see Table II), whereas the column efficiency is more modest (Table II) as in all other cases in which this support was used. It should be noted in this context that N-methyloctylamine consistently gives rise to higher k' values than does N,N-dimethyloctylamine (Table II). A typical chromatogram of the three model peptides using N,N-dimethyloctylamine is shown in Fig. 3. The first peak in the chromatogram refers to the pentapeptide, which has one negative charge less than the tetra- and tripeptides, and therefore quite likely binds one fewer molecule of hydrophobic amine. The tripeptide is obviously the most hydrophobic of the three phosphopeptides studied.

In Table II, the results of similar experiments with three additional secondary and tertiary heptyl- and nonylamines are presented, together with data on the two octylamines discussed above. As expected, within each group k' increases with the chain length of the amine. As previously shown for the two octylamines, N-methyl-

TABLE II

EFFECT OF THE AMINES ON THE RETENTION OF THREE ACIDIC HYDROPHILIC PHOSPHOSERINE PEPTIDES

For further details, see Standard chromatographic conditions, at the end of the Experimental section.

Amine	k'_1	H_1	k'_2	α_2	H_2	k'_3	α_3	H_3
N,N-Dimethylheptyl	–	–	1.91	–	0.15	2.21	–	0.14
N,N-Dimethyloctyl	3.27	0.15	5.59	1.71	0.14	8.09	2.47	0.11
N,N-Dimethylnonyl	3.44	0.17	7.67	2.23	0.13	11.21	3.26	0.10
N-Methyloctyl	4.74	0.15	9.37	1.98	0.13	12.53	2.64	0.10
N-Methylnonyl	5.82	0.15	12.40	2.13	0.13	19.15	3.29	0.13

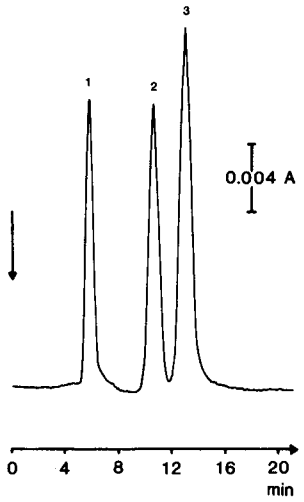

Fig. 3. Separation of three model peptides on a PLRP-S column using N,N-dimethyloctylamine. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

nonylamine gives rise to higher k' values than does N,N-dimethylnonylamine. With respect to the selectivity, the two nonylamines mentioned rank highest, but the octylamines are nearly as good. The column efficiency has approximately the same value for all amines studied, and is rather low in comparison with that of a microsilica column. For any given amine it improves with the k' value.

Table III contains some additional data on N,N-dimethylnonylamine, obtained using two different organic modifiers, acetonitrile and ethanol. As can be seen, the retention order of the three phosphopeptides is identical with both modifiers. Contrary to our previous experience of chromatography of peptides, these phosphopeptides

TABLE III

SUMMARY OF DATA ON THE SEPARATION OF THREE PHOSPHOSERINE PEPTIDES USING N,N-DIMETHYLNONYLAMINE WITH ACETONITRILE AND ETHANOL AS ORGANIC MODIFIERS

Peptide	*Acetonitrile*			*Ethanol*		
	k'	α	H	k'	α	H
Arg–Ala–Ser(P)–Val–Ala[a]	3.43	1	0.15	2.88	1	0.16
Ala–Ser(P)–Val–Ala[a]	7.30	2.13	0.12	6.00	2.08	0.13
Ser(P)–Val–Ala[a]	9.98	2.91	0.09	8.08	2.81	0.10
Arg–Ala–Ser(P)–Val–Ala[b]	3.92	1	0.16	3.44	1	0.17
Ala–Ser(P)–Val–Ala[b]	9.08	2.31	0.12	7.67	2.23	0.13
Ser(P)–Val–Ala[b]	12.80	3.27	0.08	11.21	3.26	0.10

[a] Concentration of N,N-dimethylnonylamine 0.005 *M*.

[b] Concentration of N,N-dimethylnonylamine 0.010 *M*. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

gave higher k' values with acetonitrile than with ethanol. The selectivity appears to be approximately the same for both, whereas the column efficiency is marginally higher for acetonitrile. The amount of modifier was the same, 7%, as in all previous experiments. Because of the high k' values, no further experiments were conducted with a higher concentration of the amine.

Amines have previously been applied as modifiers only in conjunction with microsilica columns[16]. Even after careful capping of their silanol groups, the residual ones can have unfavourable effects on the symmetry of the peaks. The peak symmetry could, however, be improved by minute amounts of a hydrophobic amine or quaternary ammonium salt. Compounds of the latter type were recently used in one case for the separation of amino acids[17]. Up to now, however, amines have not been exploited for the regulation of the retention of anionic peptides, and their widely variable selectivities in this context have also gone unnoticed.

The column has been in regular use for nearly a year without any loss of resolution. It made possible the convenient separation of peptides, otherwise not easily separated, by application of protonated lipophilic amines as counter-ions at a pH not compatible with standard microsilica columns.

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REFERENCES

1 W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, Vols. I and II, CRC Press, Boca Raton, FL, 1984.
2 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190–212.
3 A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Editors), *High Performance Liquid Chromatography in Biochemistry*, VCH, Weinheim, 1985.
4 G. Schill and K.-G. Wahlund, *NBS Spec. Publ. (U.S.)* 519 (1979) 509–523.
5 T. Jira, T. Beyrich and E. Lemke, *Pharmazie*, 39 (1984) 141–149.
6 R. K. Gilpin, S. S. Yang and G. Werner, *J. Chromatogr. Sci.*, 26 (1988) 388–400.
7 Ö. Zetterqvist, U. Ragnarsson, E. Humble, L. Berglund and L. Engström, *Biochem. Biophys. Res. Commun.*, 70 (1976) 696–703.
8 B. Fransson, U. Ragnarsson and Ö. Zetterqvist, *J. Chromatogr.*, 240 (1982) 165–171.
9 B. Fransson, U. Ragnarsson and Ö. Zetterqvist, *Anal. Biochem.*, 126 (1982) 174–178.
10 L. Grehn, B. Fransson and U. Ragnarsson, *J. Chem. Soc. Perkin Trans. I*, (1987) 529–535.
11 L. Grehn, B. Fransson, U. Ragnarsson and Ö. Zetterqvist, in D. Theodoropoulos (Editor), *Peptides 1986, Proc. 19th European Peptide Symposium*, de Gruyter, Berlin, 1987, pp. 235–238.
12 H. P. J. Bennet, C. A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353–1365.
13 E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50 (1978) 502–511.
14 Z. Iskandarani, R. L. Smith and D. J. Pietrzyk, *J. Liq. Chromatogr.*, 7 (1984) 111–150.
15 A. W. Frank, *CRC Crit. Rev. Biochem.*, 16 (1984) 51–101.
16 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299–316.
17 P. G. Rigas, S. J. Arvanitis and D. J. Pietrzyk, *J. Liq. Chromatogr.*, 10 (1987) 2891–2910.

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MICROPREPARATIVE SEPARATION OF PEPTIDES DERIVED FROM SODIUM DODECYL SULPHATE-SOLUBILIZED PROTEINS

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SUMMARY

A systematic investigation of the influence of the detergent sodium dodecyl sulphate (SDS) on micropreparative peptide separations on microbore reversed-phase high-performance liquid chromatographic columns is reported. A tryptic digest of bovine serum albumin and a mixture of synthetic peptides were used to monitor the separation behaviour of a 1.6 mm I.D. Nucleosil C_{18} column in the presence of various amounts of SDS. The data demonstrate that even traces of SDS in the sample reduce the separation efficiency and peptide recovery. An extraction method is presented which reduces the SDS content in peptide mixtures below the critical concentration without affecting significantly the recovery of individual peptides. After acidification of the sample, the detergent is extracted into heptane–isoamyl alcohol (4:1, v/v). In combination with chemical or enzymatic fragmentation techniques, this extraction method facilitates the sequence analysis of minute amounts of SDS-solubilized hydrophobic proteins. The applicability of the method is demonstrated on the example of the integral membrane protein bacteriorhodopsin.

INTRODUCTION

Technological advances over the past few years now permit the sequence analysis of proteins and peptides to be performed routinely at low picomole levels. However, the micropreparative isolation of microgram amounts of proteins and peptides with suitable purity for microsequence analysis appears to be a persistent problem. In particular, large hydrophobic proteins, *e.g.*, integral membrane proteins and membrane-associated proteins, pose difficulties[1]. Hydrophobic proteins require the presence of detergents such as sodium dodecyl sulphate (SDS) during the purification procedure. SDS is also required to solubilize Coomassie blue-stained and fixed proteins before electroelution from the gel[2].

Several methods for the removal of SDS from protein preparations have been published which use inverse gradients on reversed-phase columns[3], methanol–chloroform precipitation[4] or ion-pair extraction[5]. Although these methods work effectively even with minute amounts of soluble proteins, they often cannot be applied to hydrophobic proteins. After efficient removal of SDS, many hydrophobic proteins

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become insoluble in aqueous buffers and form precipitates which are inaccessible to endoproteases. The presence of small amounts of SDS is therefore required in order to keep the proteins in solution and to ensure efficient enzymatic fragmentation[6]. Accordingly, the enzymatic digests derived from these proteins and also proteins that were digested within SDS gels[7] contain various amounts of detergent. However, the data presented in this paper demonstrate that the SDS concentration in the sample is a critical parameter affecting the separation drastically when microbore reversed-phase high-performance liquid chromatography (HPLC) is used for peptide purification. Therefore, an efficient extraction procedure for removal of SDS from complex peptide mixtures was developed, which is generally applicable to the micropreparative isolation of peptides derived from SDS-solubilized proteins.

EXPERIMENTAL

High-performance liquid chromatography

Separation of the peptides was performed using a Hewlett-Packard 1090 liquid chromatograph equipped with a Kontron 430 UV detector. The peptides were detected at 215 nm. The column (250 × 1.6 mm I.D.) was Nucleosil C_{18} (Macherey, Nagel & Co.) packed by MZ-Analysentechnik (Mainz, F.R.G.). The synthetic peptides were separated at a flow-rate of 100 μl/min using a 20-min linear gradient from 5 to 50% B followed by a 10-min isocratic elution at 50% B; buffer A was 0.1% trifluoroacetic acid (TFA) in water and buffer B was acetonitrile containing 0.07% TFA. The tryptic peptides obtained from bovine serum albumin (BSA) were separated under the same conditions, but using a 60-min linear gradient from 0 to 60% B. All gradient separations of SDS containing samples were followed by a 10-min isocratic elution at 80% B. A 90-min linear gradient from 10 to 90% B was used for separation of the peptides derived from tryptic digestion of bacteriorhodopsin.

Trypsin digestion

BSA (Serva, Heidelberg, F.R.G.) was digestd in 100 m*M* ammonium hydrogencarbonate for 6 h at 37°C. The protein concentration was 2 μ*M* and the enzyme/substrate ratio was 1:5 (w/w). Bacteriorhodopsin (Serva) was solubilized by boiling in 1% SDS and was diluted with 100 m*M* ammonium hydrogencarbonate to yield a 2 μ*M* protein solution containing 0.08% (w/v) SDS. Digestion of 200-μl aliquots of this solution was performed for 12 h at 37°C and the enzyme/substrate ratio was 1:4 (w/w). Trypsin (sequencing grade) was obtained from Boehringer (Mannheim, F.R.G.).

SDS extraction

The extraction was performed on 100-μl samples containing 200 pmol of peptide mixture in 0.1% TFA–water and up to 0.05% (w/v) SDS. The sample was acidified with 5 μl of TFA and extracted with 100 μl of heptane–isoamyl alcohol (4:1, v/v) by briefly vortexing. After centrifugation in an Eppendorf centrifuge, the heptane phase was removed with a pipette and residual heptane was evaporated in a gentle stream of nitrogen. Samples containing more than 0.05% of SDS were extracted twice.

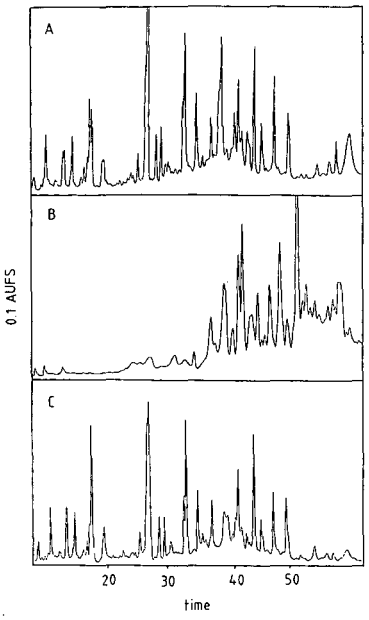

Fig. 1. High-performance liquid chromatography of tryptic peptides derived from bovine serum albumin. The peptides (200 pmol) were separated on a 250 × 1.6 mm I.D. Nucleosil C_{18} column using a 60-min linear gradient from 0 to 60% B at a flow-rate of 100 μl/min. Sample A contained no SDS, samples B and C contained 50 μg of SDS. Before injection, sample C was extracted with heptane–isoamyl alcohol (4:1, v/v). Time in min.

RESULTS AND DISCUSSION

Fig. 1A shows the elution profile of a tryptic digest of 200 pmol of BSA. The peptides were separated on the Nucleosil C_{18} microbore column. The profile in Fig. 1B shows the same separation under identical chromatographic conditions, but with 50 μg (0.05%) of SDS in the sample. In the presence of the detergent the peptides were shifted to higher retention volumes and the number of separated peaks was markedly reduced.

TABLE I

AMINO ACID SEQUENCES OF THE SYNTHETIC PEPTIDES USED IN THE SEPARATION EXPERIMENTS

Peptide	*Sequence*
	1 5 10
1	Lys-Val-Glu-Gly-Glu-Glu-Gly-Glu-Glu-Glu-Gly-Glu
	1 5 10
2	Lys-Cys-Arg-Asn-Arg-Arg-Arg-Glu-Leu-Thr-Asp-Thr-Leu-Gln
	1 5 10
3	Pro-Thr-Val-Thr-Ala-Ile-Ser-Thr-Ser-Pro-Asp-Leu-Gln
	1 5 10
4	Pro-Ala-Cys-Lys-Ile-Pro-Asp-Asp-Leu-Gly-Glu-Phe-Pro-Glu
	1 5 10
5	Ser-Glu-Glu-Pro-Ile-Tyr-Ile-Val-Thr-Glu-Tyr-Met-Ser-Lys-Gly-Ser-Leu

The injection of 100 μg (0.1%) of SDS led to the complete breakdown of the separation and the peptides eluted as a single broad peak at 50–60% acetonitrile (data not shown).

To investigate the nature of the separation mechanism in the presence of various SDS concentrations, the elution behaviour of five synthetic peptides of similar size and of graded polarity (Table I) was monitored. The separations were performed under identical chromatographic conditions, but with the addition of increasing amounts of SDS (1–50 μg) to the sample. The results are shown in Fig. 2. In the absence of SDS, the most polar peptide (peptide 1) eluted first and peptide 5, containing the highest number of apolar residues, eluted last from the reversed-phase column. In Fig. 2b–e the effect of increasing concentrations of SDS in the sample is demonstrated. Whereas the retention time and the peak area of peptide 3 remained stable up to 50 μg (0.05%) of SDS, all other peptides were affected. The peaks were shifted to longer retention times and the peak areas were reduced. Even at 5 μg (0.005%) of SDS the recovery of the basic peptide 2 was reduced to 10% and at 50 μg (0.05%) of SDS the peptide failed to elute from the column. The effect of SDS on the retention volumes of the peptides can therefore be related to the number of Lys and Arg residues present in the individual sequences (Table I). This indicates an increasing interaction of the positively charged residues with the separation matrix due either to the binding of the anionic detergent to the peptide, thus rendering them more hydrophobic, or to the binding of SDS to the reversed-phase matrix. The latter would convert the hydrophobic C_{18} phase into a negatively charged cation-exchange matrix. Therefore, as the amount of SDS applied to the column increases, the ion-exchange mechanism becomes predominant and, owing to the low ionic strength of the elution buffer, the basic peptides are retarded.

The fact that the detergent binds to the column and not to the peptides is illustrated in Fig. 3. In this experiment the detergent was applied separately to the column 5 min (B) and 15 min (C) before injecting the peptide mixture. The change in the elution profile was almost identical with the profile obtained with simultaneous

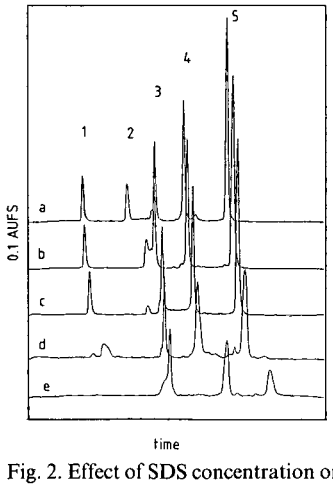

Fig. 2. Effect of SDS concentration on gradient elution of a mixture of five synthetic peptides. The peptides were separated on a 250 × 1.6 mm I.D. Nucleosil C_{18} column using a 20-min linear gradient from 0 to 50% B followed by a 10-min isocratic elution at 50% B. The flow-rate was 100 μl/min. The peptides are designated according to their numbering in Table I. In each experiment 200 pmol of peptide mixture were injected in 100 μl of 0.1% TFA. SDS content in the sample: (a) 0; (b) 1; (c) 5; (d) 20; (e) 50 μg.

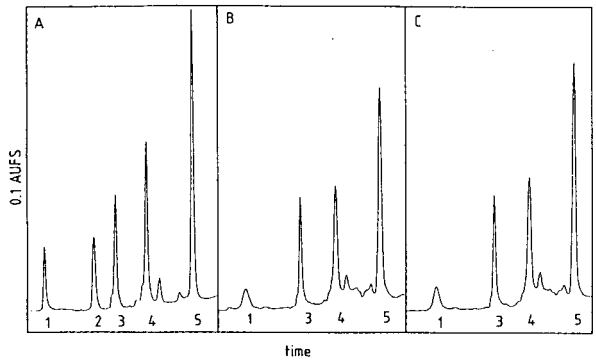

Fig. 3. Effect of SDS injection on gradient elution of peptides. In all instances (A–C) the peptides were injected in 100 ml of 0.1% TFA and eluted under the chromatographic conditions given in Fig. 2. In B and C the column was loaded with 20 mg of SDS and washed for (B) 5 min or (C) 15 min with starting buffer before sample injection.

injection of peptide and detergent. Owing to the strong binding of the detergent even after a 15-min wash with buffer A, no change in the retention behaviour was observed. To restore the separation, the detergent had to be eluted from the column with 80% acetonitrile.

From these results, it was obvious that even traces of SDS have to be removed from peptide mixtures before applying them to reversed-phase columns. As none of the cited methods for removal of SDS from proteins gave satisfactory results with peptide mixtures, we tried an extraction method which was proposed by Heukeshoven[8]. In this

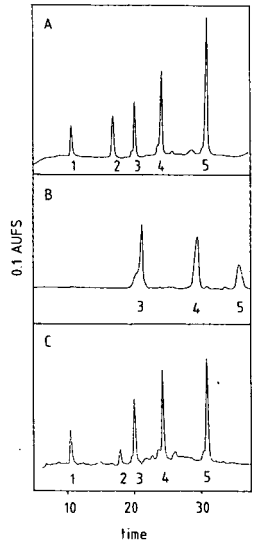

Fig. 4. Efficiency of removal of SDS from a peptide mixture (200 pmol in 100 μl of 0.1% TFA) by extraction with heptane–isoamyl alcohol (4:1, v/v). The chromatographic conditions were the same as in Fig. 2. The peptide numbering corresponds to the numbering in Table I. The peptides were separated (A) without SDS, (B) after addition of 50 μg SDS and (C) after a single extraction of B with heptane–isoamyl alcohol (4:1, v/v). Time in min.

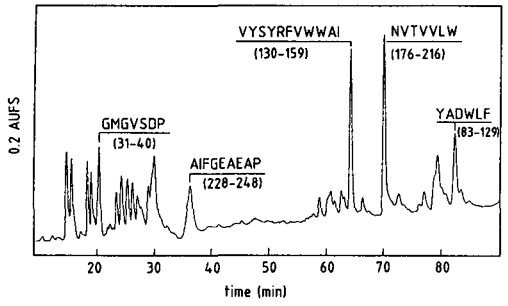

Fig. 5. Separation of tryptic peptides derived from SDS-solubilized bacteriorhodopsin (500 pmol). The protein was digested as described under Experimental and the fragments were extracted with heptane–isoamyl alcohol (4:1, v/v). The separation was performed on a 250 × 1.6 mm I.D. Nucleosil C_{18} column using a linear gradient from 0 to 90% B at a flow-rate of 100 µl/min. The amino acid sequence data obtained from individual peaks are given as one-letter codes on top of the peaks. The numbers in parentheses give the positions of the corresponding peptides in the sequence of the mature protein.

method, SDS is extracted into heptane–isoamyl alcohol (4:1, v/v) after acidification of the sample with TFA. The efficiency of the method is illustrated in Fig. 4. After a single extraction of the peptide mixture containing 50 µg of SDS per 100 µl, the elution profile which was obtained from the SDS-free sample (A) was almost completely restored (C).

As determined by the methylene blue method[9], the SDS concentration was below 0.001%. The recovery of peptides 1, 3 and 4 was near 100%. Peptide 3 was still slightly retarded and only peptide 5 was recovered with reduced yield (90%) owing to extraction losses. A similar result was obtained when a tryptic digest of 200 pmol of BSA containing 50 µg of SDS (Fig. 1B) was extracted. After extraction, the elution pattern of the SDS-free sample was restored and the recovery of only a few peptides was reduced (Fig. 1C).

The applicability of the method to hydrophobic membrane proteins is demonstrated in Fig. 5, which shows the micropreparative separation of tryptic peptides obtained from 500 pmol of SDS-solubilized bacteriorhodopsin. The protein was digested in the presence of 0.1% of SDS and the digest was extracted twice with heptane–isoamyl alcohol (4:1, v/v). Corresponding to the distribution of the lysine and arginine residues in the primary structure of bacteriorhodopsin[10], several small hydrophilic peptides (eluting at 30–45% B) and a number of large hydrophobic fragments (eluting at 75–90% B) were obtained. The identities of some of the peptides were established by microsequence analysis in a gas-phase sequencer[11]. The sequences are given in Fig. 5.

In conclusion, the data presented demonstrate the extreme sensitivity of microbore reversed-phase HPLC columns to the presence of SDS in the sample. The detergent binds to the column and converts the reversed-phase matrix into a cation-exchange matrix. The separation capacity of the column and the peptide recovery are reduced. Therefore, control of the amount of detergent present in the sample is important for efficient micropreparative isolation of peptides. As many proteases, such as trypsin and V8-protease, are not inhibited in the presence of up to 0.1% of SDS, the proposed extraction method allows the enzymatic fragmentation of SDS-solubilized proteins to be performed without prior removal of the detergent. The

extraction reduces the amount of SDS in the resulting peptide mixture to below the concentration critical for micropreparative HPLC, without affecting the recovery of peptides. This strategy significantly improves the microsequence analysis of hydrophobic membrane proteins.

REFERENCES

1 P. Tempst, D. D.-L. Woo, D. B. Teplow, R. Aebersold, L. E. Hood and S. B. H. Kent, *J. Chromatogr.*, 359 (1986) 403.
2 M. W. Hunkapiller, E. Lujan, F. Ostrander and L. E. Hood, *Methods Enzymol.*, 91 (1983) 227.
3 R. I. Simpson, R. L. Moritz, E. C. Nice and B. Grego, *Eur. J. Biochem.*, 165 (1987) 21.
4 D. Wessel and U. I. Flügge, *Anal. Biochem.*, 138 (1984) 141.
5 W. H. Konigsberg and L. Henderson, *Methods Enzymol.*, 91 (1983) 254.
6 H. Suzuki and T. Terada, *Anal. Biochem.*, 172 (1988) 259.
7 U. M. Benedum, P. A. Baeuerle, D. S. Konecki, R. Frank, J. Powell, J. Mallet and W. B. Huttner, *EMBO J.*, 5 (1986) 1495.
8 J. Heukeshoven, paper presented at the *6th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baden-Baden, October 20–22, 1986.*
9 K. Hyashi, *Anal. Biochem.*, 67 (1975) 503.
10 H. G. Khorana, E. G. Gerber, W. C. Herlihy, C. P. Gray, R. J. Anderegg, K. Nihei and K. Biemann, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5046.
11 H. Gausepohl, M. Trosin and R. Frank, in B. Wittmann-Liebold (Editor), *Advanced Methods in Protein Microsequence Analysis*, Springer, Berlin Heidelberg, p. 150.

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NON-EXTRACTABLE STATIONARY PHASES FOR GAS CHROMATOGRAPHY CROSS-LINKED BY EXPOSURE TO LOW-TEMPERATURE PLASMAS

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SUMMARY

Non-polar and medium-polarity stationary phases, such as SE-30, SE-52 and SE-54, can be completely cross-linked by exposure to low-temperature plasmas. Plasma-exposed polymers on glass coverslips resist solvent dissolution and were judged fully cross-linked. SE-30 coated on glass beads was also rendered insoluble by plasma treatment. Columns packed with treated beads show no reduction in chromatographic performance. A simple method for generating plasmas within evacuated capillaries is presented. Tests made with *in situ* cross-linked capillary columns demonstrate plasma exposure does not affect retention characteristics. The advantages of plasmas are described, and additional applications are proposed.

INTRODUCTION

In recent years, chromatographers have cross-linked polymer stationary phases to improve the performance of capillary gas chromatography (GC) columns. Only a few cross-linkages on each polymer chain are required to greatly increase the average molecular weight and render the material insoluble, thus giving rise to the term "non-extractable phase".

For capillary GC applications, cross-linked stationary phases provide several benefits[1]. First, these phases withstand large solvent injections. A second benefit is the freedom to wash columns with solvent to remove non-volatile sample components. Resistance to dissolution has also facilitated the development of capillary supercritical fluid chromatography and open-tubular liquid chromatography with partition retention mechanisms. A third feature of cross-linking is enhanced film stability. This is particularly important for polar polysiloxane phases, which are often subject to film disruption at elevated temperatures due to reductions in phases viscosity[2].

A variety of cross-linking techniques have been reported in the chromatographic literature. Siloxane polymers have received the most attention, though other phase classes have also been cross-linked. Two distinctly different types of cross-linking may be considered: with linkages formed either through the linear backbone of the polymer or through substituent groups.

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Polysiloxane stationary phases are usually linear. *In situ* concatenation of a partially polymerized siloxane results in highly stable coatings[3]. Blomberg *et al.*[4] prepared cross-linked phases by adding tri- or tetrafunctional silanes during the polymerization step to form cross-linked phases. Both approaches invariably suffered from increased stationary phase activity due to the presence of uncapped functional sites.

The application of free radical induced cross-linking to GC stationary phases[1] was a major improvement over earlier techniques. This approach, directly adapted from basic silicone chemistry developed in the fifties, creates cross-linkages through substituent groups, leaving the polymer backbone intact. Because polymers that are not based on the siloxanes can have these same substituents, free radical cross-linking has been applied to a broad range of stationary phases including polyethylene glycols[5]. In addition, this method can be performed *in situ* after the phase has been deposited on the column wall.

Several different initiators have been successfully used to stimulate the cross-linking chain reaction. Initial experiments were with organic peroxides; however, active by-products were incorporated into the stationary phase layer. Another drawback involved the elevated temperatures required to cause free radical formation. When heated, those phases exhibiting only marginal physical stability may coalesce into droplets, thus drastically reducing column efficiency. Azo compounds seem to be preferred over peroxides due to their lower decomposition temperatures and relatively clean decomposition products[6]. Ozone has also been shown to cause *in situ* cross-linking of certain phases at room temperature[7].

With each of these chemical cross-linking agents, the incorporation of residual groups into the polymer structure can cause residual activity. A second drawback is that chemical initiators, except for ozone which reacts spontaneously at room temperature, must be heated before cross-linking occurs. Even though the temperatures used to stimulate cross-linking are usually well below those encountered during separations, it may be preferable to avoid heating some polar phases before their mechanical stability has been augmented by cross-linking.

Forming free radicals directly in the stationary phase by irradiation with gamma rays has also been reported[8], but columns prepared in this manner still exhibit undesirable adsorption of polar solutes. Other problems with irradiative cross-linking include damage to the outer polyimide coating and the general inaccessibility of irradiation facilities.

Cross-linking procedures can be judged on the following criteria: (1) cross-linking should not degrade column performance as measured by selectivity and efficiency; (2) adsorptive activity should not be increased by the cross-linking procedure; and (3) cross-linking procedures should be applicable across a broad spectrum of stationary phases. At present, the phases that can be cross-linked are somewhat limited. Commercial high-polarity polysiloxanes remain resistant to existing procedures. Although part of this problem is the unavailability of highly polar gum phases, the presence of functional groups certainly affects cross-linking[9,10].

In this work, a new agent for initiating cross-linking in GC stationary phases is described: the low-temperature plasma. The ability of low-temperature plasma to cross-link polymers is well documented[11]. Low-temperature plasmas are produced when a low-pressure gas (generally *ca.* 100 Pa) is excited by strong electric fields. The

electrons in such plasmas are not in thermal equilibrium with the molecular and atomic species, with the gas temperature typically one or two orders of magnitude less than the electron temperature[12]. Energetic electrons can thus stimulate chemical reactions by transferring energy to gas molecules and forming ions, free radicals, metastable species, and atoms while the gas remains near ambient temperature. These active species then react with polymers in their vicinity.

For chromatographic purposes, plasma cross-linking presents several potential advantages. First, low-temperature plasmas are generated in a controlled, low-pressure atmosphere, therefore contamination from extraneous species should be minimal. Next, noble gases, the most common sustaining medium, are unlikely to become chemically attached to the polymer. The reactive noble-gas species serve only to initiate the free radical cross-linking chain reactions. Since the same noble gas atom can be repeatedly energized in the plasma, free radicals are created as long as external energy is supplied. Also, values for gas pressure, composition, plasma excitation energy, and exposure time can all be optimized, if necessary, for each stationary phase. Finally, plasma cross-linking should permit external energy to be selectively coupled to the gaseous plasma species, thus avoiding indiscriminate heating throughout the entire column.

The chromatographic applications of plasmas are by no means limited to cross-linking. At least three brief reports have described plasmas being used to prepare chromatographic columns. Masada *et al.*[13] not only showed scanning electron micrographs of glass capillaries etched with plasmas of organo-fluorine compounds, but also claimed to have plasma-polymerized dimethyldichlorosilane on the column wall, though no chromatograms were produced. Such films are probably inferior to conventional polysiloxanes for separating organic solutes, as studies of plasma-polymerized siloxane monomers have shown the resulting polymers to exhibit a marked inorganic character[14]. Two other groups[15,16] have treated column packing materials with plasmas to improve performance.

A preliminary demonstration of stationary-phase cross-linking by means of exposure to a low temperature plasma is reported here. Several other novel uses of plasmas will also be discussed as they apply to fused-silica capillary columns.

EXPERIMENTAL

The effectiveness of low-temperature plasmas for cross-linking GC stationary phases was initially evaluated using glass microscope coverslips coated with a thin film of stationary phase. The coverslips were first cleaned in a boiling solution of dilute hydrochloric acid. After thorough washing with deionized water, the plates were dried in air overnight at 110°C. Stationary phase was applied to tared microscope coverslips as a dilute solution that was allowed to dry. The amount of phase deposited was determined gravimetrically. This system resembles the thin film of stationary-phase polymer on the walls of a capillary GC column, though the layer was appreciably thicker.

Coverslips coated with polydimethylsiloxane (SE-30, GC grade, Alltech Assoc., Deerfield, IL, U.S.A.) were placed in a low-temperature argon plasma (PDC-23G Plasma Cleaner, Harrick Scientific, Ossining, NY, U.S.A.). Exposure times and argon pressures were varied systematically. Immediately following exposure, the cov-

erslips were reweighed to determine if the plasma had removed any polymer. The extent of cross-linking was taken to be the fraction of the original polymer weight remaining after a 1-h immersion in a gently rocked bath of dichloromethane.

Tests were carried out at 130 Pa of argon to determine if other stationary phases (Alltech) could be cross-linked. A series of polysiloxanes consisting of SE-30 (100% methyl), SE-52 (5% phenyl), OV-61 (33% phenyl), OV-17 (50% phenyl), and OV-25 (75% phenyl) were exposed to plasmas. SE-54 (1% vinyl, 5% phenyl) was also tested.

Next, a stationary phase coated on diatomaceous earth was cross-linked. Gas-Chrom P, 80–100 mesh, coated with 10% polydimethylsiloxane (OV-101, Applied Science Labs., State College, PA, U.S.A.) was placed in a shallow boat and exposed to a 130-Pa argon plasma within the generator chamber. After each successive 1-min interval, the material was thoroughly stirred and a small portion removed for gravimetric evaluation of cross-linking following a 1-h solvent extraction with dichloromethane. A sample exposed for a total of 1 h was then evaluated chromatographically in a well-packed, 1.85 m × 0.216 cm stainless-steel column. Retention and efficiency for the plasma exposed material were compared with an identical column packed with unexposed material.

Based on the results with conventional packing material, similar tests were performed with coated glass beads (*ca.* 0.10–0.11 mm diameter, Sargent-Welch, Springfield, NJ, U.S.A.). These beads supported a loading of 0.5 w/w% SE-30. Assuming uniform coverage, this corresponds to a coating thickness of 0.2 μm. Plasma exposure with 130 Pa of argon consisted of two 5-min periods separated by stirring. Again, the same column tubing was used to compare the performance of untreated and plasma-treated glass beads, based on retention and efficiency measured under identical conditions. Following these tests, the plasma-exposed beads were removed from the column, extracted in dichloromethane for 1 h with stirring, and repacked to form the third column. This column was used to evaluate the effect of extraction on chromatographic performance.

Polysiloxane-phase capillary columns were prepared following the basic procedure of Woolley *et al.*[17]. Column lengths were generally 4–6 m with an I.D. of 250 μm. Untreated fused silica was obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). Capillaries were purged with helium for 2 h at 250°C before use. Columns to be coated with SE-30 were deactivated with polymethylhydrosilane (Petrarch Systems, Bristol, PA, U.S.A.) before coating. An OV-215 column was first deactivated with mixed cyclics of trifluoropropylmethylsiloxanes (Petrarch) at 420°C based on the procedure of Blomberg *et al.*[18]. A column coated with SuperOx 0.1 (Alltech) was not deactivated. Columns were statically coated with a 0.4 w/v% (0.6 w/v% for OV-215) solution of the stationary phase dissolved in an appropriate solvent. After coating, columns were briefly conditioned before testing.

The ends of coated capillary columns which were to be cross-linked were first sealed into 0.636 cm O.D. glass tubing. The glass tubing was then connected by means of a simple vacuum fitting to a vacuum system with a liquid-nitrogen trapped diffusion pump capable of pressures less than 10^{-6} Pa. Before evacuation, columns were purged extensively with argon (minimum purity 99.998%, MG Industries, Valley Forge, PA, U.S.A.). Due to the inherently high flow impedance of capillaries, pumping times on the order of 1–2 h were used for these columns. Following evacuation, the columns were gently back-filled with the desired pressure of argon (130 Pa) as

measured at the column head by a thermocouple pressure gauge. Columns were then sealed by melting the glass tubing. Sealing in this manner provides a low-pressure reservoir at the column end.

Several *in situ* methods of producing plasmas in capillary columns were explored. An ordinary Tesla-coil leak detector (Fisher Scientific, Pittsburgh, PA, U.S.A.) generates a glow discharge in low-pressure glass chambers by delivering an electrical arc of 50 kV and *ca.* 0.5 MHz. Several "electrodeless" systems were surveyed for suitability. In these cases, the column was first removed from its metal cage. Microwave (Little Litton, Model 1146, Litton Systems, Minneapolis, MN, U.S.A.), radiofrequency (Rf generator from a Low Temperature Dry Asher, Tracerlab, Richmond, CA, U.S.A.), and audio-frequency (Leco Induction Furnace No. 537, Laboratory Equipment, Saint Joseph, MI, U.S.A.) electrical fields were applied to the low-pressure argon within columns. In the case of radio frequency excitation at 13.56 MHz, both capacitive and inductive coupling were examined. Finally, attempts were made to strike a plasma by means of a 7500-V neon sign transformer connected to tungsten electrodes sealed into the column ends.

The ability to cross-link stationary phases for chromatographic separations was assessed for both packed and capillary columns. Chromatographic evaluation was done using a Varian VISTA 4600 GC (Varian Instruments, Walnut Creek, CA, U.S.A.) equipped with an on-column injector for packed columns and a split injector for capillaries. Detection was by flame ionization detector. Volumetric flow-rates through the packed columns were maintained at 10 ml/min (t_o = 16 s), as measured by a soap bubble flowmeter, and were not optimized. Linear flow-rates of helium carrier gas through the capillary columns were between 20 and 30 cm/s and were not optimized. When comparing columns before and after plasma treatment, the flow-rate was kept constant. All columns were evaluated based on efficiency and retention. Capillaries were also assessed by the selectivity and activity shown for a mixture of polar solutes separated at 100°C based on the method of Grob[19]. Column activity refers to undesirable secondary retention mechanisms that cause polar compounds to elute as skewed peaks and can also negatively affect minimum detection limits. Column performance parameters were measured from the statistical moments of solutes separated isothermally at 100°C.

Chromatograms were digitally recorded using a CHROM-1 A-to-D board (Metrabyte, Taunton, MA, U.S.A.) operating within a PC's Limited 286 microcomputer (PC's Limited, Austin, TX, U.S.A.). The software controlling the data acquisition was LABTECH NOTEBOOK (Laboratory Technologies Corporation, Wilmington, MA, U.S.A.). For packed column separations, the sample size was *ca.* 500 ng. For capillary work, detector sensitivity was set so less than 1 ng of an alkane produced a full-scale response ($8 \cdot 10^{-12}$ A.f.s.). Detector output was captured at 10 points-per-second with a height resolution of *ca.* 2000 points for a full-scale response. Moment analysis of the digital data was accomplished with software written in Pascal (Borland International, Scotts Valley, CA, U.S.A.).

Chromatographic evaluation of capillaries was done both before and after *in situ* plasma exposure. A third evaluation was performed after the column had been rinsed with *ca.* 10–20 column volumes of dichloromethane [or ethyl acetate–diethyl ether (1:4) in the case of OV-215]. As with the packed columns, the extent of cross-linking in a capillary column was considered to be the adjusted retention time of an

alkane (dodecane for SE-30, pentadecane for OV-215 and SuperOx 0.1) on an extracted column divided by the retention of that compound on the same column prior to extraction.

RESULTS AND DISCUSSION

In Fig. 1, the average fraction of stationary phase remaining on its coverslip after extraction is plotted *versus* time of plasma exposure for three different argon pressures. After 3-min exposure at any of the three pressures, the polymer is insoluble and is considered to be completely cross-linked. Except for a small number of cov-

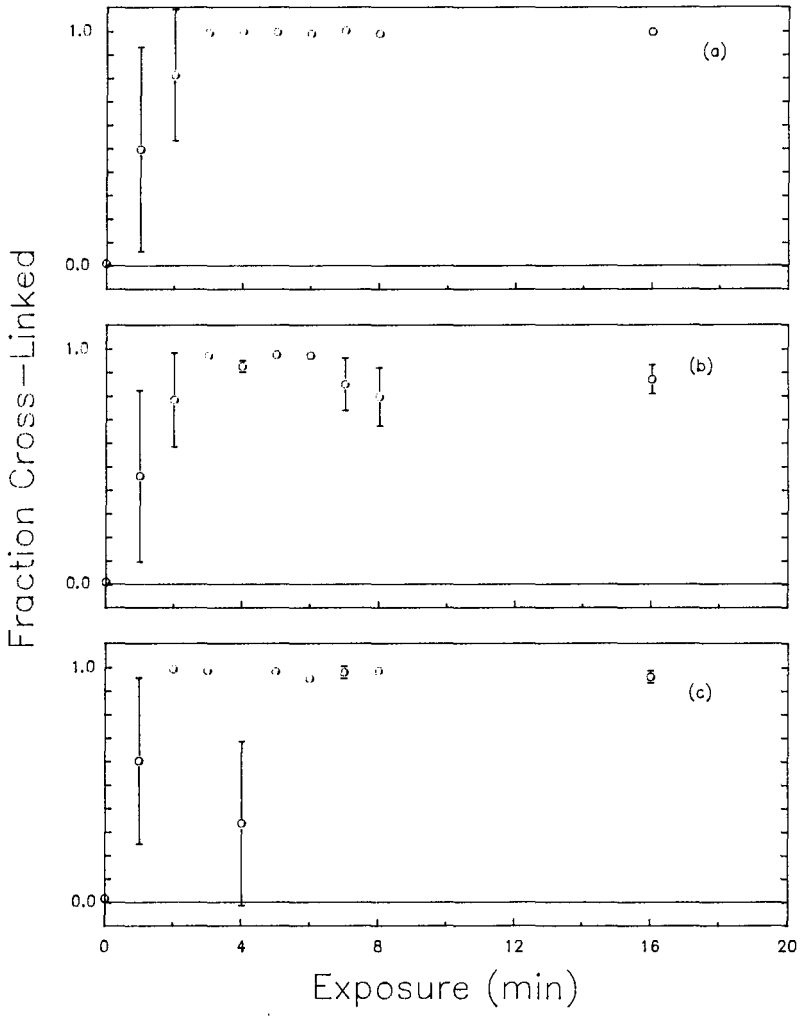

Fig. 1. Plot of polymer fraction cross-linked (mean ± S.D., $n = 3$) *vs.* plasma exposure time at three different pressures for SE-30 deposited on coverslips. Phase loss determined gravimetrically after 1-h agitation in dichloromethane. (a) 13-Pa Ar; (b) 130-Pa Ar; (c) 670-Pa Ar.

erslips, well over 95% of the polymer remained on the coverslip, even after extensive extraction. In a few cases, pieces of highly cross-linked material were dislodged from the glass and were noted in the extraction vessel. These plots confirm that a polysiloxane stationary phase can be converted into a non-extractable form by relatively brief exposure to a low-temperature plasma. These data also indicate plasma pressure only marginally affects the cross-linking process.

Considerable differences were observed between the stationary phases tested. In general, the degree of cross-linking decreases with increasing phenyl substitution. SE-30, SE-52, and SE-54 all show complete cross-linking after 240 s of plasma exposure (more than 98% of the phase resisted extraction). The more polar polysiloxanes remain partially soluble even after 16-min contact, but display evidence of some cross-linking. A 16-min treatment resulted in 20% cross-linking for OV-61 and *ca.* 10% for OV-17 and OV-25. All phases tested could be quantitatively extracted if not subjected to plasma exposure.

Phenyl substitution has been reported to hinder chemically induced cross-linking[9,10]. A similar trend shown by plasma cross-linking is consistent with the hypothesis that free radicals formed in the plasma initiate stationary-phase cross-linking. In these experiments, other factors, such as polymer chain length, polymer purity, and surface wettability, are likely to contribute to the observed differences in cross-linking for different phases and may obscure important distinctions between the two techniques. Further investigations are required to adequately determine the range of stationary phases that plasmas can cross-link.

Both packed and capillary chromatographic columns were prepared with plasma cross-linked stationary phases to determine if the plasma corrupts the inert nature of the phase. The two different geometries presented different obstacles for successful plasma treatment. Diatomaceous earth column packings were subjected to plasma exposure in the same chamber as the coverslips to allow similar treatment conditions. One concern was that the plasma would not penetrate the material evenly. Fig. 2

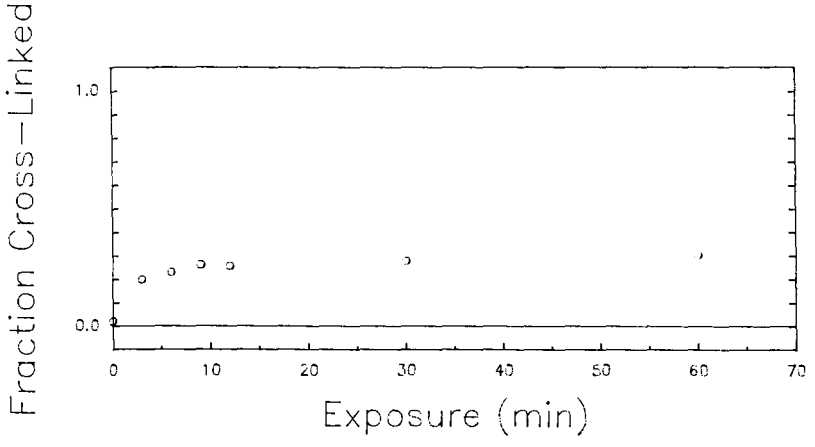

Fig. 2. Plot of polymer fraction cross-linked *vs.* plasma exposure time at 130-Pa Ar for 10% OV-101 on Gas-Chrom P, 80–100 mesh. Material was thoroughly stirred after each exposure interval. Phase loss determined after 1-h agitation by weighing polymer extracted in 10 ml of dichloromethane.

shows the fraction of stationary phase remaining on extracted packing material as a function of exposure time. The weight of polymer phase isolated from the extraction solvent and the weight loss measured for the packing material were equivalent in all cases.

After exposure for 360 s, the material was 23% cross-linked. This value did not increase significantly even after repeated extended exposures interspersed with mixing. From these data, it was concluded reactive plasma components penetrate the interparticulate spaces. If penetration had not occurred, the fraction cross-linked should increase markedly at each exposure interval after the material was thoroughly stirred, presumably exposing particles coated with virgin polymer. However, though the plasma apparently penetrates between particles, the phase was not completely cross-linked even after prolonged treatment. The porous nature of diatomaceous earth supports is suspected as the cause of this failure. These pores form highly tortuous paths which reactive plasma species do not seem to penetrate, and thus a fraction of polymer that remains soluble is shielded from the plasma.

Both the cross-linked and non-cross-linked columns displayed similar performance for separating alkanes. At 100°C, the control column generated 2400 effective plates for decane at $k' = 22.5$. Under identical conditions, a column packed with plasma-treated material produced 2200 effective plates at $k' = 22.2$. Sample retention is unchanged, confirming that the plasma does not significantly alter solute solubility. Because of the large amount of surface activity present on the packing material, polar solutes eluted with distorted peak shapes on both columns.

The inability to completely cross-link the stationary phase on ordinary packings prompted experiments with glass beads. With no internal pores available, the stationary phase is confined to the surface. Low liquid phase loadings and reduced surface area allow the film thickness of capillary columns to be approximated. A third advantage of glass bead solid supports is their mechanical strength. Unlike Gas-Chrom P, coated beads can be removed from the column, extracted with solvent, and repacked without damage. This property allows the fraction of polymer cross-linked to be assessed chromatographically as well as gravimetrically.

Coated glass beads proved more amenable to cross-linking than diatomaceous earth, but still less than expected from the coverslip experiments. After two 300-s exposures at 130 Pa, samples were 60% cross-linked based on gravimetric analysis of the extracted phase. As shown in Table I, column performance was virtually unaffected by plasma treatment.

From the measured decrease in chromatographic retention following extraction, 55% of the phase withstood extraction, in agreement with the gravimetric measurement. This value, though higher than for the diatomaceous earth support, was significantly below the fraction cross-linked during the coverslip studies. This loss may arise from abrasive action during the stirred extraction removing fully cross-linked polymer from the exposed bead surface. Phase pooling at contact points between glass beads is well documented[20] and may also account for incomplete cross-linking of SE-30 on glass beads.

Several schemes designed to strike a plasma in a capillary were tried using low-pressure argon in various diameter tubing. Experiments were first performed with 4-mm I.D. glass tubing, then the bore was reduced to 1 mm. The next step was to attempt striking a plasma in a 250-μm capillary. In every case, decreasing the volume

TABLE I

CHARACTERISTICS OF PACKED COLUMNS TREATED WITH PLASMAS

Column dimensions, 1.85 m × 0.216 cm I.D.; 0.10–0.11 mm glass beads. Height equivalent to an effective plate (HEEP) and capacity ratio for dodecane at 100°C.

Glass-bead column No.	*Stationary phase and treatment*	*Capacity ratio*	*HEEP (mm)*
1	0.5% (w/v) SE-30, no treatment	20.3	0.85
2	0.5% (w/v) SE-30, 2 × 5 min 130-Pa Ar plasma	19.6	0.95
3	Beads from column 2, extracted 60 min	11.1	0.72

to surface area ratio made it progressively more difficult to generate and sustain the plasma.

The neon-sign transformer successfully lit only the larger glass tubes and contamination was observed from the internal electrodes. The discharge tubes quickly became hot. The induction furnace easily ignited plasmas at 20 kHz in the glass tubing without significant warming over a 1-h excitation. Capillaries resisted plasma excitation. Brief flashes occurred only when a short section of column was held near the induction coil. Radio frequency excitation was applied with two reactor designs. Using a capacitive reactor as described by Masada *et al.*[13], no plasmas could be ignited in capillaries after repeated attempts. An induction-coil reactor sustained a plasma in the glass tubes, but not in a capillary. Heating was more pronounced with this system than with the 20-kHz furnace. Microwave excitation sustained plasmas only in the glass tubing and the tubes rapidly became too hot to touch.

Of the plasma generation methods tested to date, the Tesla coil provides the most reliable plasma generation. Energy can be coupled from the coil to the capillary using two approaches. In the first, a column containing argon at 130 Pa is unwrapped from it metal cage. A column section is held centered in a 2-cm I.D. glass tube wrapped with ten turns of bare copper wire. By touching the wire with the Tesla coil, a plasma is ignited over a 20-cm length within the straight capillary. This discharge is quite bright. By pulling the column through the outer tube at *ca.* 20 cm/s, the entire column is briefly exposed to the plasma. Columns cross-linked by this process displayed poor chromatographic performance although they were completely resistant to solvent extraction. The intense plasma appears to damage the polymeric stationary phase.

The second Tesla coil excitation method is even simpler and produces a less vigorous plasma over the entire column. The coil is brought close to a metal cage holding a column containing argon at 130 Pa. As the tip approaches the cage, a faint discharge is visible throughout the entire column. This discharge flickers somewhat and appears a pale greenish orange when viewed through the outer polyimide layer.

The small cross-sectional area of capillaries, coupled with their extreme length, leads to slow evacuation. When an entire column is simultaneously excited, plasma

inhomogeneities due to atmospheric contamination are easily observed as color variations across the column length. To minimize contamination by air, capillaries were extensively purged with argon prior to evacuation. Severe pressure gradients cause dramatic changes in plasma intensity over the column length. Data from coverslip experiments indicate pressure gradients within a more moderate range should not interfere with *in situ* cross-linking of capillary columns. Successful cross-linking of a polydimethylsiloxane stationary phase within a capillary column is illustrated in Table II. Utilization of theoretical efficiency (U.T.E.) is reported relative to an ideal column of the same diameter providing identical solute retention at optimal flow velocity. The U.T.E. values reported here are conservative. Both detector and injector volume contribute slightly to band broadening as evidenced by a 5–10% increase in U.T.E. for 10-m columns. Using statistical moments to calculate column performance is more sensitive to instrumental distortions than traditional measures of peak-width at half-height. The ability to measure U.T.E. values consistently between 80 and 90% [height equivalent to an effective plate (HEEP) = 0.31 mm at capacity ratio = 7.5] for untreated SE-30 capillaries confirms proper system operation.

Capillary column 1 is an untreated control. The dramatic loss in retention following extraction confirms the phase is largely soluble. A second solvent wash of 10 ml removed an additional 3% of the original phase. The decrease in U.T.E. upon extraction is largely due to the decrease in retention. At greatly reduced capacity ratios, ordinarily minimal bandbroadening contributions from dead volume, injection plug width, and detector time constant significantly degrade the relative efficiency for weakly retained solutes on short capillaries. (Similar U.T.E. values were measured for decane prior to extraction, where decane eluted with the same capacity ratio as dodecane following extraction.)

TABLE II
CHARACTERISTICS OF CAPILLARY COLUMNS TREATED WITH PLASMAS

Column dimensions, *ca.* 5 m × 0.25 mm I.D. Utilization of theoretical efficiency (U.T.E.) measured for dodecane on columns 1 and 2. Pentadecane used for columns 3 and 4. (a) Before plasma treatment; (b) after plasma treatment; (c) after solvent extraction.

Capillary column No.		*Stationary phase*	*U.T.E. (%)*	*Fraction cross-linked (%)*
1	a	0.4% (w/v) SE-30	82	–
	c		39	23
2[a]	a	0.4% (w/v) SE-30	87±4	–
	b		37±3	–
	c		40±1	96±3
3	a	0.6% (w/v) OV-215	72	–
	b		67	–
	c		31	36
4	a	0.4% (w/v) SuperOx 0.1	68	–
	b		38	–
	c		32	12

[a] Average of three columns ± S.D.

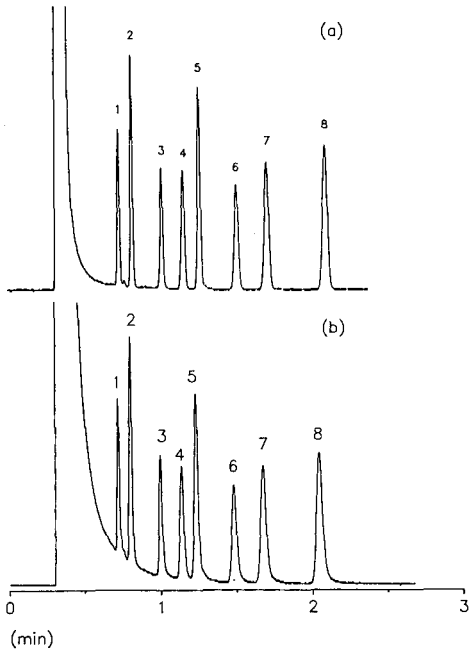

Fig. 3. Typical gas chromatogram of a test mixture at 100°C for capillary column 2. Peaks: 1 = 2-octanone; 2 = decane; 3 = octanol; 4 = dimethylphenol; 5 = undecane; 6 = dimethylaniline; 7 = naphthalene; 8 = dodecane. (a) Before plasma exposure, column length = 5.2 m; (b) after 4-s plasma exposure at 130-Pa Ar and extensive extraction, column length = 4.7 m.

The data for capillary column 2 are averages from three columns. Solute retention diminished only slightly after cross-linking. This minor reduction probably arose from conditioning before testing rather than the plasma itself. Solute retention was completely maintained after solvent extraction. A second solvent wash of 10 ml did not measurably alter retention. These data confirm such columns are effectively cross-linked.

Unfortunately, the present plasma generation method markedly reduces column efficiency. As shown in chromatogram b of Fig. 3, the test solute peaks all tail appreciably, with pronounced tailing in the solvent peak. The second statistical moment, used here to measure column efficiency, is highly sensitive to tailing peaks. This sensitivity is reflected in the low U.T.E. values for the cross-linked capillaries.

Interestingly, alkanes tail to the same extent as polar solutes. Plasma exposure does not change the peak areas for polar compounds relative to the alkanes. For properly deactivated columns, even the highly polar test solutes described by Lee *et al.*[17] elute with the proper peak area relative to the alkanes. Furthermore, peak shape does not change significantly when the sample size is increased by a factor of ten to 10 ng per compound on column. These observations are inconsistent with the usual cause of tailing, exposed active sites.

If present, active sites affect polar solutes much more than alkanes, therefore we suggest the loss in efficiency is not due to chemical changes in the polymer, but to

physical disruption of the stationary phase during plasma generation. Normally, low-temperature plasmas do not disturb delicate structures. Inductively generated plasmas did not diminish column efficiency when either column packing or glass beads were treated. Even exposure times well in excess of those needed for cross-linking showed no deleterious effects on packed columns. The present method of exciting plasmas in capillaries, however, involves coupling an energetic electrical arc from a Tesla coil through the column to the plasma. Multiple tiny arcs, where the metal cage contacts the column, penetrate the fused-silica tubing and the thin polymer layer before reaching the plasma in the column interior. It is suspected that the inner film is damaged only at these localized areas.

Capillary column 3, coated with a trifluoropropyl-substituted polysiloxane, was partially cross-linked *in situ*. Unlike the polydimethylsiloxane column, plasma exposure by itself did not greatly affect column efficiency. Extraction with the coating solvent removed 64% of the phase. Tests with the extracted column show reduced efficiency, although the peak shape remained symmetrical in this instance. The reasons why OV-215 behaved differently than SE-30 are not clear.

The polyethylene glycol phase, SuperOx 0.1 (capillary column 4), exhibited the least propensity for cross-linking. Chemically induced cross-linking at elevated temperatures has been effective for these polymers[5]. The problem with plasma induced cross-linking of SuperOx is suspected to be the polymer's physical state. At room temperature, this phase is a solid. Methylene radicals created on the polymer chain may not exist long enough to come into proximity with an adjacent chain. At the cross-linking temperature, all of the other phases tested in this work were either liquids or gums where molecular motion is greater than in the solid state. Other factors may also contribute to the failure with SuperOx.

Efforts are being made to cross-link columns without reducing their efficiency. High-frequency electromagnetic fields are commonly used to create plasmas. Matsuzawa and Yasuda[21] described a capacitively-coupled plasma polymerization apparatus for coating the inside of plastic tubing as small as 1.5-mm I.D. A slightly modified version of their device has been constructed and initial trials on 0.25-mm I.D. fused-silica capillaries have been promising.

In addition to initiating cross-linking in capillaries, low-temperature plasmas may be uniquely applicable for other column preparation steps. Oxidizing plasmas produced within glow discharges have long been used for cleaning glass and other surfaces[22]. Similar treatments should be equally useful for fused-silica capillaries. The principal advantage over aqueous and non-aqueous cleaning solutions is the reduced opportunity for contamination by trace impurities.

In the past, the surface of glass capillaries was often chemically roughened to improve the wettability of polar stationary phases. Most of these techniques are not applicable to fused silica. The introduction of organo-fluorine compounds into a plasma forms reactive fluorine species which etch fused silica[23]. Although roughening invariably increases surface activity, there may be applications where etched surfaces are desirable. It may be possible to create highly deactivated, fused-silica surfaces which remain wettable for polar films by plasma deposition of appropriate materials. The doping of plasmas with certain polar compounds may also greatly increase the polarity of previously coated stationary phases. As these films are already mechanically stable due to cross-linking, the *in situ* addition of polar moieties should not

disrupt the film's structure. In this manner it is may be feasible to prepare highly polar phases which normally could not be coated on low-energy, deactivated surfaces. The enhanced retention of polar compounds relative to the alkanes has already been observed when air inadvertently contaminated the plasma during cross-linking. A final possibility is the preparation of porous-layer open-tubular (PLOT) columns through plasma deposition of aluminum oxide or other adsorbent layers. Plasma deposition of these materials at low temperatures has been reported within the semiconductor industry[24].

At this early stage, capillary plasma applications other than cross-linking are speculative extrapolations based on existing processes. These potential uses do serve, however, to illustrate the broad utility of low-temperature plasmas. Investigations into these areas are being conducted.

CONCLUSIONS

Low-temperature plasmas cause cross-linking of GC stationary phases in a variety of geometries. Exposing the stationary phase to plasmas in a large chamber does not seem to affect chromatographic performance. Plasmas can also be generated within coated capillary columns with relatively simple equipment. These columns exhibit cross-linked behavior, but show diminished efficiency. Chromatographic tests reveal no gross chemical transformations have occurred to alter retention patterns. It is suggested that the polymer layer is physically disrupted by the discharge as it passes through the capillary wall. A suitable method of generating plasmas without disturbing the interior coating is essential for further developments.

The fundamental properties of low-temperature plasmas make them attractive for a variety of column fabrication steps. Many of the potential applications, including cleaning, deactivation, chemical modification, and material deposition, are predicated on the widespread use of such plasmas in a variety of technological areas.

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REFERENCES

1 K. Grob, G. Grob and K. Grob, Jr., *J. Chromatogr.*, 211 (1981) 243.
2 B. W. Wright, P. A. Peaden and M. L. Lee, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 413.
3 C. Madani, E. M. Chambaz, M. Rigaud, J. Durand and P. Chebroux, *J. Chromatogr.*, 126 (1976) 161.
4 L. Blomberg and T. Wännman, *J. Chromatogr.*, 168 (1979) 81.
5 P. Sandra, M. Van Roelenbosch, I. Temmerman and M. Verzele, *Chromatographia*, 16 (1982) 63.
6 B. W. Wright. P. A. Peaden, M. L. Lee and T. J. Stark, *J. Chromatogr.*, 248 (1982) 17.
7 J. Buijten, L. Blomberg, S. Hoffman, K. Markides and T. Wännman, *J. Chromatogr.*, 283 (1984) 341.
8 G. Schomburg, H. Husmann, S. Ruthe and M. Herraiz, *Chromatographia*, 15 (1982) 599.
9 K. Grob and G. Grob, *J. Chromatogr.*, 213 (1981) 211.
10 P. A. Peaden, B. W. Wright and M. L. Lee, *Chromatographia*, 15 (1982) 335.
11 M. Shen and A. T. Bell, in M. Shen and A. T. Bell (Editors), *Plasma Polymerization (ACS Symp. Ser. 108)*, American Chemical Society, Washington, DC, 1979, Ch. 1.

12 A. T. Bell, in J. R. Hollahan and A. T. Bell (Editors), *Techniques and Applications of Plasma Chemistry*, Wiley-Interscience, New York, 1974, Ch. 1.
13 Y. Masada, K. Hashimoto, T. Inoue, Y. Sumida, T. Kishi and Y. Suwa, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 400.
14 A. M. Wrobel, M. R. Wertheimer, J. Dib and H. P. Schreiber, *J. Macromol. Sci.-Chem.*, A14 (1980) 321.
15 Y. Yamamoto, S. Oka and K. Hayashi, *Chem. Lett.*, (1981) 221.
16 S. A. Volkov, K. I. Sakodynskii, A. B. Gil'man, V. M. Kolotyrkin, N. F. Zelenkova, T. A. Zakharova and V. K. Potapov, *J. Chromatogr.*, 242 (1982) 166.
17 C. L. Woolley, R. C. Kong, B. E. Richter and M. L. Lee, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 329.
18 L. Blomberg, K. Markides and T. Wännman, in R. E. Kaiser (Editor), *Proceedings of the Fourth International Symposium on Capillary Chromatography-Hindelang IV*, Huthig, Heidelberg, 1981, p. 73.
19 K. Grob, Jr., G. Grob and K. Grob, *J. Chromatogr.*, 156 (1978) 1.
20 J. C. Giddings, *Anal Chem.*, 34 (1962) 458.
21 Y. Matsuzawa and H. Yasuda, *J. Appl. Polym. Sci., Appl. Polym. Symp.*, 38 (1984) 65.
22 L. Holland, *The Properties of Glass Surfaces*, John Wiley, New York, 1964, pp. 310–335.
23 B. Chapman, *Glow Discharge Processes*, Wiley-Interscience, New York, 1980, Ch. 7.
24 R. W. Kirk, in J. R. Hollahan and A. T. Bell (Editors), *Techniques and Applications of Plasma Chemistry*, Wiley-Interscience, New York, 1974, Ch. 9.

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NEW PERSPECTIVES IN CAPILLARY CHROMATOGRAPHY[a]

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SUMMARY

A coating procedure for preparing fused-silica capillary columns with graphitized carbon black, modified with a liquid stationary phase, is described. This yields reproducible results in terms of column efficiency, separation power and retention parameters. The factors that affect the final results are discussed and some analytical applications are reported.

INTRODUCTION

For several years, our group has been investigating the preparation of fused-silica capillary columns with the inner walls coated with graphitized carbon black and treated with suitable amounts of liquid stationary phases[1,2], in an attempt to enhance the selectivity of capillary columns by using the peculiar stationary phases yielded by gas–liquid–solid chromatography (GLSC)[3]. Faster analysis is also obtained with these capillary columns.

In a previous paper[1] we reported a method for the preparation of GLSC capillary columns, based on making a slurry of graphitized carbon black and the liquid phase to be used, in a suitable solvent [generally pentane–dichloromethane (1:1, v/v)]. The column is then filled with the slurry, closed at one end and the solvent is evaporated slowly from the other end by keeping the column in a water-bath at a temperature higher than the boiling point of the solvent mixture.

Although good results were obtained with the preparation and use of such columns and the theoretical expectations concerning their properties were confirmed, many questions remained open, in particular those concerning reproducibility. The preparation of a good column is affected by several critical parameters: the dimensions of the carbon black particles; the surface area of the particular carbon black used; the concentration of the slurry, both in carbon black and the liquid phase; and the final amount of stationary phase on the carbon black surface. These parameters also influence each other, so that the final result is the best compromise among them.

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In this paper, we present a method of preparation intended to obtain good column reproducibility. The influence of the carbon black concentration on different columns is shown and some applications to environmental analysis are reported.

EXPERIMENTAL

Preparation of the slurry and coating

The carbon black (Carbopack B or F, obtained from Supelco, Bellefonte, PA, U.S.A.) is first crushed and sieved to less than 200 mesh. In order to use the full power of the sonicator (Model 450; Branson, Danbury, CT, U.S.A.), the material is dispersed in water, which is the best medium for sonication, and maximum power is maintained for 30 min. Then the water is evaporated and a suitable organic solvent (dichloromethane) is added to the dry material. By using water, a much higher subdivision of the carbon black particles is obtained, so that the slurry is more homogeneous. The mixture of the organic solvents and the carbon black is again sonicated for 15 min and a homogeneous slurry is again obtained. The column is then coated, following the procedure described previously[1], and after the solvent has completely evaporated the column is dried under a stream of nitrogen.

Adjusting the amount of stationary phase and column conditioning

The initial amount of stationary phase added to the slurry is usually greater than needed for optimum conditions of GLSC, so that, prior to conditioning, the column is again placed in a water-bath kept at 25°C and washed with the solvent previously used for the slurry. In this way, some of the stationary phase is washed out, and the percentage that remains in the column depends on the amount of solvent passed through the column. The column is then dried and conditioned overnight. The silica tubes were not specially treated before coating.

Chromatographic measurements

All analyses were performed with a Carlo Erba (Milan, Italy) Mega Series HRGC 5160 gas chromatograph, with flame ionization detection (FID) and using a split/splitless injector, and with a DANI (Monza, Italy) Model 6500 chromatograph, with electron-capture detection (ECD) and using a programmed-temperature vaporizer (PTV) injector. The heat of adsorption of *n*-pentane was measured for the isosteres by plotting capacity ratio (k') against reciprocal temperature. The separation factor, α, between *n*-heptanol and *n*-octane was measured using the corrected retention times. As the heptanol peak is tailed, care was taken always to inject the same amount of this compound for the sake of reproducibility. The peak maximum is considered as the retention time.

Hydrogen was used as carried gas with FID and nitrogen with ECD measurements.

RESULTS AND DISCUSSION

Determination of the surface coverage

Because of the coating procedure, the actual percentage of stationary phase on the carbon black cannot be established directly, because by micronizing the carbon

particles, the surface area is increased with respect to the accepted value of 90 m^2/g given for Carbopack B and 6 m^2/g for Carbopack F in the 40–120-mesh range. However, the surface coverage can be established from the inflection point of the curve obtained by plotting the heat of adsorption of *n*-pentane against the nominal percentage[3]. This experiment was carried out by preparing a series of packed columns, containing Carbopack B coated with various amounts of SP-1000 liquid phase, and the results are shown in Fig. 1a. The change in the retention volume ratio between an aliphatic saturated linear hydrocarbon (*n*-octane) and an aliphatic saturated linear alcohol of analogous molecular weight (*n*-heptanol) was also measured on the packed columns, giving Fig. 1b.

As the retention volume ratio is linearly related to the surface coverage, by measuring this ratio on the capillary column its surface coverage can be found by comparing the value obtained with those for the packed column.

In this way, the actual surface coverage (θ) of three capillary columns was calculated to be 0.6, which corresponds to about 2.2% for the packed columns. However, it should be understood that we do not know the percentage for the capillary column, as the surface area of the carbon black is not known for the reasons stated above.

In fact, the coverage obtained approximately corresponds to the maximum value of the heat of adsorption on the curve, where the maximum of the "lateral interactions" of the eluate with the stationary phase takes place. This corresponds to a maximum of the column selectivity for hydrocarbons. The surface coverage is ob-

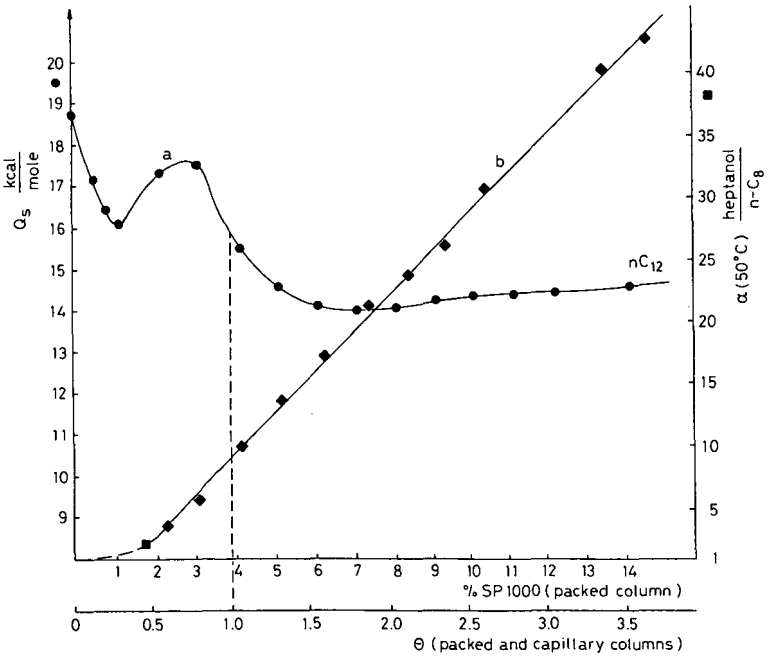

Fig. 1. Plots of (a) the chromatographic heat of adsorption and (b) separation factors against the surface coverage, θ, and the percentage (w/w) of liquid phase SP-1000 on Carbopack B.

tained by assinging a value of unity to the inflection point of the curve of the heat of adsorption[3,4]. Washing of the capillary column was carried out to such an extent on purpose, in order to obtain maximum selectivity for hydrocarbons.

This method seems to be the most convenient for establishing the extent of surface coverage by the stationary phase, as it is found from GC measurements and because measurements of the surface area with the Brunauer–Emmett–Teller (BET) method have only a relative meaning and may lead to different evaluations that have little significance for chromatographic purposes.

Column reproducibility

Once the optimum procedure had been established, we prepared three columns under the same conditions in order to test the reproducibility of the coating procedure. The results are summarized in Table I.

The reproducibility is very good in terms of the efficiency. The three columns show a maximum of 18 000 theoretical plates with respect to n-C_{12}. The same reproducibility is observed for the ratio of the retention volumes for an alcohol and a hydrocarbon of similar structure and molecular weight. The two compounds were chosen because the carbon black and the stationary phase have counteracting effects on polar and non-polar compounds. At zero surface coverage, the two compounds should have very similar retention volumes, as their polarizabilities are similar, as has been shown previously[3]. The retention of the polar compounds increases with increasing surface coverage by a polar stationary phase, such as SP-1000, whereas it decreases for the hydrocarbon. Hence, the ratio of the retention volumes is a very good index of the surface coverage by the stationary phase. Of course, this parameter is critical, and very small variations in the percentage of stationary phase may lead to considerable differences in the relative retentions. The three columns show a difference of about 10% in relative retention; it is our opinion that such a result is satisfactory and does not affect substantially the chromatographic characteristics of the columns.

Fig. 2 shows a practical example of the reproducibility of the columns. The characteristics of columns A, B, and C are the same and are reported in Table I. Some of the peaks show slight tailing, but the separations reported have no analytical

TABLE I

COMPARISON OF SOME PARAMETERS FOR THREE COLUMNS (A–C), PREPARED BY THE SAME PROCEDURE AND FOR A COLUMN WITH A HIGHER CONTENT OF CARBON BLACK IN THE SLURRY (D)

Column dimensions 7 m × 0.25 mm I.D.

Column	t_0 *(s)*[a]	*Flow-rate (ml/min)*	k' *(140°C),* n-C_{12}	α *(90°C), n-heptanol/*n-C_8	H_{min} *(mm)*
A	18	1.5	49	3.8	0.40
B	18	1.5	52	3.8	0.38
C	18	1.5	56	3.7	0.40
D	18	1.5	125	4.0	0.40

[a] t_0 (dead time) = retention time of methane.

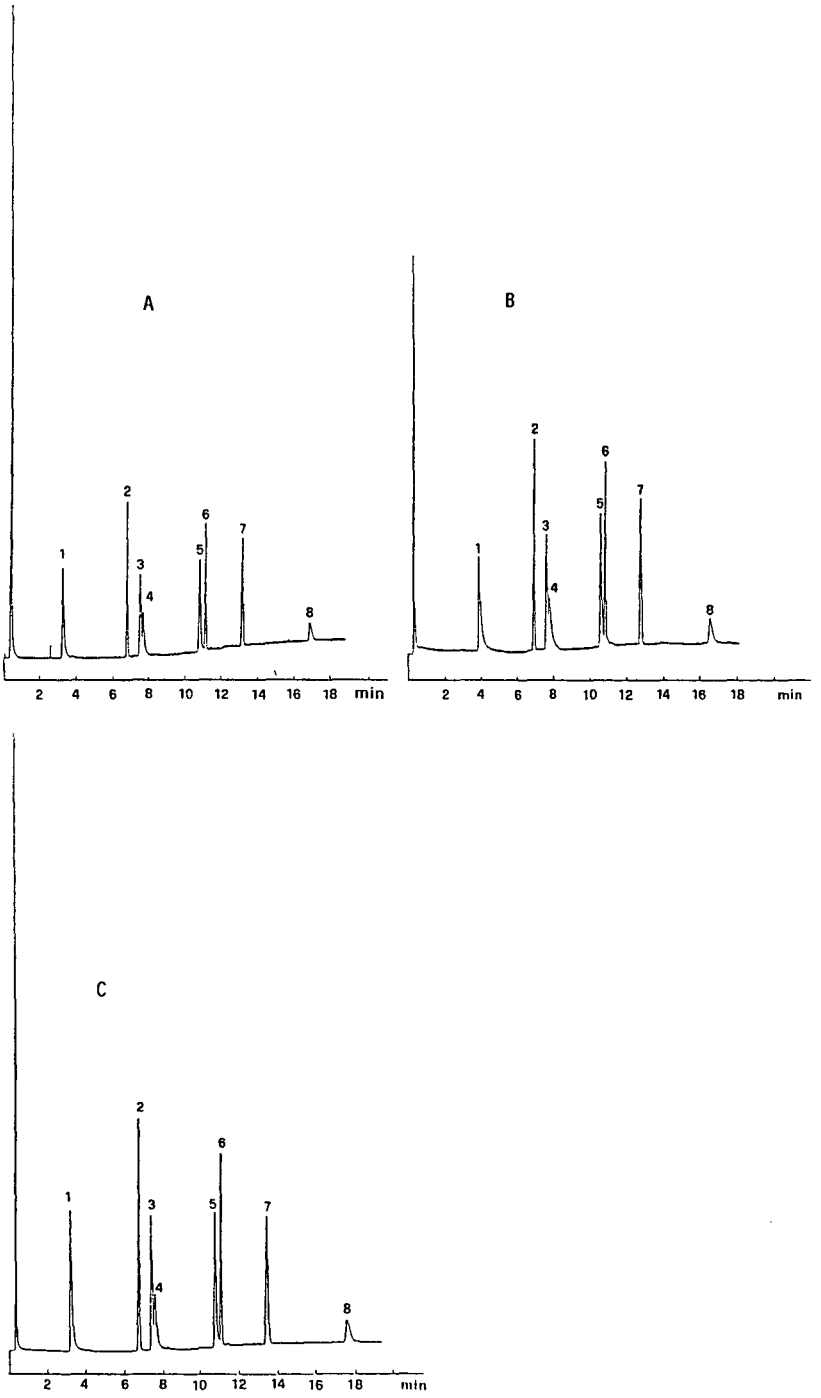

Fig. 2. Chromatograms showing the reproducibility of three columns (A, B and C) of the same length and diameter, coated by the same procedure and under identical conditions. Fused-silica capillary columns (7 m × 0.25 mm I.D.) coated by the procedure dexcribed in the text. 1 = 1, 2, 4-Trichlorobenzene; 2 = *n*-dodecane; 3 = 1-methylnaphthalene; 4 = 2-methylnaphthalene; 5 = 1,3-dimethylnaphthalene; 6 = *n*-tetradecane; 7 = *n*-pentadecane; 8 = hexachlorobenzene. Carrier gas, hydrogen; flow-rate, 1.5 ml/min at 140°C; temperature programme, 2 min at 140°C, then increased at 10°C/min to 240°C.

meaning, the mixture being made for the sake of comparison, not for solving actual analytical problems. A fourth column (D) was prepared with the same carbon black and the same stationary phase, but by changing the concentration of carbon black in the slurry while keeping the same ratio of the stationary phase to carbon black (30:1). The results are also given in Table I.

The data show that the column efficiency is very similar to the previous results, whereas a slight increase in the relative retentions of the alcohol and the hydrocarbon is observed. In contrast the retention of the n-C_{12} hydrocarbon increases substantially. This can only be explained by assuming that an increase in carbon black concentration in the slurry leads to a more extensive coating of the silica surface, whereas the surface coverage of the carbon black by the stationary phase is not affected to a large extent. In fact, the ratio of the SP-1000 to Carbopack B was kept unchanged for the three columns.

It is worth noting that scanning electron microscope photographs of the inner surface of the coated capillary show that the carbon black particles cover only 50% of the silica surface. This introduces a third variable into the column characteristics that should be taken into account for obtaining a more or less retentive layer while maintaining a very similar selectivity. We think this parameter is extremely important and is a peculiar feature of this type of column. In fact, making a comparison with the thickness of the liquid film in conventional GLC capillary columns, an increase in the film thickness produces the same effect as the increase in the surface coating of our columns. This is possible because the silica surface is not completely coated. Other-

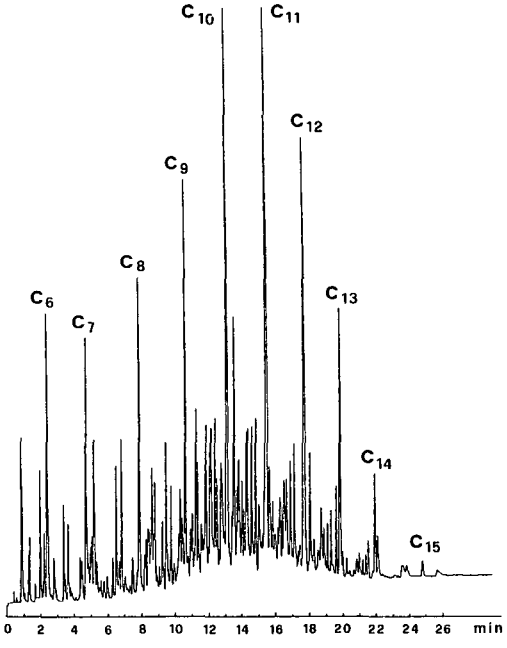

Fig. 3. Chromatogram of a virgin naphtha (80–250°C). Column D (see text). Carrier gas, hydrogen; flow-rate, 1.5 ml/min at 35°C; temperature programme, 35°C for 1 min, then increased at 12°C/min to 240°C.

wise, the thickness of an adsorptive layer does not affect the retention, which in this instance is due only to the surface phenomena.

We realize that the absorbent–stationary phase–silica surface system is very complex and that the retention mechanism may be different from that for classical packed columns with liquid-modified graphitized carbon. However, from the values and the behaviour of the heat of adsorption, measured with the capillary column, it can be concluded that the silica surface contributes to retention to a very minor extent and may only be responsible for some peak tailing of polar compounds. This is worth investigating, and some surface treatment prior to coating may be useful in eliminating irreversible adsorption phenomena due to the silica active sites.

A fifth column was prepared with the more concentrated slurry, but this time the washing was omitted. This is important in order to understand the modifications induced by the solvent treatment. In fact, the retention time ratio between *n*-heptanol and *n*-octane is 56:1, which shows that the carbon surface is now covered by at least three or four monolayers of SP-1000. The column efficiency is also much lower, the minimum plate height (H_{min}) being *ca.* 1 mm.

The conclusion from these experiments is that, although a large amount of SP-1000 must be added to the slurry to make it stable, washing with the solvent is necessary to ensure both column reproducibility and better efficiency. Also, it has

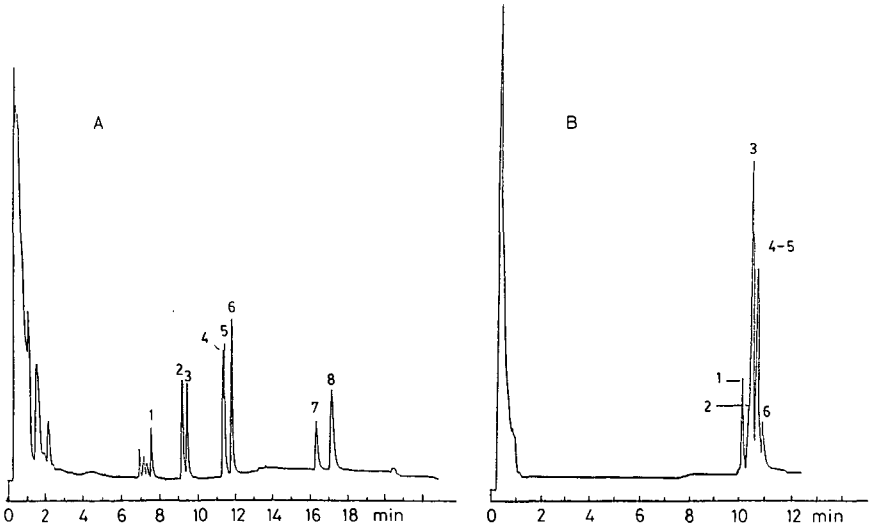

Fig. 4. (A) Separation of chlorodibenzodioxins and benzofurans. 1 = 2,3,7,8-Tetrachlorodibenzodioxin; 2 = 1,2,3,7,8-polychlorodibenzofuran; 3 = 1,2,3,7,8-polychlorodibenzodioxin; 4 = 1,2,3,6,7,8-hexachlorodibenzofuran; 5 = 1,2,3,6,7,8-hexachlorodibenzodioxin; 6 = 1,2,3,7,8,9-hexachlorodibenzodioxin; 7 = octachlorodibenzofuran; 8 = octachlorodibenzodioxin. Fused-silica capillary column (8 m × 0.25 mm I.D.); coating, Carbopack F + SP-1000; carrier gas, hydrogen; flow-rate, 1.5 ml/min at 150°C; temperature programme, 1.5 min at 150°C, then increased at 10°C/min to 280°C. (B) Separation of tetrachlorodibenzodioxin isomers: 1 = 1,4,7,8-tetrachlorodibenzodioxin; 2 = 1,2,3,4-tetrachlorodibenzodioxin; 3 = 1,2,3,7-tetrachlorodibenzodioxin; 4 = 1,2,7,8-tetrachlorodibenzodioxin; 5 = 1,2,3,8-tetrachlorodibenzodioxin; 6 = 2,3,7,8-tetrachlorodibenzodioxin. Fused-silica capillary column (8 m × 0.25 mm I.D.); coating, Carbopack F + SP-1000; carrier gas, hydrogen; flow-rate, 1.5 ml/min at 150°C; temperature programme, 1.5 min at 150°C, then increased at 8°C/min to 250°C.

been observed that, once the slurry has been prepared, it should be used within a short time. As time passes between preparation and use, the surface coverage by the stationary phase increases, probably because the graphitized carbon adsorbs some stationary phase from the solvent during storage. Hence, the time between preparation and use of the slurry is another parameter to be taken into account for column reproducibility.

Analytical applications

In Fig. 3 the chromatogram of a virgin naphtha, obtained on the fourth column (D), made with a more concentrated slurry, is shown. The analysis was performed in 22 min. The complexity of the mixture in terms of number of peaks and separation power is analogous to that obtained with a much longer column by GLC. This is due to the ability of GLSC columns with carbon black coated with a polar stationary phase, to separate hydrocarbon isomers.

Fig. 4A shows the separation of some chlorodibenzodioxins and chlorodibenzofurans. This separation was obtained with a column only 8 m long, coated with Carbopack F covered by SP-1000. In Fig. 4B the same column was used with the specific purpose of separating the isomers of tetrachlorodibenzodioxins. It is interesting that, owing to the selectivity of this particular column, a separation analogous to

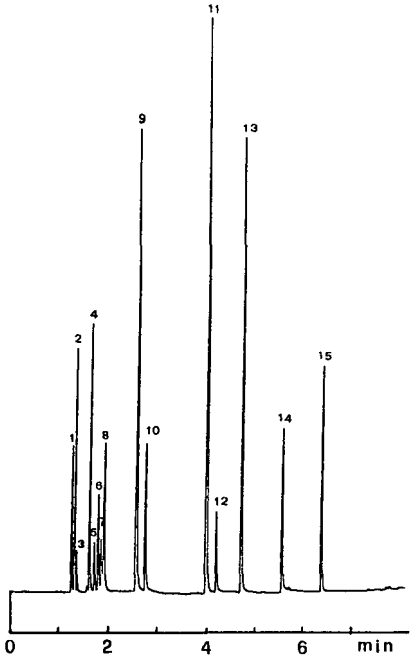

Fig. 5. Separation of some chlorinated priority pollutants. 1 = Dichloromethane; 2 = *trans*-1,2-dichloroethylene; 3 = 1,1,1-trichloroethane; 4 = carbon tetrachloride; 5 = bromochloromethane; 6 = chloroform; 7 = trichloroethylene; 8 = bromochloromethane; 9 = *cis*-1,3-dichloropropene; 10 = 1,1,2-trichloroethane; 11 = tetrachloroethylene; 12 = dibromochloromethane; 13 = bromoform; 14 = 1,4-dichlorobutane; 15 = 1,1,2,2-tetrachloroethane. Fused-silica capillary column (20 m × 0.25 mm I.D.), coated with Carbopack B + SP-1000; temperature programme, 2 min at 30°C, then increased at 20°C/min to 120°C.

that obtained with a 60-m capillary column of the standard GLC type was obtained with an 8-m column within 12 min.

In Fig. 5 the separation of some halogenated low-molecular-weight compounds of environmental interest is shown. Very fast analysis is obtained.

CONCLUSIONS

The results help to clarify the mechanism of operation of capillary columns coated with liquid-modified cabron black. We have now found a reproducible method of preparing such columns, and their chromatographic properties can be predicted as a function of the various parameters investigated. As has been pointed out, a better performance for polar compounds should be obtained by appropriate treatment of the silica surface. Different stationary phases should be tested according to preliminary experiments performed previously. We believe that much more work is needed to exploit fully the analytical potential of these columns, which have the advantage that they couple a high selectivity with the high efficiency typical of capillary columns. As a final consideration, we think that previous attempts to obtain capillary columns coated with carbon black[5,6] remained interesting experiments but were not exploited further because of the difficulties encountered reproducing the results. Reproducibility depends on many parameters that need to be considered, especially those which mutually interact.

REFERENCES

1 F. Bruner, G. Crescentini, F. Mangani, P. Palma and M. Xiang, *J. Chromatogr.*, 399 (1987) 87.
2 F. Bruner, G. Crescentini, F. Mangani and M. Xiang, *Ann. Chim. (Rome)*, 77 (1987) 745.
3 F. Bruner, P. Ciccioli, G. Crescentini and M. T. Pistolesi, *Anal. Chem.*, 45 (1973) 1851.
4 G. Crescentini, F. Mangani, A. R. Mastriogiacomo and P. Palma, *J. Chromatogr.*, 392 (1987) 83.
5 C. Vidal Madjar, S. Bekassy, M. F. Gonnord, P. Arpino and G. Guiochon, *Anal. Chem.*, 49 (1977) 768.
6 G. Goretti, A. Liberti and G. Pili, *J. High. Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1978) 143.

Journal of Chromatography, 473 (1989) 103–113
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SYNTHESIS AND STRUCTURAL CONSIDERATIONS OF OLIGOETHYLENE OXIDE-CONTAINING POLYSILOXANE STATIONARY PHASES IN CAPILLARY GAS AND SUPERCRITICAL-FLUID CHROMATOGRAPHY

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SUMMARY

A series of oligoethylene oxide-containing polysiloxanes were prepared by hydrosilylating the appropriate oligoethylene oxide-containing alkene onto a well-defined polymethylhydrosiloxane polymer. The most convenient oligoethylene oxide-containing alkenes were prepared from eugenol and the tosylate of di- or triethylene glycol monomethyl ether. The polysiloxane phase prepared from 1-(4-allyl-2-methoxyphenoxy)-5-methoxy-3-oxapentane was found to possess desirable chromatographic properties when used in capillary supercritical-fluid chromatography and is suggested as a substitute for Carbowax 20M. The phase was usable at temperatures from 20°C to 300°C. The synthetic rationale and the effects of structural changes in the polymers on chromatographic performance are discussed in this paper. This paper also contains a systematic study of the influence of chemical structure on chromatographic properties of the resulting polysiloxane phases.

INTRODUCTION

Nearly all of the presently used stationary phases for capillary gas chromatography (GC) are based on the polysiloxane backbone. Thermal stability as well as good diffusion of solutes in the stationary phase are necessary requirements for preferred stationary phases. Polysiloxanes have the best diffusion properties of the polymeric materials known today[1] and they are also very stable. Substitution on the polysiloxane backbone has been diverse, depending on the chromatographic need. Stationary phases range from polar cyanophenyl-substituted[2], to non-polar alkyl-substituted polysiloxanes[3].

The polyethylene glycol (PEG) phases, such as Carbowax, are popular because of their moderate polarities which lead to selective separations of polar and polarizable solutes without exhibiting strong retention of these compounds. A number of studies have been made to modify Carbowax for use at both higher and lower oper-

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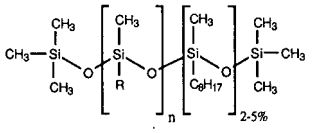
ating temperatures[4]. In a previous paper[5], a comparison was made between a PEG phase (Carbowax 20M) and a phenyl-containing oligo(ethylene oxide)-substituted polysiloxane (phase **1a**, Fig. 1). The new phase was found to have a selectivity similar to Carbowax 20M. In addition, the phase could be crosslinked and was usable at temperatures from 20°C to 300°C in GC. These desirable chromatographic properties of polymer **1a** led us to prepare a series of similar phases shown in Fig. 1, and to study the effects of different structural features on gas and supercritical-fluid chromatographic (SFC) selectivity and stability. Our objective was to prepare a medium polar phase that could be used in SFC as well as in the typical full temperature range of GC.

EXPERIMENTAL

Synthetic procedure for the preparation of oligoethylene oxide-containing polysiloxane phases (Scheme 1)

The new phenyl- and non-phenyl-containing oligoethylene oxide-substituted polysiloxanes were prepared by hydrosilylating the appropriate oligoethylene oxide-containing alkene onto a previously prepared polymethylhydrosiloxane of known composition (Scheme 1). The hydrosilation reaction used to prepare these polymers and the synthesis of the polymethylhydrosiloxanes have been reported previously[5,6]. The number of Si–H units in the polymethylhydrosiloxane determined the extent of substitution on the polymer. For example, a polymer with 50% Si–H (every silicon atom had a methyl group and a hydrogen atom) would produce a final polymer



1a, R = $(CH_2)_3$–C6H3(OCH3)–O–O–OCH_3

2a, R = $(CH_2)_3$–C6H3(OCH3)–O–O–O–OCH_3

3a, R = $(CH_2)_3$–C6H3(OCH3)(OCH3)–O–O–OCH_3

4a, R = $(CH_2)_3$–C6H3(OCH3)–O–O–O–OCH_3

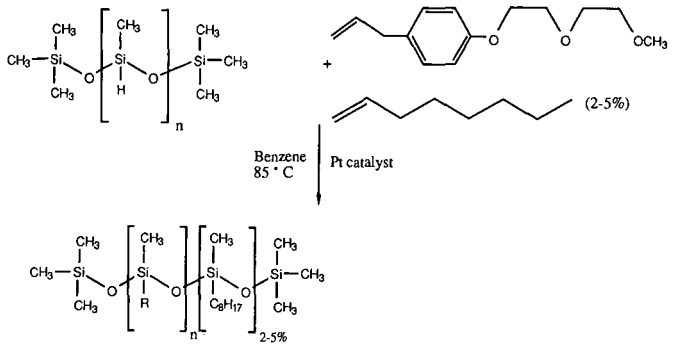
5a, R = $(CH_2)_3$–C6H3(OCH3)(OCH3)–O–O–OCH_3

6a, R = $(CH_2)_3$–O–O–OCH_2CH_3

7a, R = $(CH_2)_3$–O–O–O–OCH_3

8a, R = $(CH_2)_4$(OCH3)–O–O–O–O–OCH_3

Fig. 1. Oligoethylene oxide-containing polysiloxane gum phases.



Scheme 1. Hydrosilylation of a polar substituent onto a preformed polysiloxane. Where R = the above oligoethylene oxide-containing alkene.

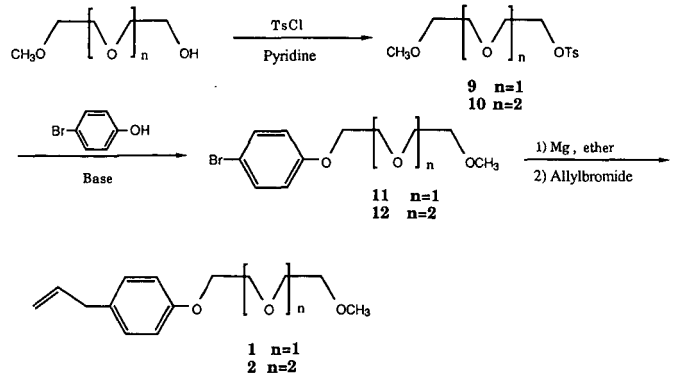
containing approximately 50% substitution. The polymers in this study contained about 47% of the oligoethylene oxide substituent and about 3% octyl substituent. The oligoethylene oxide-containing alkenes were prepared by several different methods as presented below.

Preparation of oligoethylene oxide-containing alkenes (Schemes 2–4)

*1-(4-Bromophenoxy)-5-methoxy-3-oxapentane (**11**) (Scheme 2).* Compound **11** was prepared as reported[5]. In an alternative method, 4-bromophenol was dissolved in 75 ml of dry dimethyl sulphoxide (DMSO), and a slight excess of sodium hydride was added. The tosylate **9** was added over a period of about 20 min and the mixture was allowed to react overnight while keeping the temperature around 40°C. Product **11** was then isolated as reported[5] in a 79% yield.

*1-(4-Allylphenoxy)-5-methoxy-3-oxapentane (**1**) (Scheme 2).* Compound **1** was prepared from **11** as reported[5].

*1-(4-Bromophenoxy)-8-methoxy-3,6-dioxaoctane (**12**) (Scheme 2).* This material was prepared as above for compound **11**[5] except that tosylate **10** (prepared from the monomethyl ether of triethylene glycol and tosyl chloride) was added to the



Scheme 2. Synthesis of alkenes using *p*-bromophenol.

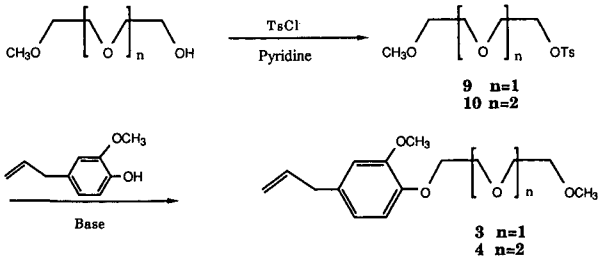
phenoxide. The product was purified by distillation to give 55% of **12**; b.p. 120–125°C/0.07 mm; NMR (δ): 3.4 (3H, s), 3.72 (10H, bm), 4.1 (2H, m), 6.8 (2H, d), 7.36 (2H, d).

*1-(4-Allylphenoxy)-8-methoxy-3,6-dioxaoctane (**2**) (Scheme 2).* Compound **2** was prepared as above for **8** using 1.31 g (0.054 mol) of magnesium turnings, 15 g (0.047 mol) of **12** and 70 ml of anhydrous tetrahydrofuran (THF). After the magnesium was consumed, 5.3 ml of 3-bromopropene in 15 ml of anhydrous THF were added and the mixture was refluxed overnight. The product was purified by distillation to give 0.79 g (6%) of **2**; b.p. 123–125°C/0.04 mm; NMR (δ): 3.40 (3H, s), 3.72 (10H, bm), 4.1 (2H, m), 5.0 (2H, m), 5.95 (1H, m), 6.86 (2H, d), 7.12 (2H, d).

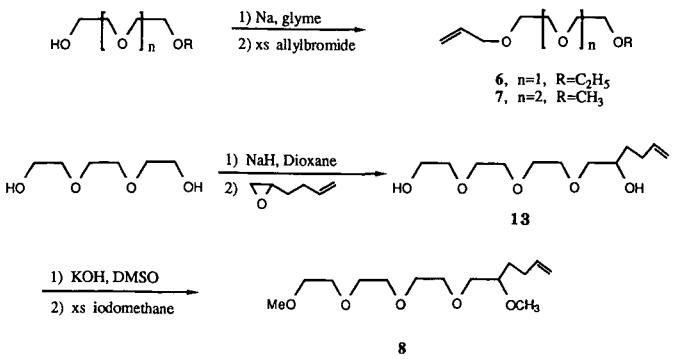
*1-(4-Allyl-2-methoxyphenoxy)-5-methoxy-3-oxapentane (**3**) (Scheme 3).* The potassium alkoxide of eugenol was first prepared by treating 16.91 g (0.103 mol) of eugenol with 5.78 g (0.103 mol) of potassium hydroxide (in as little water as possible) and evaporating the salt to dryness on a rotary evaporator. The salt was then dissolved in DMSO and heated to 40°C while 27.43 g (1.10 mol) of tosylate **9** was slowly added. The mixture was stirred overnight at 40°C and extracted with ether. The ether layer was washed with 5% sodium bicarbonate, dried over anhydrous magnesium sulfate and evaporated. The resulting oil was distilled to give 19.44 g (73%) of **3**; b.p. 127–131°C/0.06 mm; NMR (δ): 3.30 (2H, d), 3.38 (3H, s), 3.5 (2H m) 3.70 (4H, m), 3.85 (3H, s), 4.15 (2H, t), 5.15 (2H, m), 5.95 (1H, m), 6.7 (2H, s) 6.85 (1H, d). Analysis for $C_{15}H_{22}O_4$; calculated: C, 67.66; H, 8.33; found: C, 67.54; H, 8.42.

*1-(4-Allyl-2-methoxyphenoxy)-8-methoxy-3,6-dioxaoctane (**4**) (Scheme 3).* This material was prepared in the same manner as **3** above from 7.36 g (0.045 mol) of eugenol and 10.93 g (0.034 mol) of tosylate **10**. The product was distilled to give 7.32 g (54%) of **4**; b.p. 154–157°C/1.2 mm; NMR (δ): 3.31 (2H, d), 3.38 (3H, s), 3.54 (2H, m), 3.7 (6H, m), 3.81 (3H, s), 3.38 (2H, t), 4.15 (2H, t). 5.06 (2H, dd), 5.92 (1H, m), 6.7 (2H, bs), 6.85 (1H, d). Analysis for $C_{17}H_{26}O_5$; calculated: C 65.79; H, 8.44; found: C, 65.65; H, 8.54.

*1-(4-Allyl-2,6-dimethoxyphenoxy)-5-methoxy-3-oxapentane (**5**).* Compound **5** was prepared as above for **3** from 1.92 g (0.01 mol) of 2,6-dimethoxy-4-allylphenol and 1.37 g (0.01 mol) of 1-chloro-5-methoxy-3-oxapentane (**9** with a chlorine substituted for the tosylate group) using N,N-dimethylformamide (DMF) rather than DMSO as the solvent. The mixture was then heated to 120–135°C overnight. After the reaction mixture had been extracted, product **5** was isolated by distillation to give 1 g (34%) of **5**; b.p. 110–120°C/0.75 mm; NMR (δ): 3.3 (2H, d), 3.4 (3H, s), 3.8 (10H,



Scheme 3. Synthesis of alkenes starting from eugenol.



Scheme 4. Synthesis of oligoethylene oxide alkenes without phenyl rings (xs = in excess).

m), 4.57 (2H, t), 4.15 (2H, t), 5.1 (2H, dd), 6.0 (1H, m), 6.4 (2H, bs). Analysis for $C_{16}H_{24}O_5$; calculated: C, 64.86; H, 8.33; found: C, 64.54; H, 8.42.

*3,6,9-Trioxa-11-dodecene (**6**) (Scheme 4).* Freshly distilled diethylene glycol monoethyl ether (15 g, 0.112 mol) and 2.57 g of sodium were stirred and refluxed overnight in 50 ml of glyme. Allyl bromide (12.6 ml, 0.146 mol) was then added and the mixture was heated at reflux overnight. The resulting mixture was cooled and extracted with 200 ml of ether. The ether layer was washed with 100-ml portions of brine until the aqueous layer was neutral. The ether layer was dried over anhydrous magnesium sulfate and the solvent was removed under vacuum to give 13.5 g of an oil. The oil was distilled to give 8.5 g (43%) of **6**; b.p. 44°C/0.225 mm; NMR (δ): 1.05 (3H, t), 3.4 (10H, bm), 3.85 (2H, d), 5.05 (2H, m), 5.75 (1H, m). Analysis for $C_9H_{18}O_3$; calculated: C, 62.04; H, 10.41; found: C, 61.94; H, 10.47.

*2,5,8,11-Tetraoxa-13-tetradecene (**7**) (Scheme 4).* Compound **7** was prepared as **6** above using 10 g (0.061 mol) of triethylene glycol monomethyl ether (Fluka) and 1.4 g of sodium. The solution was refluxed about 3 h, and 6.9 ml (0.079 mol) of allyl bromide was added. This crude product (5.5 g) was distilled to give 4.1 g (33%) of **7**; b.p. 57°C/0.05 mm; NMR (δ): 3.1 (3H, s), 3.35 (12H, bm), 3.74 (2H, d), 5.05 (2H, m), 5.65 (1H, m). Analysis for $C_{10}H_{20}O_4$; calculated: C, 58.80; H, 9.87; found: C, 58.61; H, 9.64.

*1-(3-Butenyl)-3,6,9-trioxaundecan-1,11-diol (**13**) (Scheme 4).* Dry dioxane (250 ml) and 1.54 g of sodium hydride (60% dispersion) were placed in a large three-necked round bottom flask with 140 ml of triethylene glycol (1.1 mol). The solution was allowed to stir at room temperature until the sodium hydride was dissolved, and then the solution was heated to reflux. 1,2-Epoxy-5-hexene (28.7 ml, 0.26 mol) was added over a 1.5-h period. The mixture was stirred for two days at reflux temperature, after which it was cooled and made slightly acidic with dilute hydrochloric acid, and then neutral with saturated sodium bicarbonate solution. The mixture was then filtered and the solvent was removed to give an oil which was distilled to give 16.65 g (26%) of 1-(3-butenyl)-3,6,9-trioxaundecan-1,11-diol; b.p. 144°C/0.20 mm.

*3-(3-Butenyl)-2,5,8,11,14-pentaoxapentadecane (**8**) (Scheme 4).* DMSO (stored over molecular sieves) (80 ml) and 18 g of powdered potassium hydroxide were placed in a round bottom flask and stirred for 20 min. Compound **13** (10 g, 0.04

mol) was added along with 10 ml (0.080 mol) of methyl iodide. The solution was stirred overnight, poured into 300 ml of water, and extracted with three 200-ml portions of dichloromethane. The combined extracts were washed with four 200-ml portions of dichloromethane and four 200-ml portions of water. The solution was dried over anhydrous magnesium sulfate and the solvent was removed to yield 10.7 g (96%) of an oil. NMR: 1.58 (2H, m), 2.10 (2H, m), 3.30 (3H, s), 3.40 (3H, s), 3.6 (14H, bm), 5.05 (2H, bm), 5.3 (1H, s), 5.8 (1H, bm). Analysis for $C_{14}H_{28}O_5$; calculated: C, 60.84; H 10.21; found: C, 60.67; H, 10.33.

Column preparation and evaluation

Capillary columns for GC and SFC were prepared according to the method described by Jones *et al.*[7]. The fused-silica columns were deactivated with cyanopropylhydrosiloxane deactivation reagent[8], and the oligoethylene oxide stationary phases were immobilized using azo-*tert.*-butane or dicumyl peroxide free radical initiator. GC column evaluations were performed with a Carlo Erba 5160 Mega Series GC using hydrogen gas at 50 cm s^{-1} starting linear velocity and a flame ionization detector. SFC column evalutations were performed using a Lee Scientific 501 supercritical-fluid chromatograph with a flame ionization detector using CO_2 as carrier fluid at 2 cm s^{-1} starting linear velocity and using a frit restrictor (Lee Scientific, Salt Lake City, UT, U.S.A.). The injected sample amounts and the detector attenuation settings were adjusted to give 1–10 ng full-scale response.

RESULTS AND DISCUSSION

The search for a stationary phase which possesses many of the desirable properties of a PEG such as Carbowax, led to the investigation of a series of oligoethylene oxide-containing polysiloxane stationary phases (see Fig. 1). The objectives in preparing the oligoethylene oxide-substituted polysiloxanes were: first, to lower the glass transition temperature (t_g), making the phase useful at lower temperatures; second, to extend the upper temperature limit, increasing the utility of the phase in GC; third, to produce a phase that was inherently crosslinkable, allowing the phases to be highly reproducible from batch to batch and allowing the crosslinked phase to be physically stable for both high temperature GC and SFC use; and finally to find the optimum structure to give the best selectivity for chosen test solutes.

The first two objectives were achieved by grafting the alkenes onto preformed polysiloxanes containing Si–H sites (see Scheme 1). This provided a route to many structurally similar compounds which all possessed the advantages of the polysiloxane backbone, *i.e.* thermal stability and high flexibility, which tends to lower the glass transition temperature. The chromatographic testing of phase **1a** indicated that in fact, these new materials possessed superior physical properties over PEGs. The temperature range for this material was found to be 20–300°C.[5] The low bleeding of this stationary phase is demonstrated in Fig. 2 in which a paraffin wax extract was eluted during a temperature program up to 300°C, resulting in a baseline rise of only 1 pA.

The third objective, to have a crosslinked phase, was achieved by including a measured amount of 1-octene in the hydrosilylation reaction along with the oligoethylene oxide-containing alkene (see Scheme 1). This results in a polymer which contains from 2–5% octyl substitution and greatly enhances the crosslinkability of

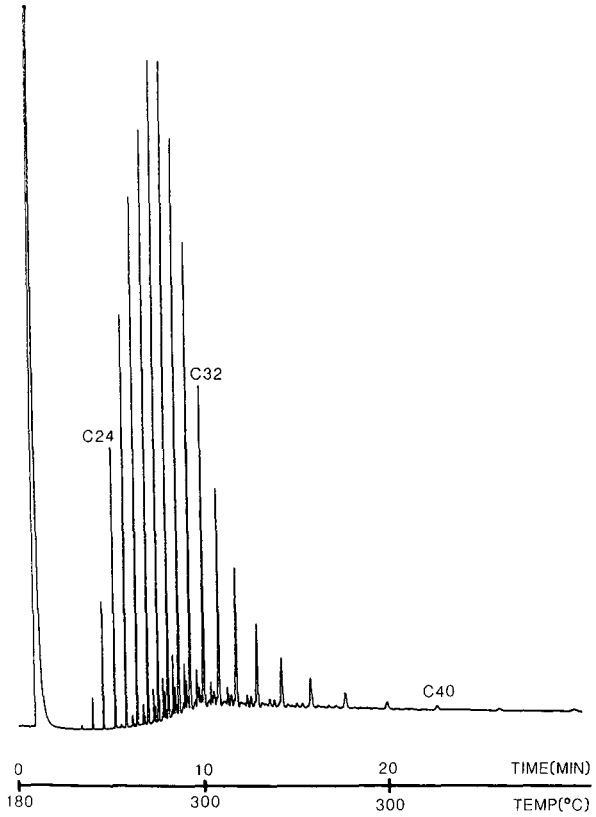


Fig. 2. Gas chromatogram of a paraffin wax oil extract on oligoethylene oxide stationary phase **1a**. Conditions: 10 m × 200 μm I.D. column with a 0.25-μm film thickness.

the phase[6], therefore providing much needed long term physical stability when the phase is used in both GC and SFC. The octane side chain had another beneficial effect. When the polymer was prepared by hydrolysation without octene, the polymer precipitated from the hot reaction mixture[5]. Even lower molecular weight polymers, when hydrosilylated without some octene present, behaved in a similar manner. One of the gelled polymers was pressure filtered through a 2-μm filter, leaving no residue, and the solution gelled again upon standing at room temperature for several hours. Even though the solution could be filtered, it could not be coated. This polymer property could be due to the strong interactions between individual substituents on the polysiloxane backbone, resulting in a thixotropic gel. The addition of octane substituents made the polymers dissolve nicely and retain a homogeneous consistency. The inclusion of the octene in slightly different amounts from batch to batch was found to have no measurable effect on the chromatographic properties of the phases[7].

The major differences in the chemical structures that were studied included: (1) presence or absence of a phenyl group between the hydrocarbon spacer and the oligoethylene oxide chain, (2) presence or absence of a methoxy group on the above

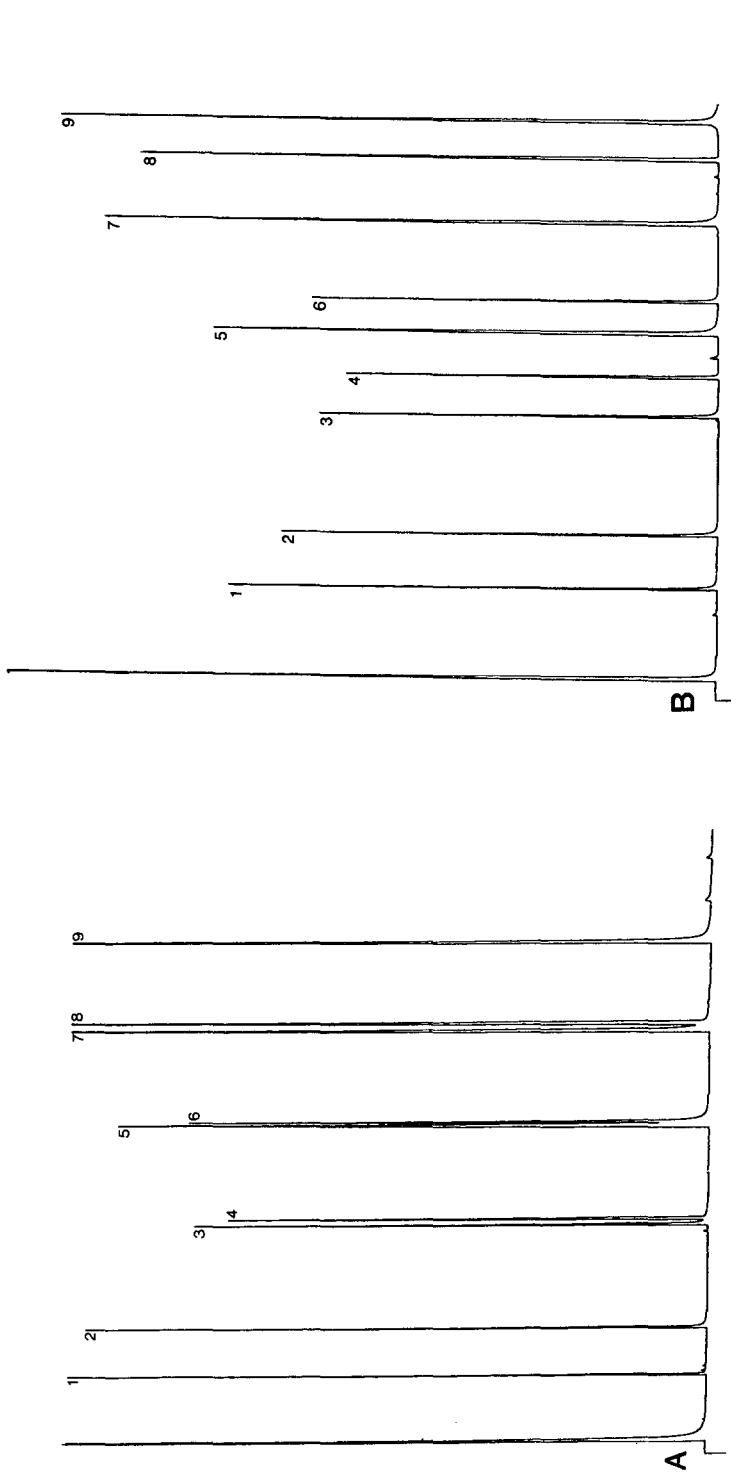


Fig. 3. Gas chromatogram of an alcohol standard mixture on oligoethylene oxide stationary phases **3a** (A) and **1a** (B). Conditions: 10 m × 200 μm I.D. columns with 0.25 μm film thickness. Peak identifications: 1 = 2-octanol; 2 = 1-octanol; 3 = 1-decanol; 4 = hexadecane; 5 = 1,6-hexanediol; 6 = 1-dodecanol; 7 = 1,8-octanediol; 8 = eicosane; and 9 = 1,10-decanediol.

mentioned phenyl group, (3) number of oxygens in the oligoethylene oxide chain, and (4) methyl or ethyl end groups on the oligoethylene oxide chain. The preferred oligoethylene oxide-containing polysiloxanes have a phenyl unit in the side chain (**1a–5a**). These materials were found to have higher thermal stability than those that did not have a phenyl unit in the chain (*e.g.* phases **6a–8a**). These latter phases showed serious degradation above 240°C. The higher stability is due to many factors, however it is possible that the phenyl groups afford some steric protection to the polysiloxane backbone.

The alkenes needed to prepare the different polysiloxane phases shown in Fig. 1 were prepared as shown in Schemes 2–4. The best method to join the oligoethylene oxide group to the benzene ring employed sodium or potassium hydroxide in DMSO to remove the proton from 4-bromophenol, and then slow addition of the tosylate (Scheme 2). It was possible to run the complete set of reactions in one pot with mechanical stirring as follows: the tosylate of the monomethyl ether was formed using hydroxide and DMSO, the phenoxide was prepared in a separate container also in DMSO and slowly added to the tosylate. The resulting solution was allowed to react at 45–65°C for 3–5 h. The allyl group was then attached by forming a Grignard reagent in THF and trapping the Grignard with allyl bromide.

The procedure shown in Scheme 2 to prepare the alkene containing both a benzene ring and the oligoethylene oxide unit is not synthetically convenient because of the need to use a Grignard reaction to attach the allyl group. A more convenient method is to prepare the oligoethylene oxide-containing alkenes from eugenol (4-allyl-2-methoxyphenol) as shown in Scheme 3. Oxide materials **3** and **4** are similar to **1** and **2**, respectively, except for the added methoxy substituent on the benzene ring. Excellent yields of both **3** and **4** were realized. Care must be taken to exclude all acid before the final distillation step in the purification of **3** and **4**. Acid causes the allyl group to isomerize to form a methylvinyl substituent.

Polysiloxane phase **3a**, which was prepared using the more convenient synthetic pathway, was found to provide somewhat different performance than phase **1a**. The only structural difference between **1a** and **3a** is the methoxy substituent on the benzene ring of **3a**. Phase **3a** was tested in detail and compared to phase **1a**.

Fig. 3 shows chromatograms of an alcohol standard mixture separated on phases **3a** (A) and **1a** (B), respectively. Both phases demonstrate comparable retention of polar solutes. This is shown by the retention times of the diols of peak numbers 5, 7 and 9. The alkenes (peaks 4 and 8) on the other hand, are less retained on methoxy-substituted phase **3a**. High-molecular-weight alcohols are also less retained on phase **3a** as their alkane chains get longer. Phase **3a** would indicate higher polarity than phase **1a** if the phases were compared using a retention index scale.

Phase **3a** was found to have low thermostability before it was crosslinked. At 210°C the bleed from the uncrosslinked phase was 2 pA using a flame ionization detector and a 10 m × 200 μm I.D. column having a 0.15-μm polymer film thickness. A dynamic free radical initiated crosslinking procedure using dicumyl peroxide improved the thermal stability of phase **3a** to 260–270°C. The solute retention properties of crosslinked **3a** was changed unless mild crosslinking conditions were used. Phase **3a** is not as thermally stable as **1a** and, therefore, has no advantage over **1a** except for its ease of preparation. No further tests of phase **3a** for use in GC were carried out.

Phase **5a**, with two methoxy groups on the benzene ring, exhibited poor GC

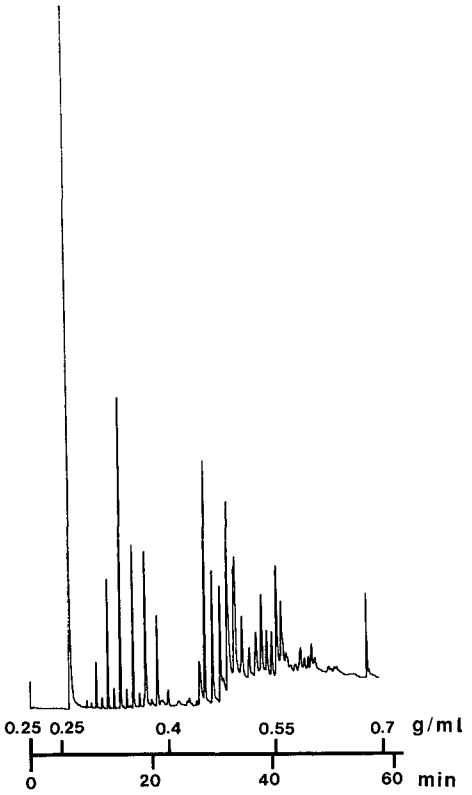


Fig. 4. Supercritical-fluid chromatogram of a beeswax extract on oligoethylene oxide stationary phase **3a**. Conditions: CO_2 at 120°C, 9 m × 50 μm I.D. capillary column with 0.25 μm film thickness.

performance in all aspects of efficiency, selectivity and thermostability and was not tested further. It is obvious, from the above results, that having methoxy groups on the benzene ring in phases **3a** and **5a** significantly affects the selectivity and thermal stability of the phase.

Fig. 4 shows a capillary supercritical-fluid chromatogram of a beeswax extract using phase **3a**. After crosslinking, this phase remained stable under stringent SFC conditions for several weeks. Also, the limited thermostability of the phase as mentioned above is not a problem for use in SFC. The non-polar hydrocarbon series, in the early part of the chromatogram, and the medium polar long chain ester series starting to elute after 25 min in the chromatogram, are both well resolved.

Removal of the benzene ring from these phases greatly lowers their stability as shown by the fact that phases **6a–8a** could not be used above 240°C and showed severe bleeding from around 180°C. Also, the addition of another ethylene oxide unit at the end of the arm as in phases **2a** and **4a** resulted in decreased thermostability without any measurable gain in selectivity.

The importance of having a benzene ring in a medium polar oligoethylene oxide-substituted polysiloxane phase has been demonstrated in this systematic study of the structural influence on the chromatographic performance of these oligoethylene

oxide phases. New phases containing different substituents at the end of the oligoethylene oxide chain are presently under study to see if stable phases with unique selectivities can be prepared based on the phenyl-containing oligoethylene oxide polysiloxane structure.

ACKNOWLEDGEMENTS

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REFERENCES

1 W. Millen and S. Hawkes, *J. Chromatogr. Sci.*, 15 (1977) 148.
2 J. S. Bradshaw, R. S. Johnson, N. W. Adams, M. A. Pulsipher, K. E. Markides and M. L. Lee, *J. Chromatogr.*, 357 (1986) 69.
3 J. C. Kuei, B. J. Tarbet, W. P. Jackson, J. S. Bradshaw, K. E. Markides and M. L. Lee, *Chromatographia*, 20 (1985) 25.
4 P. Silvis, J. W. Walsh and D. M. Shelow, *Am. Lab. (Fairfield, Conn.)*, 19 (1987) 41 (Review).
5 C. A. Rouse, A. C. Finlinson, B. J. Tarbet, J. C. Pixton, N. M. Djordjevic, K. E. Markides, J. S. Bradshaw and M. L. Lee, *Anal. Chem.*, 60 (1988) 901.
6 J. M. Bayona, B. J. Tarbet, H.-C. Chang, C. M. Schregenberger, M. Nishioka, K. E. Markides, J. S. Bradshaw and M. L. Lee, *Int. J. Environ. Anal. Chem.*, 28 (1987) 263.
7 K. Jones, C. A. Rouse, A. C. Finlinson, B. J. Tarbet, K. E. Markides, J. S. Bradshaw and M. L. Lee, in preparation.
8 K. E. Markides, B. J. Tarbet, C. M. Schregenberger, J. S. Bradshaw, M. L. Lee and K. D. Bartle, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 741.

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COMPARISON OF VARIOUS NON-POLAR STATIONARY PHASES USED FOR ASSESSING LIPOPHILICITY

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SUMMARY

Two non-polar stationary phases, *i.e.*, a novel octadecylpolyvinyl (ODP) packing and a poly(styrene–divinylbenzene) (PLRP-S) gel, were compared with a standard phase (octadecylsilane, ODS) for their usefulness in determining lipophilicity. Various monosubstituted benzenes and neuroleptic drugs were used as solutes. The usefulness of the polystyrene divinylbenzene phase was limited by physical problems, long retention times and capacity factors which did not appear to express the same partitioning behaviour as in an octanol–water system. In contrast, the ODP phase proved very interesting. Like the ODS phase, it showed high selectivity and fast elution, and yielded retention data which reflect a partitioning behaviour comparable to that seen in an octanol–water system. It also proved superior to the ODS phase in that it did not require the addition of a masking agent.

INTRODUCTION

Since the pioneering work of Meyer[1] and Overton[2] on the narcosis of tadpoles, lipophilicity has become a major parameter in quantitative structure–activity relationships[3–6]. Although several calculation methods have been used to determine lipophilicity[7–9], its experimental determination remains crucial. A number of methods have been used to measure lipophilicity but only two are in common use today, namely the shake-flask method and reversed-phase high-performance liquid chromatography[10] (RP-HPLC). In the former method, the biphasic *n*-octanol–water system has been chosen as a standard owing to some analogies with biological systems[11]. The equilibrium of a solute between an organic and an aqueous phase defines the partition coefficient (expressed as log *P*). Time consumption, large errors caused by small impurities with strong chromophores and limitations to log *P* values in the range −2 to 4 are the main practical disadvantages of the shake-flask method. For extensive details on these limitations and shortcomings, the reader is referred to a review by Dearden and Bresnen[12].

In recent years, RP-HPLC has proved to be a successful alternative for assessing

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lipophilicity of drugs and other compounds. Alkylsilane-bonded phases, particularly the octadecyl type, are the non-polar stationary phases most frequently used. They are prepared by reacting the silanol groups of silica gel with organochloro- or organoalkoxysilanes[13]. However, a high proportion of silanol groups (up to 50%) remain unreacted owing to steric hindrance[14]. During the chromatographic process, such residual silanol groups can interact with basic solutes by means of so-called silanophilic interactions, resulting in severe peak tailing and a decreased number of theoretical plates[15]. These phenomena severely disturb the partitioning behaviour[16,17]. The addition of a masking agent, usually an amine modifier, attenuates the silanophilic interactions without always suppressing them. Triethylamine, *n*-decylamine and N,N-dimethyloctylamine are masking agents in common use[16–21].

Other types of bonded phases have been proposed for the RP-HPLC determination of lipophilicity, *e.g.*, phenyl-modified silica gels, which were suggested to be superior to the ODS phase for measuring the partition coefficients of aromatic compounds[22]. Despite these efforts, the octadecylsilane (ODS) stationary phase is still considered to be the most convenient[23], and is indeed the most frequently used.

In order to avoid the silanophilic interactions, a poly(styrene–divinylbenzene) packing (PLRP-S) was used to assess the lipophilicity of 30 aromatic acids, yielding values which were well correlated with partition coefficients calculated according to Rekker's method[24]. The hydrophobic matrix of the PLRP-S is formed by a network of linear chains of polstyrene interconnected with divinylbenzene molecules[25–27]. Steric exclusion, which is claimed to govern the mechanism of retention[28], in fact appears restricted to relatively large molecules; this implies that retention of solutes of ordinary size should be based solely on partitioning. A poly(styrene–divinyl) stationary phase has the advantage of being stable over a broad pH range, allowing the determination of capacity factors and dissociation constants of compounds with comparatively high pK_a values (above 7)[29,30]. On the other hand, PLRP-S columns have a comparatively small number of theoretical plates and undergo considerably shrinking and swelling, which shorten their life.

A rigid macropourous polyacrylamide-based packing having a chemically bonded octadecyl phase has recently been developed. According to Dawkins *et al.*[31], this stationary phase, like ODS and unlike PLRP-S, may be viewed as a hydrophobic layer attached to a rigid hydrophilic support. This material is considered to be a direct replacement for C_{18} silica packings with the additional advantage of providing long-term physical and chemical stability over a wide pH range (1–13).

Recently, an octadecylpolyvinyl copolymer packing (ODP) became commercially available. The ODP gel is obtained by reaction of stearoyl chloride with the hydroxyl group of a vinyl alcohol copolymer gel[32,33]. It is claimed to offer the advantages of both the ODS and the polystyrene phases without their respective disadvantages, in other words, to afford high resolution, a large number of theoretical plates, expanded applicability with efficient separation of basic substances, stability over a wide eluent pH range and absence of silanophilic interactions, swelling and shrinkage. This phase is considered to be very stable chemically but physically weaker than silica gels.

To the best of our knowledge, the above-mentioned phases have never been objectively compared for their ability to assess lipophilicity. This study was therefore undertaken with the aim of comparing ODS, PLRP-S and ODP stationary phases for

their ability to measure lipophilicity. A correlation between retention data and octanol–water partition coefficients was established using a wide range of solutes of known lipophilicity. In addition, the ODS packing was used with and without a masking agent (*n*-decylamine).

EXPERIMENTAL

Chemicals

Monosubstituted benzenes (28 compounds) of the best available purity were obtained from Fluka (Buchs, Switzerland). Samples of neuroleptic drugs of pharmaceutical purity were kindly donated by various pharmaceutical companies. Analytical-reagent grade methanol and 3-morpholinopropanesulphonic acid (MPS) were obtained from Merck (Darmstadt, F.R.G.).

Chromatographic equipment

A Siemens S101 chromatograph equipped with an Orlita DMP-AE 10.4 pump was used. The detector was a Uvikon 740 LC (Kontron) operating at 254 nm. A Hewlett-Packard integrator was used for peak registration and calculation of retention times.

Columns

The ODS column (25 cm × 4 mm I.D.) was prepacked with LiChrosorb RP-18, particle size 10 μm (Knauer, Berlin, F.R.G.). The PLRP-S column (15 cm × 4.6 mm I.D.) was filled with macroporous, rigid spherical particles (5 μm) made of poly(styrene–divinylbenzene). The ODP column (15 cm × 6 mm I.D.) was prepacked with the octadecyl copolymer gel, particle size 5 μm (Asahi Chemicals, Kawasaki, Japan).

Mobile phase preparation

The mobile phases were prepared volumetrically from combinations of methanol and aqueous MPS buffer (0.02 *M*, pH 7.4) in the range 10–90%. All solutions were purified by filtration using a Milli-Q system (Millipore). All measurements were performed at room temperature (21 ± 1°C). The flow-rate was 1.5 ml/min for the ODS column and 0.8 ml/min for the PLRP-S column; for the ODP column it was 1.5 ml/min with the monosubstituted benzenes and 1.3 ml/min with the neuroleptic drugs. Isocratic capacity factors, k_i, were defined as

$$k_i = (t_R - t_0)/t_0 \quad (1)$$

where t_R is the retention time of the solute and t_0 is the column dead time determined using methanol as the non-retained compound.

RESULTS AND DISCUSSION

In agreement with previous results[34], the increase in log k_i values with decreasing methanol concentration in the eluent was linear with the ODS packing. Indeed, the straight lines of log k_i *versus* methanol concentration display r^2 values ranging from

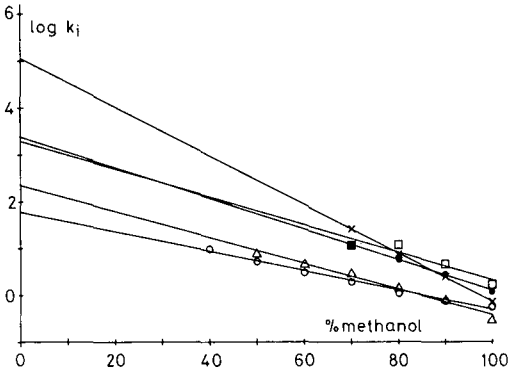

Fig. 1. Linear relationship between the isocratic capacity factors and the percentage of methanol in the mobile phase for five typical monosubstituted benzenes using the PLRP-S column. ×, Trifluoromethylbenzene; ●, N-methylaniline; □, chlorobenzene; △, aniline; ○, methyl phenyl sulphoxide.

0.9846 to 0.9983 without added decylamine, and from 0.9879 to 0.9996 in the presence of decylamine. Figs. 1 and 2 show that a linear increase in log k_i values is also observed with the PLRP-S and ODP phases, with r^2 values ranging from 0.9570 to 0.9995 with the former and from 0.9644 to 0.9986 with the latter phase. The log k_i values could thus be extrapolated linearily to 100% water content, yielding the log k_w values reported in Table I for the three stationary phases. Partition coefficients in the *n*-octanol–water system are also reported.

ODS stationary phase

The relationship between *n*-octanol–water partition coefficients (log *P* values) and the extrapolated capacity factors (log k_w values) determined using the ODS packing wih (log k_w[ODS/d] values) and without (log k_w[ODS] values) a masking agent are reported in eqns. 2 and 3, respectively.

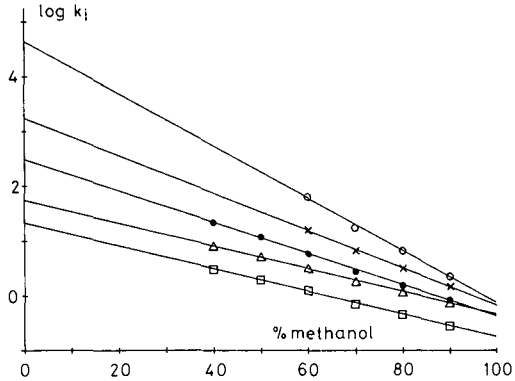

Fig. 2. Linear relationship between the isocratic capacity factors and the percentage of methanol in the mobile phase for five typical monosubstituted benzenes using the ODP column. ○, Biphenyl; ×, thioanisole; ●, methyl benzoate; △, benzaldehyde; □, benzyl alcohol.

$$\log P = 1.07(\pm 0.08) \log k_w[\text{ODS/d}] - 0.124(\pm 0.176) \quad (2)$$
$$n = 28; \quad r = 0.982; \quad s = 0.185; \quad F = 721$$

$$\log P = 1.21(\pm 0.12) \log k_w[\text{ODS}] - 0.587(\pm 0.270) \quad (3)$$
$$n = 28; \quad r = 0.971; \quad s = 0.236; \quad F = 430$$

The good relationship between these log k_w values and log P values[35] confirms previous findings[34,36]. As expected with neutral solutes, the use of *n*-decylamine only slightly improved the correlation. Indeed, the relationship would not be so good in the absence of a masking agent had basic compounds been included in the study (see later). Further, several workers have claimed that the polar character of silanol groups is essential for a non-polar stationary phase to mimic the properties of *n*-octanol by adsorbing both water and methanol molecules[37,38], as the stationary phase is viewed as a dynamic system made of silica substrate, linked organic chains and associated solvent molecules[39–41]. This reasoning ignores the abnormal behaviour of sulphonyl-containing solutes, which may interact very strongly with silanol groups and yield misleading log k_w values[34]. An additional shortcoming is that protonated masking agents must be avoided when investigating ionized acidic compounds (none of which was incorporated in the present study) giving either to ion-pair formation or to an ion-exchange mechanism[42].

PLRP-S stationary phase

The PLRP-S phase was characterized by a comparatively small number of theoretical plates and suffered from excessive shrinkage and swelling. The flow-rate was limited to 0.8 ml/min and the pressure increased dramatically at the column inlet. Retention times were inconveniently long, and only eluents with high methanol proportions could be used. Thus, no more than five compounds could be investigated with eluents containing down to 40% methanol (see Fig. 1). A dense structure and small particle size (5 μm) render PLRP-S an extremely compact stationary phase, and a pressure superior to 300 bar would be necessary to reach a flow-rate of 1.5 ml/min. It can be observed in Table I that the PLRP-S phase yields retention times that are significantly longer than those obtained with the other stationary phases. This may be related to problems of mass transfer due to the highly compact stationary phase.

The extrapolated capacity factors (log k_w[PLRP-S]) obtained with the PLRP-S stationary phase gave the following relationship with log P values:

$$\log P = 0.771(\pm 0.107) \log k_w[\text{PLRP-S}] - 0.708(\pm 0.391) \quad (4)$$
$$n = 28; \quad r = 0.946; \quad s = 0.321; \quad F = 221$$

This is a relatively unsatisfactory correlation, which can perhaps be partly explained by the technical problems mentioned above. An additional explanation may be that the PLRP-S phase does not possess polar groups such as the silanol groups in the ODS phase. As a consequence, the partitioning process will be limited to a hydrophobic mechanism, whereas lipophilicity is the sum of polar and hydrophobic contributions[43].

TABLE I

CAPACITY FACTORS EXTRAPOLATED TO 100% WATER OF 28 MONOSUBSTITUTED BENZENES DETERMINED WITH VARIOUS REVERSED-PHASE COLUMNS

No.	*Compound*	*Log k_w[ODS/d]*[a]	*Log k_w[ODS]*[b]	*Log k_w[PLRP-S]*[c]	*Log k_w[ODP]*[d]	*Log P*[e]
1	Aniline	0.95	1.13	2.34	1.46	0.90
2	Phenol	1.29	1.23	2.35	1.81	1.46
3	Benzaldehyde	1.54	1.67	3.03	1.74	1.45
4	Acetanilide	1.17	1.45	2.11	1.52	1.16
5	Benzamide	0.81	1.04	1.55	1.24	0.64
6	Benzyl alcohol	1.05	1.32	2.11	1.33	1.10
7	2-Phenylethanol	1.43	1.73	2.69	1.93	1.36
8	Benzonitrile	1.45	1.85	3.33	2.35	1.56
9	Benzenesulphonamide	0.78	0.90	1.61	1.12	0.31
10	Nitrobenzene	1.70	2.00	3.62	2.62	1.85
11	N-Methylaniline	1.51	1.74	3.38	2.26	1.66
12	N,N-Dimethylaniline	2.28	2.36	4.02	2.87	2.31
13	Phenyl acetate	1.57	2.04	3.28	2.20	1.49
14	Methyl benzoate	2.15	2.26	3.81	2.48	2.12
15	Thioanisole	2.72	2.71	4.76	3.23	2.74
16	Methyl phenyl sulphoxide	0.76	1.33	1.77	0.68	0.55
17	Methyl phenyl sulphone	0.89	1.29	2.20	1.18	0.49
18	Anisole	2.01	2.20	3.59	2.46	2.11
19	Benzene	1.91	2.08	3.88	2.40	2.13
20	Fluorobenzene	2.07	2.18	4.09	2.93	2.27
21	Chlorobenzene	2.72	2.75	4.54	3.25	2.84
22	Bromobenzene	2.89	2.89	4.76	3.63	2.99
23	Iodobenzene	3.14	3.16	5.06	3.89	3.25
24	Toluene	2.62	2.62	4.54	3.25	2.73
25	Trifluoromethylbenzene	3.11	3.11	3.16	3.68	2.79
26	Biphenyl	3.92	3.88	5.99	4.63	4.09
27	Benzophenone	3.03	3.11	4.58	3.71	3.18
28	Naphthalene	3.29	3.22	5.15	3.94	3.30

[a] Log k_w[ODS/d] is the lipophilic index extrapolated linearly to 100% water using the ODS column, with *n*-decylamine as a masking agent.
[b] Log k_w[ODS] is the lipophilic index extrapolated linearly to 100% water using the ODS column without any masking agent.
[c] Log k_w[PLRP-S] is the lipophilic index extrapolated linearly to 100% water using the PLRP-S column.
[d] Log k_w[ODP] is the lipophilic index extrapolated linearly to 100% water using the ODP column.
[e] Log *P* is the logarithm of the *n*-octanol–water partition coefficient (data from Hansch and Leo[35]).

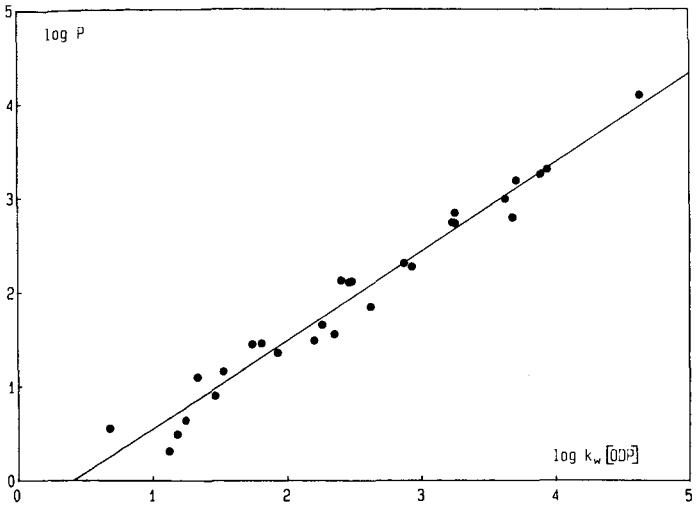

Fig. 3. Relationship between log k_w values of 28 monosubstituted benzenes (determined with the ODP phase) and partition coefficients (log P values) in the *n*-octanol–water system[29].

ODP stationary phase

The vinyl alcohol copolymer gel is very polar and is surface-modified with hydrophobic octadecyl groups[33]. Adsorption of both water and methanol can therefore be expected, resulting in a stationary phase that is viewed as being of dynamic nature[41,44]. Flow-rates of 1.3 and 1.5 ml/min were easily reached without too much pressure increase at the column inlet, and were chosen to obtain log k_w[ODP] values that were higher than log k_w[ODS] but lower than log k_w[PLRP-S] values (Table I). For thirteen compounds, log k_i values could be measured with eluents down to 40% methanol (Fig. 2).

The relationship between *n*-octanol–water partition coefficients and log k_w[ODP] values (Fig. 3) is given by the equation

$$\log P = 0.930(\pm 0.080) \log k_w[\text{ODP}] - 0.347(\pm 0.215) \quad (5)$$
$$n = 28; \quad r = 0.978; \quad s = 0.206; \quad F = 573$$

The correlation is as good as that obtained with the ODS phase (eqn. 3). Many advantages are therefore associated with the ODP phase. To assess its usefulness further, it was examined using a number of drugs the lipophilicity of which had previously been determined with the ODS column in the presence of *n*-decylamine[18].

Comparison of the lipophilicity of neuroleptics determined using the ODP and ODS phases

The ODS stationary phase has proved to be ineffective without a masking agent when studying basic compounds such as neuroleptics[18]. Although necessary in such instances, a masking agent has the disadvantages of introducing an additional variable into a standardized protocol.

TABLE II

LIPOPHILIC INDICES OF TWELVE NEUROLEPTIC DRUGS USING THE ODP AND ODS STATIONARY PHASES

Compound	*Log k_w[ODP]*	*Log k_w[ODS/d]*
Benperidol	3.84	3.65
Chlorpromazine	4.37	3.36
Clozapine	3.82	2.99
Flupentixol	5.21	4.33
Fluphenazine	4.87	4.31
Haloperidol	3.95	3.11
Mezilamine	2.83	2.64
Pipamperone	2.58	2.38
Sulpiride	1.53	0.61
Tefludazine	4.72	4.16
Thioridazine	4.82	3.90
Zetidoline	2.54	2.15

The ODP phase does not require a masking agent. In order to verify that the ODP phase is indeed capable of yielding a true measure of lipophilicity for basic compounds, we measured the log k_w[ODP] values of twelve neuroleptics and compared the results with log k_w[ODS/d] values (Table II):

$$\log k_w[\text{ODP}] = 1.02(\pm 0.21) \log k_w[\text{ODS/d}] + 0.567(\pm 0.676) \quad (6)$$
$$n = 12; \quad r = 0.962; \quad s = 0.331; \quad F = 122$$

Eqn. 6 indicates a reasonably good correlation, as also shown in Fig. 4. A better correlation was not to be expected as the two lipophilic phases are not totally identical

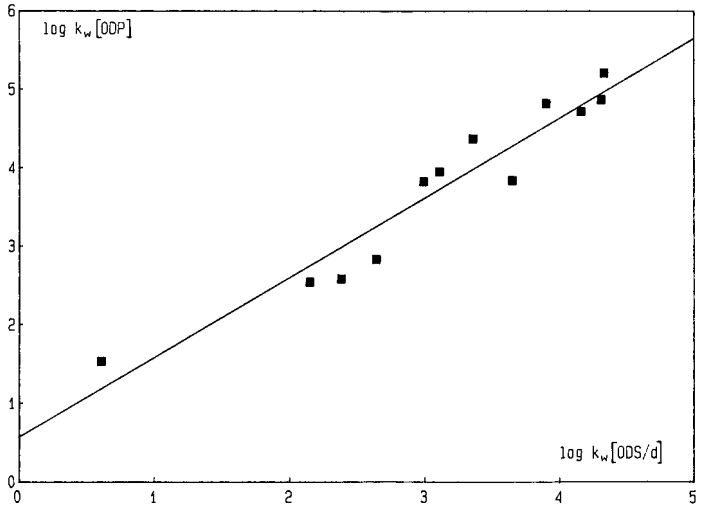

Fig. 4. Relationship between log k_w values of 12 neuroleptics determined with the ODP and ODS columns.

in terms of interactions between solute and stationary phase owing to the presence of a masking agent in one system only. Hence the log k_w values obtained with the two phases cannot be identical because the two sets of underlying intermolecular forces that they express are not identical. In this context, an open question concerns the compared hydrogen-bonding ability of the two phases towards solute molecules. The ODS phase is a strong hydrogen-bond donor and also a hydrogen-bond acceptor; the ODP phase, on the other hand, is a good hydrogen-bond acceptor, but its proportion of free hydroxyl groups and hence its hydrogen-bond donating capacity appear unknown at present and warrant specific studies. These questions, however, do not affect our conclusion that the ODP column is a valuable tool for determining the lipophilicity of basic drugs without the need to add a masking agent. A number of perturbative interactions can thus be avoided.

CONCLUSION

The ODS stationary phase is a selective and useful tool for assessing lipophilicity, and indeed is used extensively. However, its useable pH range is narrow, which restricts its applicability. In addition, the ODS phase can give rise to undesirable silanophilic interactions with ionized and basic compounds, thus necessitating a masking agent which imposes restrictions of its own.

The PLRP-S phase avoids silanophilic interactions and expands the usable pH range. However, of the three phases investigated, PLRP-S proved the least satisfactory for assessing lipophilicity owing to physical restrictions and because its retention mechanism differs from that of the two other phases. Indeed, its mechanism is based purely on hydrophobic interactions with the solute, whereas lipophilicity implies a combination of hydrophobic and electrostatic (polar) interactions[43,45].

The ODP packing indeed appears to combine the advantages of both the ODS and the PLRP-S phases without the disadvantages of either, *i.e.*, sharp resolution with a large number of theoretical plates, efficient separation of basic compounds without the need for a masking agent, stability over a wide pH range, reduced swelling and shrinkage and the possibility of having a reasonable flow-rate without undesirable pressure increases at the column inlet. The ODP stationary phase therefore offers a promising alternative to the ODS packing.

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REFERENCES

1 H. Meyer, *Arch. Exp. Pathol. Pharmakol.*, 42 (1899) 109.
2 E. Overton, *Z. Phys. Chem.*, 22 (1897) 189.
3 N. El Tayar, G. J. Kilpatrick, H. van de Waterbeemd, B. Testa, P. Jenner and C. D. Marsden, *Eur. J. Med. Chem.*, 23 (1988) 173.
4 J. K. Baker, D. O. Rauls and R. F. Borne, *J. Med. Chem.*, 22 (1979) 11.
5 T. Braumann, G. Weber and L. H. Grimme, *J. Chromatogr.*, 261 (1983) 329.
6 H. Terada, *Quant. Struct. Act. Relat.*, 5 (1986) 81.

7 R. F. Rekker and H. M. de Kort, *Eur. J. Med. Chem.*, 14 (1979) 479.
8 C. Hansch and T. Fujita, *J. Am. Chem. Soc.*, 86 (1964) 1616.
9 C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, Wiley, New York, 1979.
10 T. Braumann, *J. Chromatogr.*, 373 (1986) 191.
11 H. Kubinyi, *Prog. Drug Res.*, 23 (1979) 97.
12 J. C. Dearden and G. Bresnen, *Quant. Struct. Act. Relat.*, 7 (1988) 133.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979.
14 A. Nahum and Cs. Horvath, *J. Chromatogr.*, 203 (1981) 53.
15 D. Chan Leach, M. A. Stadalius, J. S. Berus and L. R. Snyder, *LC · GC Int. Mag. Liq. Gas Chromatogr.*, 1 (1988) 22.
16 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
17 R. N. Nikolov, *J. Chromatogr.*, 286 (1984) 147.
18 N. El Tayar, H. van de Waterbeemd and B. Testa, *J. Chromatogr.*, 320 (1985) 305.
19 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
20 R. Gill, S. P. Alexander and A. C. Moffat, *J. Chromatogr.*, 247 (1982) 39.
21 S. H. Hansen, P. Helboe and M. Thomsen, *J. Chromatogr.*, 409 (1987) 71.
22 J. L. G. Thus and J. C. Kraak, *J. Chromatogr.*, 320 (1985) 271.
23 M. C. Pietrogrande, F. Dondi, G. Blo, P. A. Borea and C. Bighi, *J. Liq. Chromatogr.*, 10 (1987) 1065.
24 T. Hanai, K. C. Tran and J. Hubert, *J. Chromatogr.*, 239 (1982) 385.
25 S. Coppi, A. Betti, C. Bighi, G. P. Cartoni and F. Coccioli, *J. Chromatogr.*, 442 (1988) 97.
26 F. Šmejkal, M. Popl, A. Číhová and M. Zázvorková, *J. Chromatogr.*, 197 (1980) 147.
27 D. J. Pietrzyck, E. P. Kroeff and T. D. Rotsch, *Anal. Chem.*, 50 (1978) 497.
28 M. K. L. Bicking and S. J. Serwon, *J. Liq. Chromatogr.*, 10 (1987) 1369.
29 V. de Biasi, W. J. Lough and M. B. Evans, *J. Chromatogr.*, 353 (1986) 279.
30 H. Miyake, F. Kitaura, N. Mizuno and H. Terada, *Chem. Pharm. Bull.*, 35 (1987) 377.
31 J. V. Dawkins, N. P. Gabbott, L. L. Loyd, J. A. McConville and F. P. Warner, *J. Chromatogr.*, 452 (1988) 145.
32 K. Yasukawa, Y. Tamura, T. Uchida, Y. Yanaghiara and K. Noguchi, *J. Chromatogr.*, 410 (1987) 129.
33 Y. Arai, M. Hirukawa and T. Hanai, *J. Liq. Chromatogr.*, 10 (1987) 634.
34 N. El Tayar, A. Tsantili-Kakoulidou, T. Röthlisberger, B. Testa and J. Gal, *J. Chromatogr.*, 439 (1988) 237.
35 C. Hansch and A. Leo, *Pomona College Medicinal Chemistry Project Log P and Parameter Database*, Issue 23, Comtex Scientific, New York, 1983.
36 K. Jinno and K. Kawasaki, *Chromatographia*, 18 (1984) 90.
37 T. L. Hafkenscheid and E. T. Tomlinson, *Int. J. Pharm.*, 16 (1983) 225.
38 W. E. Hammers, G. J. Meurs and C. L. de Ligny, *J. Chromatogr.*, 246 (1982) 169.
39 E. Slaats, W. Markowski, J. Fekete and H. Poppe, *J. Chromatogr.*, 207 (1981) 299.
40 C. R. Yonker, T. A. Zwier and M. F. Burke, *J. Chromatogr.*, 241 (1982) 257.
41 R. K. Gilpin, *J. Chromatogr. Sci.*, 22 (1984) 371.
42 J. L. M. van de Venne and J. L. H. M. Hendricks, *J. Chromatogr.*, 167 (1978) 1.
43 H. van de Waterbeemd and B. Testa, *Adv. Drug Res.*, 16 (1987) 85.
44 P. Jandera, *J. Chromatogr.*, 352 (1986) 91.
45 Ch. Repond, J. M. Mayer, H. van de Waterbeemd, B. Testa and W. Linert, *Int. J. Pharm.*, 38 (1987) 47.

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SILYLALDONITRILE DERIVATIVES FOR THE DETERMINATION OF SUGARS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A novel procedure for the derivatization of aldoses prior to gas chromatography–mass spectrometry is proposed. Reaction of the sugars with hydroxylamino-O-sulphonic acid and silylation of the hydroxyl groups yields silylaldonitrile derivatives, which give single chromatographic peaks and favourable mass spectrometric properties. The silylaldonitrile derivatives are easily separated by capillary gas chromatography and are suitable for both identification and quantification purposes.

INTRODUCTION

The determination of sugars by gas chromatography (GC) and GC coupled with mass spectrometry (GC–MS) has been accomplished with a number of derivatives, each possessing distinct advantages and drawbacks. In principle, an ideal derivative for GC–MS should yield a product eluting as a single peak, with intense and diagnostic high-mass ions in its mass spectrum. An additional useful feature in the spectra of polyhydroxy compounds is the occurrence of even-electron ion series, arising from α-cleavage next to each heteroatom-bearing carbon, which facilitates the recognition of functional groups on the carbon atoms. Among the commonly employed derivatives of simple sugars are methyl[1,2], silyl-[3] or acyl-protected[4,5] closed-ring acetals, which yield anomeric mixtures, their proportion depending largely on the reaction conditions. Some of these derivatives have been widely applied in the past for both structural and quantitative analysis.

Conversion of the oxo function to a suitable oxime derivative and silylation of the hydroxyl groups yields a mixture of *syn* and *anti* products[6,7], which can be separated by GC and give rise to intense C_6-containing ions in their mass spectra. A serious drawback of the methoxime-silyl (MO-TMS) derivatives is the excessive length of the GC run if the two isomers of each of glucose, mannose, galactose and fructose are to be separated[8].

Acetylated aldonitriles are obtained through dehydration of the corresponding aldoximes by means of acetic anhydride[9,10]. A single derivative is obtained for each aldose, and several can be separated in a chromatographic run, but the electron-

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impact (EI) mass spectra contain only two low-intensity, high-mass ions, resulting from the loss of the C_6 and C_6–C_5 fragments. This is of particular disadvantage if [6,6-$^2H_2$]glucose is to be employed in isotope dilution mass spectrometry (IDMS) or as a tracer in biochemical studies[11], as neither of these ions retains the carbon atom carrying the isotopic label.

Boronate cyclic esters[12] of many sugars are useful derivatives for GC–MS work, characterized by intense high-mass ions retaining the cyclic acetal moiety. The number of derivatives formed in the boroacetylation reaction is, however, strongly related to the stereochemistry and solution conformation of the molecule, and therefore the method does not appear to be suitable for general use in sugar derivatization.

Silylated aldonitriles were first described in the course of an investigation on cyanide addition to pentoses[13]. Such derivatives were reported to be not very stable, and no attempt was made to employ them for analytical purposes.

The aim of this work was to combine the advantages of two of the aforementioned derivatives, namely the single-peak sugar aldonitriles and the methyloxime-trimethylsilyl ethers, with C_6-containing fragments in their mass spectra. The silylated aldonitriles of various simple sugars were therefore synthetized and subjected to GC and GC–MS analyses.

EXPERIMENTAL

All solvents and reagents were of analytical-reagent or reagent grade and were used as received.

The sugars 2-deoxy-D-ribose (1), D-arabinose (2), D-xylose (3), D-lixose (4), D-ribose (5), 2-deoxy-D-glucose (6), D-galactose (7), D-mannose (8), D-gulose (9), D-allose (10), D-idose (11), D-altrose (12), D-talose (13), D-glucose (14) and D-glucuronic acid (15) were purchased from Sigma or obtained as pure standards.

Lyophilization and evaporation under vacuum were performed in a SpeedVac concentrator (Savant). All heating was accomplished with a Blok heater (Supelco).

GC analyses were performed on a Carlo Erba 4160 capillary gas chromatograph equipped with a split–splitless injector and flame ionization detector. A Waters 740 integrator was employed for recording the data. GC–MS analyses were performed on a Finnigan 4021 instrument with a Super-INCOS data system.

The capillary columns employed were of the bonded-phase fused-silica type: a Mega 25 m × 0.25 mm I.D. column coated with (5% phenyl–methyl)polysiloxane (SE-54; film thickness 0.4 μm) and a Chrompack 25 m × 0.32 mm I.D. column (50% phenyl–methyl)polysiloxane (CP-Sil-19CB; film thickness 0.1 μm). More polar columns were not tested, as the compromise between resolution, analysis time and column lifetime was judged to be sufficient with the above columns.

Synthesis of derivatives

An appropriate amount (50 μg–1 mg) of individual sugars or sugar mixture from water or methanol stock solutions was lyophilized in cone-bottomed glass tubes with PTFE-lined screw-caps. A 50–100-μl volume of the derivatizing reagent (a 1 *M* solution of hydroxylamine-O-sulphonic acid in methanol containing an equimolecular amount of triethylamine) was added and the solution reacted at room temperature for 30 min. The solvent was evaporated, 50 μl of bistrimethylsilyltrifluoroacet-

amide (BSTFA) and 50 μl of pyridine or acetonitrile were added and the suspension was heated at 80°C for 30 min.

When an internal standard was needed, as in the measurement of conversion efficiencies or retention time measurements, hexachlorobenzene (5 mg/ml in pyridine) was added in the silylation step.

RESULTS AND DISCUSSION

Derivatization

The silylated aldonitrile derivatives of several aldoses were prepared by a modification of the procedure of Fizet and co-workers[14,15], employing hydroxylamine-O-sulphonic acid, which they had applied to a variety of aliphatic, aromatic and heterocyclic aldehydes. The reaction scheme, exemplified for glucose, is depicted in Fig. 1. An earlier attempt to reproduce Fizet and co-workers' conditions, *i.e.*, carrying out the reaction in water at room temperature to 50°C, produced the silylated epimeric mixture as the only peaks in the GC run. Moreover, the reagent is scarcely, if at all, soluble in pyridine, and when a suspension was added to the sugars and reacted at 80°C for 30 min, as under the usual oxime formation conditions, a mixture containing the silylated anomers, in addition to the *syn–anti* isomers, was obtained. A very minor peak, eluting immediately before the oxime isomers, was also detected, and was also present when the silylation step was carried at temperatures higher than 100°C. It was therefore assumed that such a peak could be the expected product, formed through a thermally induced loss of silanol from the silyloxime moiety.

When an equimolar amount of a tertiary amine (pyridine or triethylamine) was added to a 1 *M* solution of hydroxylamine-O-sulphonic acid in methanol or acetonitrile, the pH of the solution increased to 5, in comparison with the value of 1 measured in water or methanol. This reagent was therefore employed for the derivatization,under a variety of conditions, which all led to the expected aldonitrile compounds, although in greatly different yields and with different rates.

Conversion efficiencies were measured for glucose in methanol and in acetonitrile and showed the conversion to be quantitative within 15 min in the former

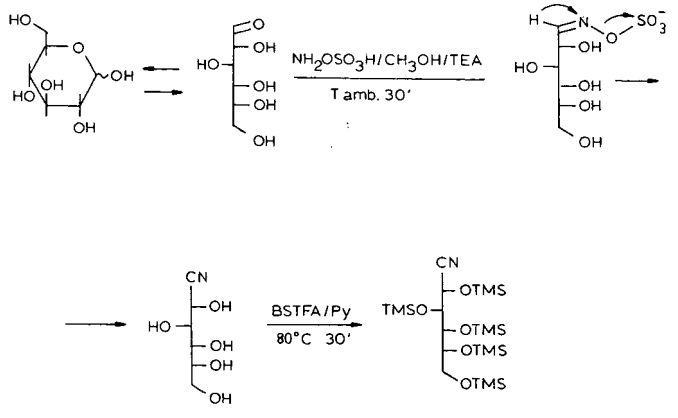

Fig. 1. Scheme of the derivatization reaction, exemplified for D-glucose.

solvent, whereas 16–24 h were necessary when the reaction was carried in acetonitrile. However, an advantage of employing acetonitrile as the reaction solvent is that lyophilization prior to silylation is unnecessary, and much less tanning of the reaction mixture occurs on storage of the samples, both at room temperature and at 4°C, in tightly closed vessels.

The reagent and the oxime-O-sulphonic acid intermediate are very prone to methanolysis when a molar excess of triethylamine is present, the measured pH being higher than 5, or when the reaction is carried out at temperatures over 40°C. Under these conditions, the silyloxime couple is formed instead of the silylaldonitrile. Further evidence of this explanation is that with aged solutions a sticky precipitate separates and the desired reaction does not occur. The derivatizing solution should therefore be used within a few hours after preparation.

The derivatized samples are stable (and can be employed for quantitative measurements) for several weeks even at room temperature, provided that the screw-capped vial is not opened.

The stability of glycosidic bonds to the reaction conditions was tested by subjecting β-methyl-D-mannopyranoside to the derivatization steps and analysing the products by gas chromatography. No mannose silylaldonitrile peak was detected, and the silyl derivative of the substrate compound was detected as the only product.

Gas chromatography

GC separation of several different aldohexoses and pentoses was accomplished on a moderately polar capillary column operated isothermally at 170–180°C (Fig. 2). At a 50:1 splitting ratio even the earliest eluting 2-deoxyribose derivative was well

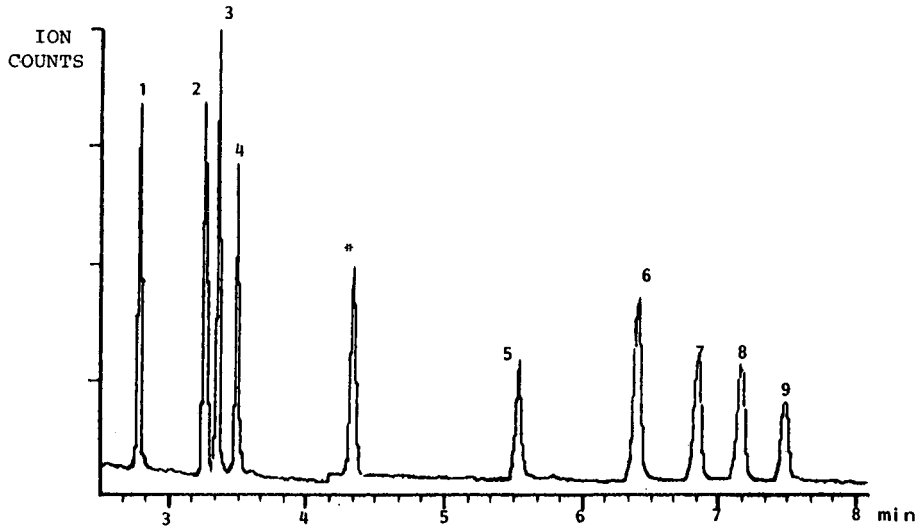

Fig. 2. Capillary GC separation of silylaldonitriles: 1, deoxyribose; 2, arabinose; 3, xylose; 4, ribose; 5, 2-deoxyglucose; 6, galactose; 7, mannose; 8, idose; 9, glucose. The peak marked with an asterisk is the internal standard (hexachlorobenzene). CP-Sil-19CB capillary column (25 m × 0.32 mm I.D., film thickness 0.1–0.15 μm); helium, 8 p.s.i.; injection temperature, 280°C; oven temperature, 180°C; coupled to a Finnigan 4021 quadrupole mass spectrometer continuously scanned between 33 and 650 u in 0.95 + 0.05 s; electron energy, 70 eV; emission current, 250 μA; multiplier voltage, 950 V.

TABLE I

RELATIVE RETENTION TIMES OF THE SILYLALDONITRILE DERIVATIVES OF VARIOUS SUGARS ON TWO COLUMNS

Isothermal operation at 180°C.

Compound	*SE-54*[a]	*CP-Sil-19CB*[b]
1	0.537	0.644
2	0.755	0.751
3	0.756	0.774
4	0.769	0.769
5	0.790	0.804
6	1.158	1.276
7	1.700	1.475
8	1.706	1.575
9	1.752	1.631
10	1.759	1.609
11	1.792	1.647
12	1.838	1.610
13	1.862	1.631
14	1.876	1.724
15	2.098	2.109

[a] Retention time of internal standard 4.525 min.
[b] Retention time of internal standard 4.537 min.

separated from the solvent peak. Differences in the elution order of the eight aldohexose steroisomers were noticed between the two phases, whereas all sugars were more retarded on the SE-54 column (Table I). Mannose and galactose could not be separated on the SE-54 column, whereas the three physiologically occurring aldohexoses

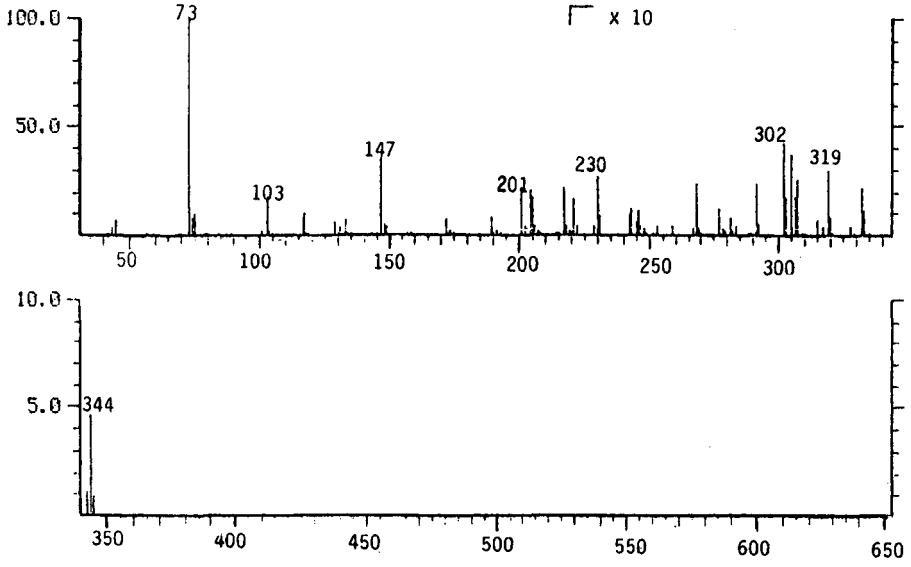

Fig. 3. 70-eV mass spectrum of D-glucose. GC–MS conditions as in Fig. 2.

(galactose, mannose and glucose), together with a diastereoisomeric internal standard (idose or gulose), were separately eluted from the CP-Sil-19CB column within 8 min. Temperature-programmed runs did not improve the separation of too closely eluting peaks.

Under the conditions used, less than 10 ng of glucose could be detected with flame ionization detection.

Mass spectrometry

The 70-eV EI mass spectrum of the glucose derivative is shown as an example in Fig. 3. The main features of the EI fragmentation parallel that of the corresponding MO-TMS derivatives, as reported by Laine and Sweeley[6,7], and their nomenclature for the fragment ions is employed here (Fig. 4 and Table II). Cleavages of the carbon skeleton α to each silyl-ether oxygen atom generate two series of even-electron oxonium ions, which can further eliminate trimethylsilanol, yielding the corresponding "prime"(') ions.

The difference in the behaviour of the two derivatives lies principally in the lower abundance of A′ and B′ fragments in the aldonitrile compounds. The smaller number of degrees of freedom (a cyano group *vs.* a methoxime) at C-1 can, of course, play a role in the more extensive fragmentation of these compounds in comparison with the MO-TMS derivatives. The derivatives of uronic acids yield ions that retain the silyl ester moiety, in addition to those related to the common part of the molecule. Other fragments, related to more complex cleavages of the silylated polyol chain, are also present (Fig. 5 and Table III).

In the spectrum of [6,6-$^2H_2$]glucose the 217/219 and 103/105 ion ratios are very close to that observed by Laine and Sweeley for the MO-TMS derivatives, thus confirming that a substantial non-specific hydrogen rearrangement takes place. This is also confirmed by the occurrence of the ion at *m/z* 103 in the derivative of the uronic acids. Spectra obtained at low electron energy (30–40 eV) show a prominent ion at *m/z* 201, which is probably generated from a weak precursor at *m/z* 291, by loss of

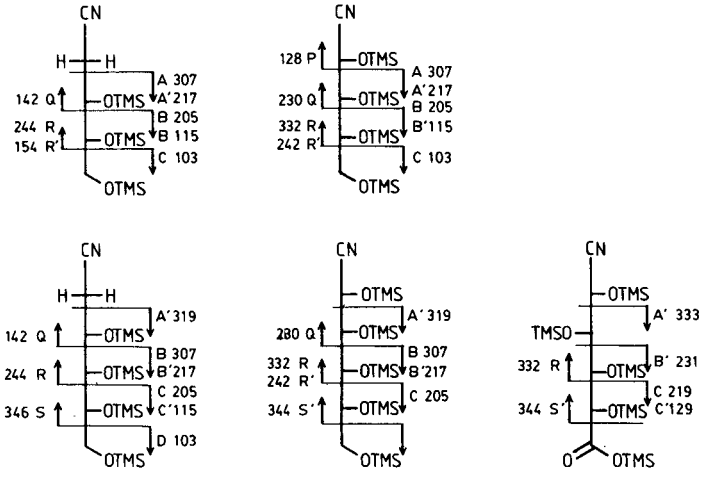

Fig. 4. Simple cleavage and silanol loss (primes) fragments in the spectra of silylaldonitrile derivatives.

TABLE II

FRAGMENT IONS FROM SIMPLE CHAIN FISSION AND SILANOL LOSS (PRIMES)

For the mass values of the fragments, see Fig. 4; the reported intensities are relative to *m/z* 73 = 100%.

Compound	*A*	*A'*	*B*	*B'*	*C*	*C'*	*D*	*Q*	*R*	*R'*	*S*	*S'*
1	–	5.72	13.30	–	6.91	–	–	6.11	4.20	–	–	–
2	1.62	11.57	17.62	–	15.76	–	–	0.67	1.20	0.76	–	–
3	1.70	12.88	14.64	–	16.11	–	–	0.84	1.22	0.66	–	–
4	2.40	11.60	10.82	–	19.51	–	–	0.65	0.93	–	–	–
5	3.42	15.11	13.23	1.01	19.63	–	–	0.78	0.55	1.01	–	–
6	–	–	2.16	13.91	17.26	–	15.29	5.75	4.18	–	2.70	–
7	–	3.98	2.53	24.54	16.29	–	16.82	3.12	1.05	–	–	7.29
8	–	5.71	2.59	21.16	18.66	–	16.83	3.12	1.61	–	–	6.61
9	–	–	1.23	10.50	11.24	–	16.14	1.79	–	0.42	–	4.26
10	–	6.84	1.42	14.34	16.47	–	13.87	3.18	0.56	0.69	–	1.63
11	–	3.84	3.55	23.32	15.98	–	18.04	2.83	3.07	–	–	6.00
12	–	4.14	1.48	13.31	15.62	–	15.34	2.61	0.61	0.67	–	4.43
13	–	2.99	1.71	11.50	11.45	–	14.23	2.76	0.51	0.46	–	4.67
14	–	2.99	2.63	21.31	17.52	–	17.23	2.70	2.19	–	–	4.67
15	–	7.15	–	0.69	2.47	2.94	–	–	2.65	–	–	0.88

trimethylsilanol. Such fragmentation is not shared by 2-deoxyaldoses, uronic acids and silylated polyols.

Applications

In order to explore the suitability of these derivatives to assist in the characterization of polysaccharides, lactose was hydrolysed under acidic conditions and the resulting sugar mixture was derivatized and subjected to GC analysis. Two peaks appeared in the chromatogram, corresponding to the expected galactose and glucose derivatives. A calibration graph ($r = 0.9989$) in the physiological range 50–350 mg per 100 ml was obtained for serum glucose, employing D-idose as the internal standard and flame ionization detection. This method could be suitable as a reference method

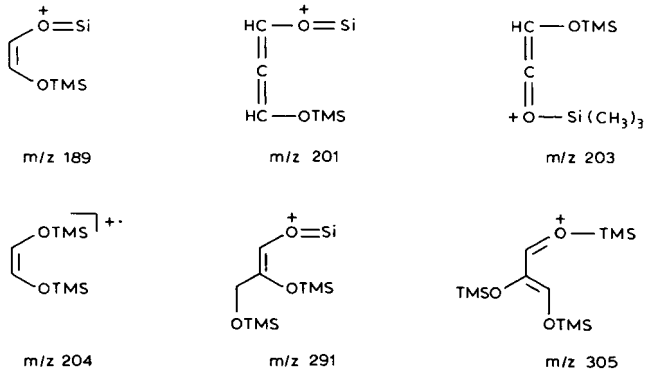

Fig. 5. Other important fragments in the spectra of silylaldonitriles.

TABLE III

OTHER IMPORTANT FRAGMENTS

For structures, see Fig. 5; intensity is relative to m/z 73 = 100%.

Compound	*Fragment ions (m/z)*					
	189	*201*	*203*	*204*	*291*	*305*
1	1.12	–	2.43	3.31	–	–
2	8.71	7.20	1.17	38.34	5.28	0.58
3	9.03	6.72	2.06	30.78	2.52	0.47
4	8.07	9.18	2.04	22.14	2.43	0.37
5	11.86	11.08	2.57	25.28	2.84	0.55
6	6.19	–	1.23	31.76	3.44	1.23
7	8.18	22.86	2.79	21.65	2.76	4.07
8	8.75	30.36	3.48	18.79	2.32	3.57
9	3.85	14.09	2.27	13.29	1.51	0.90
10	7.37	21.88	2.62	13.04	1.27	1.93
11	9.36	18.33	2.59	22.36	2.78	4.17
12	8.26	25.88	2.82	15.51	1.51	2.11
13	4.94	15.12	1.85	13.39	0.92	1.69
14	8.61	22.04	2.99	20.44	2.41	3.72
15	7.97	1.12	1.13	15.90	1.58	3.42

to assess the precision of glucose measurements in clinical chemistry. The use of an isomer rather than an isotopically labelled analogue and of simple capillary GC in the place of GC–MS would be highly cost effective, as pointed out by Kinter *et al.*[16] in a recent paper on serum cholesterol measurement.

CONCLUSIONS

Trimethylsilyl aldonitriles can be synthesized under mild conditions from aldoses carrying a variety of functional groups, and the derivatives of several sugars can be separated as single peaks in a short isothermal GC run. The mass spectra allow identification of the substituents on the carbon chain and contain ions suitable for IDMS quantification.

REFERENCES

1 A. G. McInnes, D. H. Ball, F. P. Cooper and C. T. Bishop, *J. Chromatogr.*, 1 (1958) 557.
2 J. Karkkainen, *Carbohydr. Res.*, 14 (1970) 27.
3 D. C. DeJong, T. Radford, J. D. Hribard, S. Hanessian, M. Bieber, G. Dawson and C. C. Sweeley, *J. Am. Chem. Soc.*, 91 (1969) 1728.
4 H. Bjorndal, B. Lindberg and S. Svensson, *Carbohydr. Res.*, 5 (1967) 433.
5 W. A. Koenig, H. Bauer, W. Voelter and E. Bayer, *Chem. Ber.*, 106 (1973) 1905.
6 R. A. Laine and C. C. Sweeley, *Anal. Biochem.*, 43 (1971) 533.
7 R. A. Laine and C. C. Sweeley, *Carbohydr. Res.*, 27 (1973) 199.
8 O. Pelletier and S. Cadieux, *Biomed. Mass Spectrom.*, 10 (1983) 130.
9 R. Varma, R. S. Varma and A. H. Wardi, *J. Chromatogr.*, 77 (1973) 222.
10 C. D. Pfaffenberger, J. Szafranek, M. G. Horning and E. C. Horning, *Anal. Biochem.*, 63 (1975) 501.
11 K. Y. Tserng and S. C. Kalhan, *Am. J. Physiol.*, (1983) E 476.

12 J. Wiecko and W. R. Sherman, *J. Am. Chem. Soc.*, 98 (1976) 7631.
13 R. Varma and D. French, *Carbohydr. Res.*, 25 (1972) 71.
14 C. Fizet and J. Streith, *Tetrahedron Lett.*, (1874) 3187.
15 J. Streith, C. Fizet and H. Fritz, *Helv. Chim. Acta*, 59 (1976) 2786.
16 M. Kinter, D. A. Herold, J. Hundley, M. R. Wills and J. Savory, *Clin. Chem.*, 34 (1988) 531.

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COMPUTERISED GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SEDIMENTARY BENZOPORPHYRINS

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SUMMARY

Recently developed gas chromatographic–mass spectrometric (GC–MS) and high-performance liquid chromatographic (HPLC) procedures for the analysis of petroporphyrins have been employed in the search for benzoporphyrins in six sediments, a bitumen and a petroleum. In addition, structurally assigned monobenzocycloalkanoporphyrins (benzo-CAPs) isolated from Boscan crude oil (Cretaceous, Venezuela) have been used in a co-chromatographic study of these minor components (*ca.* 1% of total porphyrins). The petroporphyrins are generally obtained by demetallation of metalloporphyrins from samples representing a variety of geographical locations and ages ranging from Permian (*ca.* 235 million years) to Palaeocene (*ca.* 60 million years). The data show that, where the benzo-CAPs were detected, up to 24 components can occur, although the dominant member in each case is the C_{33} compound. Studies of a chromatographic fraction from Boscan crude oil, enriched in high-carbon-number benzo-CAPs, indicates that the series extends from at least C_{30} to C_{38} in this sample. The GC behaviour of the benzo-CAPs as the bis(*tert.*-butyldimethylsiloxy)Si(IV) [$(TBDMSO)_2Si(IV)$] derivatives is characterised by the high Kováts retention indices (*ca.* 3900-4300; OV-1 column). Similarly, under normal-phase HPLC conditions, the free-base benzo-CAPs elute after the major alkylporphyrins. HPLC co-chromatography of the free-bases provides a rapid means of identification of the major benzo-CAPs (C_{32} and C_{33}), while the GC–MS procedure allows more detailed characterisation and quantitation of the benzo-CAP distributions as their $(TBDMSO)_2Si(IV)$ derivatives.

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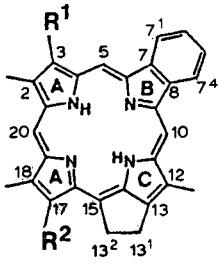
INTRODUCTION

Computerised gas chromatography–mass spectrometry (GC–MS) of porphyrins as their bis(*tert.*-butyldimethylsiloxy)Si(IV) [$(TBDMSO)_2Si(IV)$] derivatives has been demonstrated as a useful means of analysis of the complex mixtures found in sedimentary organic matter[1–5]. High-performance liquid chromatography (HPLC) of free-base porphyrins on 3-μm silica has been reported as a complementary method of analysis[6,7]. Both techniques provide a convenient means of identifying specific petroporphyrin components in complex mixtures via co-chromatography with compounds of known structure. Such an approach allows assignment of components without the necessity of time-consuming isolation of individual components. The structural assignment of selected members of the petroporphyrins is an essential step in the geochemical interpretation of the overall distributions[8].

The occurrence of so-called "rhodoporphyrins" (structural class[4] A-6, A-8) has been widely reported in petroporphyrin mixtures[9–13]. Their presence has been inferred from the distinctive rhodo-type electronic absorption spectra[9] and/or molecular ions of appropriate masses in MS[10] and GC/MS[5] studies. More recently, structural assignment of isolated rhodoporphyrin components from Boscan crude oil (Cretaceous, Venezuela), using ^1H NMR techniques, has identified them as monobenzocycloalkanoporphyrins(benzo-CAPs, 1–4, structural class[4] A-8).

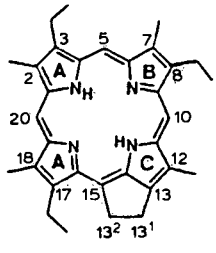


C_{32}(**2**) : R^1 = methyl , R^2=ethyl

C_{33}(**1**) : R^1 = ethyl , R^2=ethyl

C_{34}(**3**) : R^1 = ethyl , R^2=propyl

C_{35}(**4**) : R^1 = isobutyl , R^2=ethyl



(**5**)

We report here the occurrence of benzo-CAPs in a variety of petroporphyrin distributions via computerised GC–MS and HPLC analysis of the $(TBDMSO)_2Si(IV)$ derivatives and free-bases, respectively, and co-chromatography with components obtained from Boscan crude oil. Earlier studies of the petroporphyrins of Boscan oil[2,5] are extended by investigation of a fraction enriched in the high-carbon-number (> C_{34}) benzo-CAPs.

EXPERIMENTAL

Samples

The sediment samples investigated (Table I) were as follows: Gafsa chert (Palaeocene, Tunisia), Kimmeridge shale (Late Jurassic, U.K.), Serpiano shale (Middle Triassic, Switzerland), Julia Creek oil shale (Early Cretaceous, Australia), Marl slate (Permian, U.K.), El Lajjun shale (Late Cretaceous, Jordan), La Luna shale (Cretaceous, Venezuela); Gafsa chert is the least mature and La Luna the most mature sample. The other sediments are listed in a crude order of increasing maturity. The other samples were Gilsonite bitumen (Eocene, U.S.A.) and Boscan crude oil (Venezuela). The geological descriptions of the samples have been given elsewhere[15–22]. The depositional environments are all believed to be marine except for the source rock of Gilsonite bitumen, which is believed to have been deposited under saline lacustrine conditions[21].

TABLE I

GEOLOGICAL SAMPLES INVESTIGATED FOR PRESENCE OF BENZO-CAPs BY GC–MS AND/OR HPLC CO-CHROMATOGRAPHY STUDIES

N.D. = not determined; F.B. = free base.

Sample	*F.B. (%)*	*Ni (%)*	*V=O (%)*	*Fraction examined by*	
				GC–MS	*HPLC*
Boscan oil	0	5	95	Total	Total
La Luna shale	0	5	95	Total	Total
Gilsonite bitumen	0	95	5	Total	Total
Marl Slate	0	67	33	Ni, V=O	Ni
El Lajjun shale	0	57	43	Ni	N.D.
Julia Creek oil shale	0	5	95	Total	Total
Serpiano shale	0	5	95	Total	Total
Kimmeridge shale	0	95	5	Total	N.D.
Gafsa chert	95	5	0	F.B.	F.B.

Isolation of porphyrins

After pulverisation (Tema mill), the samples were Soxhlet extracted [72 h, methanol–dichloromethane (1:4, v/v)] and the extracts examined by UV–VIS (dichloromethane) spectrophotometry to determine the complexing metal (V=O, λ_{max} 530, 570 nm; Ni, λ_{max} 510, 550 nm; free-bases, λ_{max} 500, 530, 565, 615 nm. Where one species predominated (>90%; Kimmeridge, Gilsonite, Ni; Serpiano, Julia Creek, La Luna, V=O) the porphyrins were obtained as the free bases using methanesulphonic acid to remove the metal ions according to established procedures[23]. Typically, the organic extract (1 g) was heated (V=O, 100°C, 4 h; Ni, 80°C, 1 h) with a 5–10-fold excess of methanesulphonic acid (98%, *ca.* 5 ml, Aldrich). The reaction mixture was diluted with methane sulphonic acid-deionised water (20:80) (*ca.* 30 ml), decanted through a moist filter paper and extracted with dichloromethane. The extract was neutralised (saturated sodium bicarbonate), washed with water and dried (toluene azeotrope). The

free bases obtained were purified by thin-layer chromatography (silica gel, acetone–dichloromethane, 5:95).

Where both Ni and V=O porphyrins were detected (Marl slate, 67% Ni, 33% V=O; El Lajjun shale, 57% Ni, 43% V=O) fractionation was achieved by flash chromatography[24] over silica, eluting with 20% and 80% dichloromethane in hexane, respectively. The demetallation procedure above was then followed on the separate fractions.

Where free-base porphyrins predominated (>90%; Gafsa chert), the extract was dissolved in diethyl ether and extracted with hydrochloric acid (5 *M*; 3 × 20 ml). The aqueous phase was extracted with dichloromethane (3 × 20 ml). The extract containing the porphyrin dications was neutralised (sodium bicarbonate) and dried (toluene azeotrope).

Compounds for co-chromatography

The isolation and structural elucidation of the two major benzo-CAPs (**1** and **2**) and the tentative assignment of **3** and **4** from Boscan oil used in this study, has been described elsewhere[14,25]. In addition, a chromatographic fraction (HMW) enriched in benzo-CAPs (>90% with regard to other porphyrin types), and containing high-molecular-weight components[25], was obtained from Boscan oil. For the co-chromatography studies the derivatives of the C_{33} (**1**) and C_{32} (**2**) components were added to a derivatised aliquot of fraction HMW to give a mixture (mixture A) with the relative abundances of the benzo-CAPs approximating those in the oil.

HPLC co-injection experiments were restricted to the two major benzo-CAPs (**1** and **2**).

GC–MS analyses

Derivative formation. Established conditions[3–5] for silicon insertion into the individual demetallated benzo-CAPs from Boscan oil (25–50 μg) were slightly modified due to the relatively low solubility of the pure compounds in the solvent (toluene). The reaction was conducted at increased temperature (60°C *vs.* ambient) and time (72 h *vs.* 24 h). Formation of the complex was confirmed by UV–VIS spectrophotometry. Problems of solubility were not encountered for the free-base petroporphyrin mixtures obtained after removal of metal ions and the modified conditions were not employed.

Instrumentation. Analyses were performed on a Carlo-Erba HRGC 5160 gas chromatograph with a Grob-type, cooled, on-column injector, linked to a Finnigan 4000 mass spectrometer. Data collection and processing were performed using a Finnigan Incos 2300 data system.

A flexible fused-silica capillary column (Hewlett Packard Ultra series; 25 m × 0.31 μm I.D.) coated with cross-bonded polydimethylsiloxane (0.17 μm film thickness) and helium carrier gas (0.8 kg cm^{-1}) was used. The temperature program and mass spectrometer operating conditions were as described elsewhere[3–5]: 50–225°C at 20°C min^{-1}, 225–300°C at 3°C min^{-1} followed by an isothermal period at 300°C for 40 min. Data were acquired using a Finnigan program which allowed monitoring of the ion *m/z* 113 (*n*-alkanes, see below) in the selected ion monitoring mode and scanning of the range *m/z* 550–850.

Data processing and presentation. Details of the processing of porphyrin GC–MS

data have been described previously[3–5,26]. Pseudo-Kováts retention indices (KRI) were calculated using program RRI[26] employing *n*-alkane (C_{29}–C_{44}) standards. The program allows conversion of GC–MS time-intensity data to a KRI-intensity format. The *n*-alkane standards were supplemented by the $(TBDMSO)_2Si(IV)$ derivative of octaethylporphyrin (OEP) to allow greater reproducibility of KRI calculations[5]. The KRI of this standard was defined as 3800 and the alkane standards were accordingly offset by a constant value (generally < 10 KRI units).

Mass chromatograms of the base peak in the porphyrin spectra were used to quantify the components (%) relative to (i) the major benzo-CAP in the distribution and (ii) the major petroporphyrin, generally C_{32} cycloalkanoporphyrin (CAP), **5**. The presentation of data generally follows earlier formats[3–5].

HPLC analyses

Analyses of the free bases were performed using a Spectra-Physics SP8700 ternary solvent delivery system and a Rheodyne 7125 injector fitted with a 20-μl loop. Detection (UV–VIS; 405 nm) was obtained using an LCD 1202 Spectromonitor III variable-wavelength detector interfaced with a VG Minichrom data system. Analyses employed three columns (Spherisorb 3W; each 150 × 4.6 mm I.D.; Phase Separations) in series and a flow-rate of 1.0 ml min^{-1}. A stepped solvent program[6] involving mixtures of dichloromethane–acetone (4:1), hexane–pyridine (99:1) and hexane–acetic acid (99:1) was used.

RESULTS

Benzoporphyrins in Boscan oil

The partial mass spectrum (Fig. 1) of the $(TBDMSO)_2Si(IV)$ derivative of **1** (the major benzo-CAP), isolated from Boscan 8-E4 oil, illustrates the characteristic

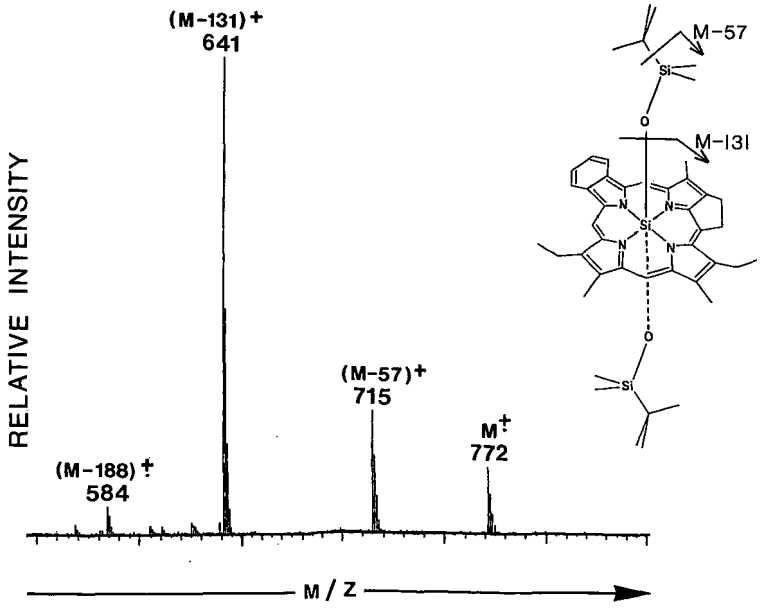


Fig. 1. Partial EI mass spectrum of the $(TBDMSO)_2Si(IV)$ derivative of C_{33} benzo-CAP (**1**).

fragment ions from cleavage of the TBDMSO ligands, as seen for these derivatives of other porphyrin types[3–5]. GC–MS analysis revealed a high KRI value (4110), which agrees closely with that obtained for the derivative corresponding formally to C_{33} benzo-CAP reported previously in the total Boscan distribution[5]. An analogous fragmentation pattern was observed for the derivative of the C_{32} homologue (**2**; KRI 4070).

Fig. 2 shows the reconstructed ion current (RIC; *m/z* 550–850) obtained for the total petroporphyrin derivatives. The general resolution and peak shape are not optimal as analysis of the minor benzo-CAP components necessitated a relatively high column loading. The inset shows the partial RIC (*m/z* 550–850) and the mass spectrum at KRI 4380 for the fraction HMW (see Experimental) enriched in benzoporphyrins, to illustrate the extended carbon number range observed. The mass spectrum at KRI 4380 shows molecular ions at *m/z* 828 and 842 and major fragments $[M-131]^+$ and $[M-57]^+$ corresponding to C_{37} and C_{38} benzo-CAPs, respectively. Analysis of the total porphyrin distribution did not detect these components (presumably because of their low abundance), as observed previously[5].

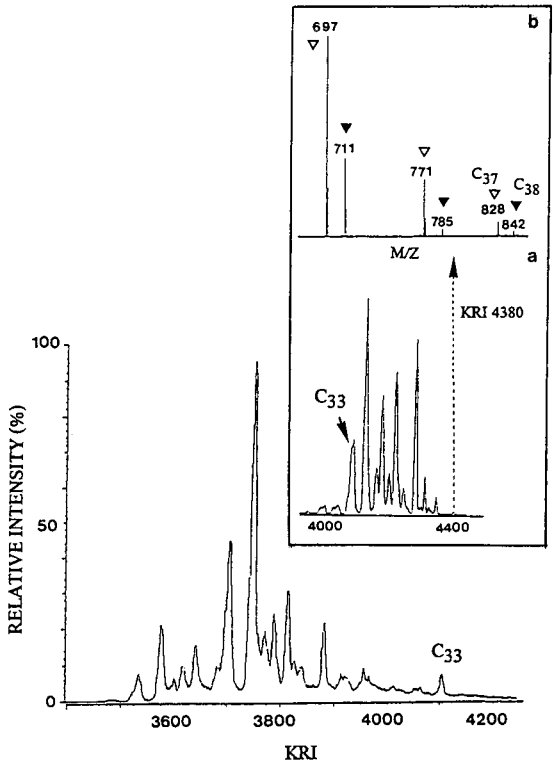


Fig. 2. Reconstructed ion current (RIC) (*m/z* 550–850) chromatogram of total Boscan petroporphyrins as the $(TBDMSO)_2Si(IV)$ derivatives. The retention time scale has been converted to a KRI scale. Annotated peak indicates compound **1**. Inset shows (a) the RIC for fraction HMW (see text) with component **1** indicated, and (b) mass spectrum from (a) at KRI 4380. Although the RIC intensity at KRI 4380 is low, the presence of components corresponding to C_{37} (▽) and C_{38} (▼) benzo-CAP derivatives is indicated by the characteristic mass spectra (*cf.* Fig. 1). ▽: $M^{+\cdot}$, 828; $[M-57]^+$, 771; $[M-131]^+$, 697). ▼: $M^{+\cdot}$, 842; $[M-57]^+$, 785; $[M-131]^+$, 711.

Single ion mass chromatograms for fraction HMW (see Experimental) we examined for the intense $[M-131]^+$ ions of all benzo-CAPs in the range C_{29}–C_{40} (Fi 3). To ensure that the peaks detected arose from "genuine" $[M-131]^+$ ions, and eliminate any possible "spurious" peaks[3], spectra were obtained, summed sequential in groups of 20 scans over the entire range of benzo-CAP elution. Thus, benzo-CAPs the range C_{30}–C_{38} were assigned. The mass chromatograms reveal the presence structural isomers at each carbon number.

The KRI values of the benzo-CAP derivatives are high (*ca.* 3900–440 compared to those of cycloalkano-type porphyrins (*e.g.* C_{32} CAP, **5**; KRI 3755; *cf.* Fi 3). A combined Kovat's plot, illustrating the benzo-CAP derivatives detected in tl total Boscan petroporphyrin distribution and the additional high-carbon-numb (C_{36}–C_{38}) isomers detected in fraction HMW, is shown in Fig. 4a. Quantification the major benzo-CAP (C_{33}, KRI 4110) as a percentage of the major C_{32} CAP (KF 3755) indicated a relative abundance of 4%. Within the error limits of determination this level and possible variations in column performance, this value agrees reasonab well with previous studies (7%)[5]. Since the abundance of other benzo-CAPs is lowe quantification of these components with respect to the total distribution of a maj component such as C_{32} CAP (**5**) is perhaps not informative. Hence, in order t

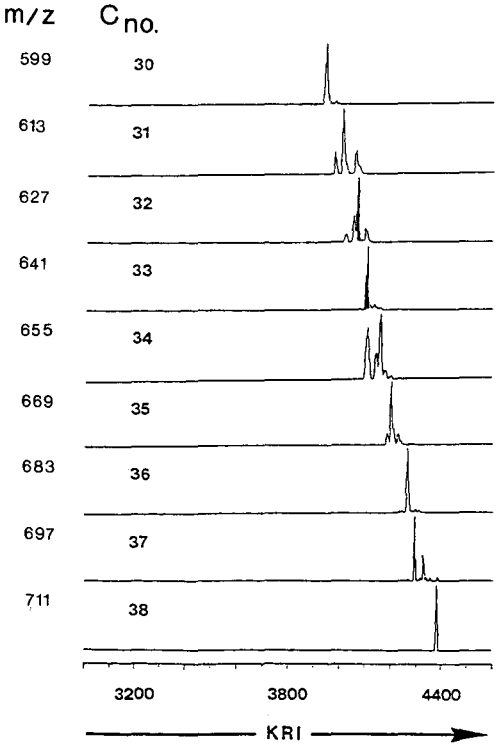


Fig. 3. Partial mass chromatograms for the $[M-131]^+$ ions from the $(TBDMSO)_2Si(IV)$ derivatives benzo-CAPs of different carbon numbers in Boscan fraction HMW. Shaded peaks indicate co-elution wi derivatives of C_{32} (**2**) and C_{33} (**1**) components[14].

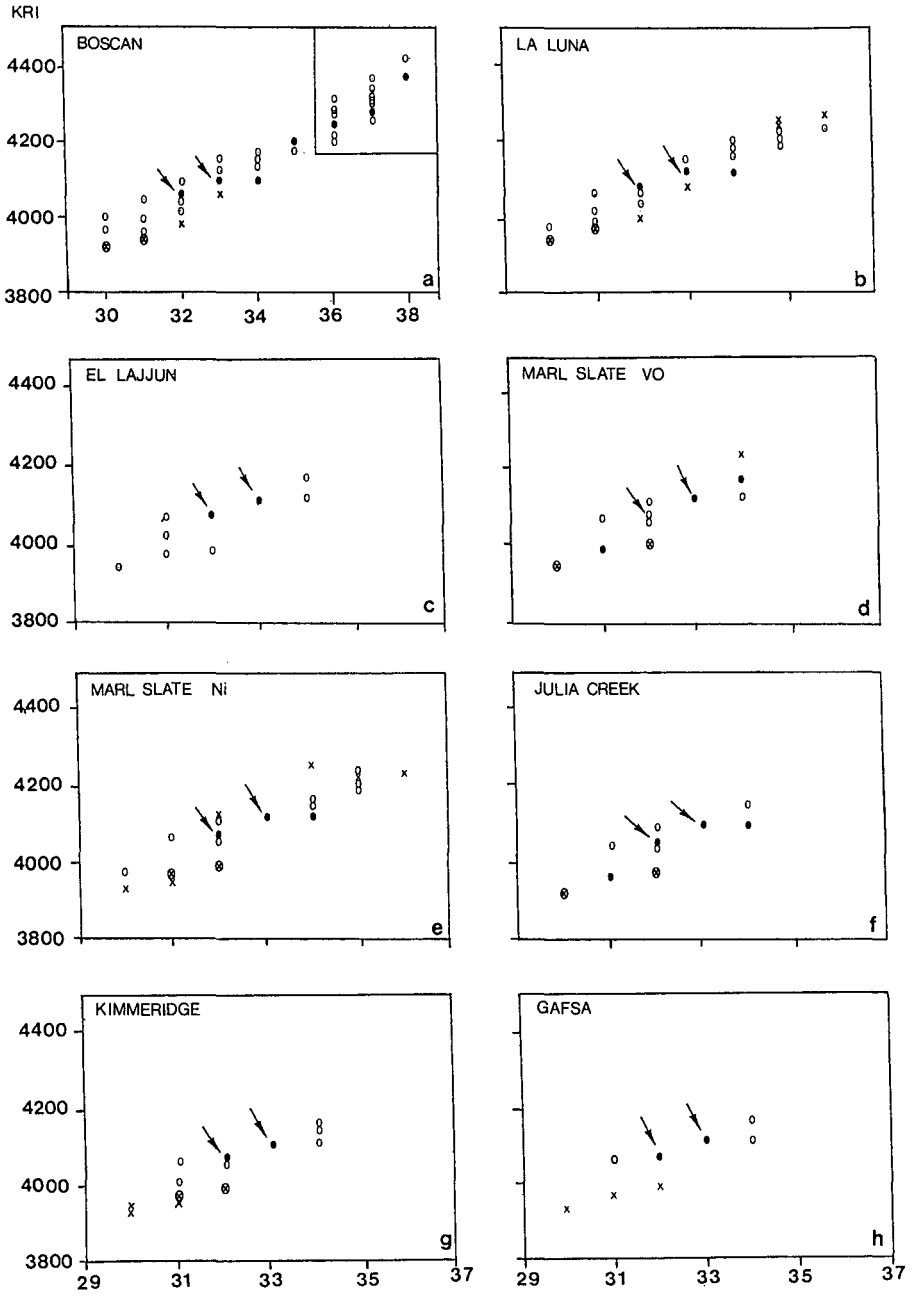


Fig. 4. Plots of KRI *vs.* carbon number for GC–MS analyses of total benzo-CAPs as $(TBDMSO)_2Si(IV)$ derivatives in (a) Boscan oil, inset shows additional carbon number range detected in fraction HMW, (b) La Luna shale, (c) El Lajjun shale Ni porphyrins, (d) Marl slate V = O porphyrins, (e) Marl slate Ni porphyrins, (f) Julia Creek oil shale, (g) Kimmeridge shale, (h) Gafsa chert. Arrows indicate co-elution of C_{32} (**2**) and C_{33} (**1**) derivatives. (○) Isomer co-elutes with a component in Boscan mixture A (see text) and is clearly most abundant at carbon number (●). (×) Isomer does not co-elute with any component in Boscan mixture A but is clearly most abundant at carbon number (⊗).

compare the benzo-CAP distributions in the samples, quantification was performed relative to the major C_{33} benzo-CAP isomer (KRI 4110). This component was then quantified relative to the major C_{32} CAP (KRI 3755) for each sample. The KRI values and relative abundances of the isomers are listed in Table II and are compared with those present in the other samples studied.

Other samples

La Luna shale. Fig. 4b shows the KRI plot of the benzo-CAPs and summarises results from co-chromatography with mixture A from Boscan (see Experimental). The major benzo-CAP co-eluted with the C_{33} derivative of **1** (KRI 4110). Hence, the structures may be assumed to be identical, or at least positionally isomeric. The second most abundant co-eluted with the C_{32} derivative of **2** (KRI 4070). The major C_{34} and C_{35} benzo-CAP isomers (KRI 4109 and 4203, respectively) co-eluted with the derivatives of Boscan components tentatively proposed[25] to have structures **3** and **4** respectively. The major C_{36} isomer (KRI 4264) co-eluted with the major C_{36} benzoporphyrin.

HPLC co-chromatography of the C_{33} (**1**) and C_{32} (**2**) standards with the total demetallated La Luna porphyrins confirmed results from the GC–MS study (Fig. 5a).

Gilsonite bitumen. Derivatives having masses corresponding formally to benzo-CAPs were not detected and this confirms earlier GC–MS studies[4] of this sample.

El Lajjun shale. GC–MS analysis was performed on the derivatised Ni(II) fraction. The Kovats' plot for the benzo-CAPs is shown in Fig. 4c and results from the co-chromatography experiment are illustrated. In contrast to the Boscan and La Luna distributions, the number of benzo-CAP isomers is small. The major component is, however, the derivative of the same C_{33} (**1**) component (KRI 4110). The abundance of this relative to the major C_{32} CAP component (KRI 3755) is *ca.* 2%.

Marl slate. GC–MS analysis of both the derivatised vanadyl and nickel porphyrin fractions was undertaken (Fig. 4d and e). The major C_{33} benzo-CAP derivative from both fractions again co-eluted with the derivative of the C_{33} (**1**) component (KRI 4110). Although the derivative of the C_{32} (**2**) benzo CAP (KRI 4070) in the Boscan mixture (mixure A) co-eluted with a C_{32} derivative from the vanadyl fraction, the latter is not the major C_{32} isomer (Table II). The major C_{32} isomer has a KRI of 3986. Although this component is not present in mixture A, it was detected in the Boscan total porphyrins in low abundance (Fig. 4a; Table II). Similarly, in the nickel fraction the C_{32} (**2**) standard (KRI 4070) co-eluted with a C_{32} benzo-CAP isomer, but again the major C_{32} isomer has a KRI of 3986 and is probably the same compound as that from the vanadyl fraction. In the nickel fraction, this isomer is not as predominant as in the vanadyl fraction.

Both the C_{31} components in the vanadyl fraction (KRIs 3980 and 4060) co-eluted with C_{31} components in the Boscan mixture. However, neither correspond to the major C_{31} isomer in Boscan oil (KRI 3970). In contrast, the major C_{31} isomer in the nickel fraction (KRI 3970) corresponds to the major C_{31} isomer in Boscan oil (Table II).

The major C_{34} isomer (KRI 4109) in the nickel fraction co-eluted with a compound in mixture A proposed[25] to correspond to the derivative of structure **3** (Table II). In contrast, the major C_{34} benzo-CAP isomer in the vanadyl fraction was at KRI 4160 (Fig. 4d; Table II).

TABLE II

RETENTION INDICES AND ABUNDANCES OF BENZO-CAPs DETECTED BY GC–MS ANALYSES OF PETROPORPHYRIN DISTRIBUTIONS AS $(TBDMSO)_2Si(IV)$ DERIVATIVES

Sample	*Pseudo Kovats' retention index (abundance[a])*							*Benzo-CAP abundance[b]*
	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}	C_{35}	C_{36}	
Boscan oil	3930(3) 3968(1) 4008(2)	3970(9) 3980(4) 4010(3) 4060(5)	3986(4) 4022(4) 4055(7) 4070(30) 4102(4)	4074(6) 4110(100) 4120(4) 4134(3)	4109(15) 4143(7) 4160(6) 4178(4)	4184(3) 4203(6)		4
La Luna shale	3930(4) 3968(1)	3970(9) 3980(3) 4010(4) 4060(2)	3986(3) 4022(5) 4055(7) 4070(20)	4070(4) 4110(100) 4135(3)	4109(18) 4143(3) 4160(5) 4178(3) 4250(1)	4184(2) 4203(3) 4226(2) 4235(1)	4264(2) 4230(2)	6
Marl slate (V=O fraction)	3930(1)	3980(5) 4060(2)	3986(17) 4055(3) 4070(4) 4102(1)	4110(100)	4109(3) 4160(6) 4230(1)			10
Marl slate (Ni fraction)	3930(3) 3968(2)	3947(1) 3970(5) 4060(2)	3986(8) 4055(1) 4070(5) 4102(2) 4111(2)	4110(100)	4109(7) 4143(1) 4160(2) 4255(1)	4184(2) 4203(1) 4217(1) 4226(1)	4230(1)	26
El Lajjun shale (Ni fraction)	3930(1)	3970(3) 4010(2) 4060(1)	3986(6) 4070(25)	4110(100)	4109(2) 4160(1)			2
Julia Creek oil shale	3930(2)	3980(11) 4060(4)	3986(5) 4055(1) 4070(18) 4102(3)	4110(100)	4109(2) 4160(1)			5
Kimmeridge shale	3930(2) 3942(1)	3947(1) 3970(3) 4010(1) 4060(1)	3986(29) 4055(1) 4070(25)	4110(100)	4109(1) 4122(1) 4160(1)			2
Gafsa chert	3930(1)	3970(2) 4060(1)	3986(14) 4070(15)	4110(100)	4109(3) 4160(1)			2

[a] Relative (%) to the derivative of the major benzo-CAP in each distribution (C_{33}, **1**, KRI 4110).

[b] Abundance (%) of the major benzo-CAP (C_{33}, **1**) relative to the most abundant petroporphyrin in the fraction (**5** in each case).

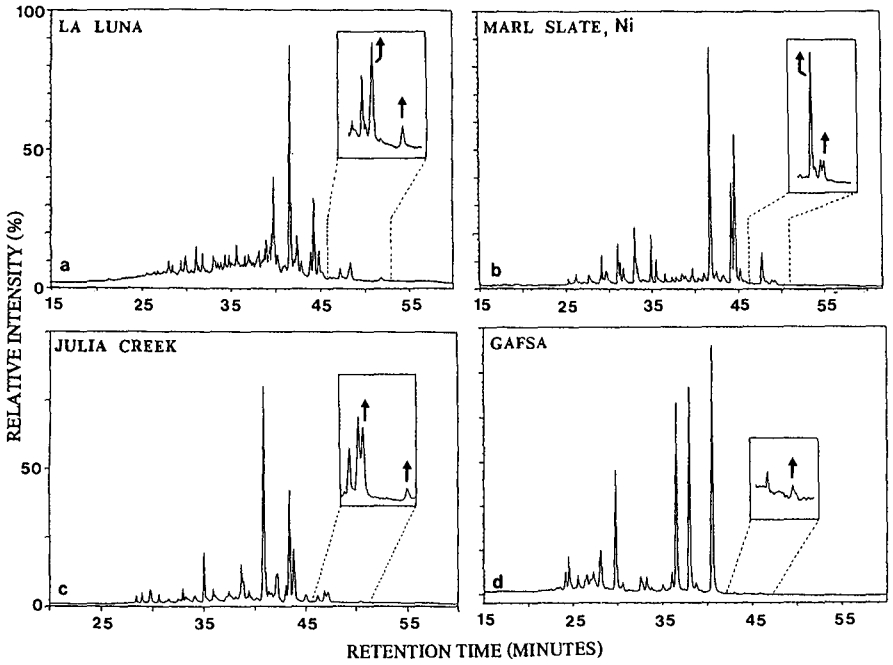


Fig. 5. HPLC chromatograms (SiO_2; 3 μm) of total petroporphyrins as free bases from (a) La Luna shale, (b) Marl slate Ni porphyrins, (c) Julia Creek oil shale, (d) Gafsa chert. Insets in each case show enlarged regions of benzo-CAP elution (not all the peaks in this region arise from benzo-CAPs). Arrows indicate co-elution with C_{33} (**1**) and C_{32} (**2**) standards (in increasing order of retention time).

Only one C_{36} isomer (KRI 4230) was detected in the nickel fraction and this did not co-chromatograph with any of the isomers present in mixture A.

The major difference between the nickel and vanadyl benzo-CAP distributions appears to be that of a simpler overall distribution for the vanadyl fraction, with fewer isomers at most of the carbon numbers. The fact that isomers were detected in the vanadyl fraction, and no isomers corresponding to C_{35} or higher may, however, be a consequence of the somewhat lower column loading in the analysis. The total benzo-CAP concentration, as a fraction of the total porphyrins, was lower in the vanadyl fraction than in the nickel fraction. Both these factors would discriminate against the detection of very minor benzo-CAP components.

In summary, the Marl slate porphyrins contain a relatively high abundance of benzo-CAPs, with the major component (C_{33}, KRI 4110) being present in *ca.* 20% of the major C_{32} CAP in the nickel porphyrins. In addition, a relatively large number of isomers were detected at each carbon number in the nickel fraction (Fig. 4e; Table II).

The assignments were confirmed in part by HPLC co-chromatography of the C_{32} (**2**) and C_{33} (**1**) benzoporphyrin standards with the demetallated nickel fraction (Fig. 5b). The demetallated vanadyl fraction was not available for HPLC analysis.

Julia Creek oil shale. Components corresponding to benzo-CAPs in the range C_{30}–C_{34} were present and the series again maximised at C_{33} (Table II). Results from the co-chromatography experiment with mixture A are summarised on the Kovats'

plot (Fig. 4f). The major benzo-CAP again co-eluted with the derivative of the C_{33} (**1**) standard, as did the C_{32} (**2**) with the major C_{32} benzo-CAP. However, a second C_{32} isomer (KRI 3986) was present in relatively high abundance (Fig. 4f; Table II). The latter was not present in mixture A, although a C_{32} component at this KRI was detected in the total Boscan porphyrins, albeit in low abundance. Two C_{31} isomers were present at KRIs 3980 and 4060, the isomer at KRI 3980 being more abundant. Both co-eluted with C_{31} isomers in mixture A and are, therefore, almost certainly the same as those present in Boscan. The C_{34} components were present in much smaller abundance, the isomer at KRI 4109 co-eluting with the derivative of the component of proposed structure **3**[25]. The overall distribution in Julia Creek oil shale is relatively simple in terms of the number of isomers at each carbon number, with the largest number of isomers at C_{32}, where there are four (Fig. 4f; Table II).

HPLC co-chromatography confirmed the presence of the components corresponding to the C_{32} (**2**) and C_{33} (**1**) standards (Fig. 5c).

Serpiano shale. No porphyrin derivatives having masses corresponding to benzoporphyrins with a cycloalkano ring were detected by GC–MS. UV–VIS and/or electron impact (EI) probe MS analyses of LC chromatographic fractions obtained from the shale, however, have reported spectra[28] and molecular ions[29] corresponding formally to benzo-CAPs. It is possible that the relative abundance of these components in the total distribution is below the limits of detection of the GC–MS procedure. If so, this would indicate an abundance of less than *ca.* 2%, since this level was detected in other samples (Table II). A C_{34} porphyrin isolated from Serpiano shale and having a molecular mass corresponding in degree of unsaturation to a benzo-aetioporphyrin, was recently reported instead to have an unusual fused-ring structure[30].

Similarly, HPLC analysis did not detect any peaks with the expected retention times of the benzo-CAP standards.

Kimmeridge shale. Results are summarised on the Kovats' plot (Fig. 4g). Again, the major C_{33} isomer (Table II) and a C_{32} isomer co-eluted with the derivatives of C_{33} (**1**) and C_{32} (**2**) components. However, the known C_{32} component (**2**) is not the most abundant C_{32} benzo-CAP, although it is present in a significant amount (25% of the major C_{33} benzo-CAP). The most abundant C_{32} isomer has a KRI of 3986 (Table II). A C_{34} isomer (KRI 4109) again co-eluted with the derivative of the Boscan component, tentatively proposed to have structure **3**. However, two other C_{34} isomers were present in equal abundance (KRIs 4122 and 4160; Table II). The isomer at KRI 4122 was not detected in any of the other samples investigated.

The demetallated porphyrins were not available for HPLC analysis. Examination of an HPLC chromatogram from previous studies[31] suggests, however, the presence of a peak with the appropriate relative retention time for the C_{33} benzo-CAP (**1**).

Gafsa chert. The Kovats' plot (Fig. 4h) shows a relatively simple distribution with the series again maximising at C_{33} (KRI 4110; Table II). The abundance of this component relative to the C_{32} CAP (KRI 3755) is low, *ca.* 2%. Two C_{32} isomers are present (KRIs 3986 and 4070), the C_{32} (**2**) derivative co-eluting with the latter (Fig. 4h). Although the isomer at KRI 3986 was not present in mixture A, it was detected, in varying amounts, in all the benzo-CAP distributions studied, including the total Boscan benzoporphyrins (Table II). Similarly, there are two C_{31} isomers at KRI 3970 and 4060 and only one of these (KRI 4060) co-eluted with a C_{31} isomer in mixture

A (Fig. 4h). Again, the other isomer at KRI 3970 was present in a number of the samples investigated and corresponds to the major C_{31} isomer in Boscan crude oil (Table II). Both C_{34} isomers in the chert (KRIs 4109 and 4160) co-eluted with components in mixture A. The isomer at KRI 4109 is present in greater abundance (Table II) and is believed to correspond to the derivative of the C_{34} benzo-CAP (**3**) tentatively assigned in Boscan oil[25].

The HPLC chromatogram (Fig. 5d) showed the presence of a small peak at the retention time expected for the C_{33} (**1**) benzo-CAP standard. Co-chromatography confirmed the GC–MS results. A peak corresponding to the C_{32} (**2**) standard could not be detected. This is presumably a result of the very low abundance of this component in the total porphyrins.

DISCUSSION

GC–MS of the derivatised porphyrins from a range of samples has revealed the presence of benzo-CAPs in a number of them (Table II). The series were detected as free bases (Gafsa chert), nickel complexes (Kimmeridge shale), vanadyl complexes (La Luna shale, Julia Creek oil shale), or as a mixture of these metallo species (Marl slate, El Lajjun shale). The presence of components with molecular ions corresponding formally to free-base benzo-CAPs in immature sediments has been recently reported from EI-MS studies[12,13]. The confirmation of benzo-CAPs as free bases indicates that they may be formed under very mild sedimentary conditions (*i.e.* before metal chelation). This contrasts with earlier proposed theories for their formation (the full structures being unknown at the time), such as sedimentary Diels–Alder reactions[32] which might be expected to require more severe conditions.

The benzo-CAPs occur over a range of maturity, extending from the relatively immature Gafsa chert to the mature La Luna shale and Boscan oil. This fact indicates that their presence is not maturity dependent. However, they do appear to show maturity effects, as do other porphyrins, in that they are present as free bases in the most immature sample (Gafsa chert) and as metalloporphyrins in the more mature samples.

The carbon number ranges do not appear to be greatly affected over the maturity range represented by the samples (Table II; Fig. 6). The range in the more mature samples (*e.g.* La Luna shale) was *ca.* C_{30} to C_{36}, whereas in less mature samples (*e.g.* Kimmeridge shale) it was *ca.* C_{30} to C_{34}. The complexity in the more mature distributions arises more from an increase in the number of isomers at each carbon number. For example, there are five C_{32} benzo-CAP isomers in the Boscan porphyrin distribution (Fig. 4a), whereas only two are present in Gafsa chert (Fig. 4h). This can also be seen at other carbon numbers (Fig. 6). For example, there are four C_{34} isomers in La Luna shale, whereas only two are present in Julia Creek. The large number of isomers presumably results from variation in structural features, such as presence/absence of unsubstituted β-positions, the spatial relationships of the β-substituents features with the cycloalkano and benzo rings[8,25,27].

Carbon numbers ranging from C_{30} to at least C_{36} are present (*e.g.* La Luna shale; Table II; Fig. 6). The possibility of higher carbon numbers, below the limits of detection in the total distributions, was highlighted by examination of fraction HMW from Boscan where benzo-CAPs with carbon numbers up to C_{38} were detected.

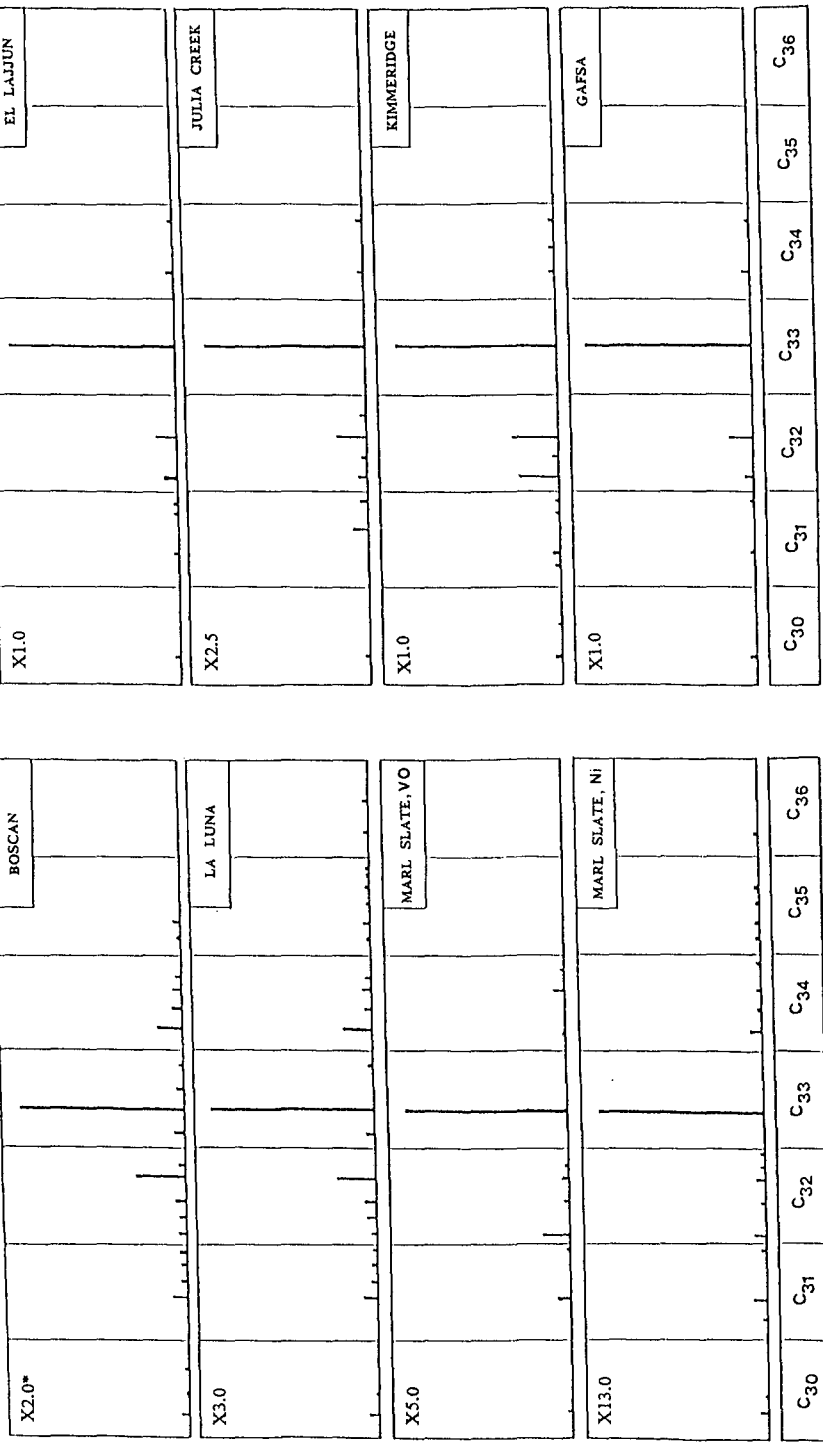


Fig. 6. Histograms illustrating relative abundances of benzo-CAP isomers at each carbon number detected by GC–MS analysis of total petroporphyrin distributions as $(TBDMSO)_2Si(IV)$ derivatives. Abundances are relative to the major benzo CAP (C_{33}, **1**) in each case. The scale on the *x*-axis is arbitrary, although in order of increasing KRI. Asterisk indicates relative to Gafsa chert = ×1.

The co-injection experiments (GC–MS and/or HPLC) reveal, remarkably, that in all the samples where benzo-CAPs were detected, the dominant member is the C_{33} component (Fig. 6) corresponding to the compound previously isolated from Boscan (**1**)[14]. In addition, although other C_{33} isomers were detected, the relative abundance of the above component (**1**) is generally >90%, *e.g.* in La Luna (Fig. 6; Table II). The abundance of this major C_{33} benzo-CAP, relative to the major porphyrin in each sample (generally C_{32} CAP) varies from *ca.* 2% (Gafsa chert) to 26% (Marl slate, nickel fraction). The next most abundant benzo-CAP in most cases is the C_{32} isomer (**2**). However, in some cases (Marl slate), an earlier-eluting C_{32} isomer (KRI 3986) was in greater abundance. Other C_{32} isomers, in varying amounts, were also detected (Table II). The very low relative abundance of most of the isomers, with respect to the total distributions, makes it difficult to compare these components quantitatively in the samples examined.

That the benzo-CAPs are widespread is clearly demonstrated by their occurrence in sedimentary samples from locations ranging from the United Kingdom (Kimmeridge shale) to Australia (Julia Creek oil shale) and of geological age ranging from Palaeocene (Gafsa chert) to Permian (Marl slate). However, they do not occur ubiquitously when porphyrins are present, being absent for example, from the Gilsonite bitumen. This offers some hope for the potential use of these components in geological applications. On the other hand, there is at present no apparent correlation with depositional environment among the samples examined. The two samples where benzo-CAPs were not detected were Gilsonite bitumen, whose source rock is from a lacustrine saline water depositional environment, and Serpiano shale, from a restricted marine environment.

Ideally, a study of geological samples from a wider range of depositional environments together with an examination of both the total petroporphyrin fractions and individual metalloporphyrin fractions would be desirable to search for significant geochemical correlations. In the present work, for convenience and due to the availability of samples, a combination of these approaches was employed. The results reveal, however, the striking predominance of compound **1** in all the distributions where benzo-CAPs were detected. This suggests an origin from a widely occurring specific precursor pigment rather than from sedimentary reactions of chlorophyll(s)[32,33]. At present, an origin from a known pigment is not obvious, although the presence of the cycloethano ring clearly indicates one from chlorophylls rather than other tetrapyrroles, such as cytohaemes. In the absence of an obvious precursor, a bacterial origin has been suggested[14].

CONCLUSIONS

The identification of benzo-CAPs in a variety of petroporphyrin distributions has been achieved via computerised GC–MS and HPLC analyses and co-chromatography.

(1) The GC retention times (KRI 3900–4400) of the $(TBDMSO)_2Si(IV)$ derivatives are long compared to those of the major alkylporphyrins (*e.g.* **5**, KRI 3755).

(2) The sensitivity of the GC–MS procedure has allowed detection of the less abundant benzo-CAPs proposed as **3** and **4**[25] in a number of samples. In addition,

minor components fractionated from Boscan oil and corresponding to benzo-CAPs, were shown to be present.

(3) Up to 24 benzo-CAP components, with multiple isomers at individual carbon numbers have been detected (*e.g.* La Luna shale; Table II).

(4) Both co-chromatographic techniques have provided a means of assignment of benzo-CAPs without the use of lengthy isolation procedures for individual compounds.

(5) Benzo-CAPs occur as minor petroporphyrin components in geological samples from a range of locations and ages.

(6) The distributions studied all maximise at C_{33} (same isomer, **1**; KRI 4110).

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REFERENCES

1 P. J. Marriott, J. P. Gill and G. Eglinton, *J. Chromatogr.*, 249 (1982) 291–310.
2 P. J. Marriott, J. P. Gill, R. P. Evershed, C. S. Hein and G. Eglinton, *J. Chromatogr.*, 301 (1984) 107–128.
3 G. Eglinton, R. P. Evershed and J. P. Gill, *Org. Geochem.*, 6 (1984) 157–165.
4 J. P. Gill, R. P. Evershed, M. I. Chicarelli, G. A. Wolff, J. R. Maxwell and G. Eglinton, *J. Chromatogr.*, 350 (1985) 37–62.
5 J. P. Gill, R. P. Evershed and G. Eglinton, *J. Chromatogr.*, 369 (1986) 281–312.
6 A. J. G. Barwise, R. P. Evershed, G. A. Wolff, G. Eglinton and J. R. Maxwell, *J. Chromatogr.*, 368 (1986) 1–9.
7 M. I. Chicarelli, G. A. Wolff and J. R. Maxwell, *J. Chromatogr.*, 368 (1986) 11–19.
8 M. I. Chicarelli, S. Kaur and J. R. Maxwell, in R. H. Filby and J. F. Brauthaver (Editors), *Metal Complexes in Fossil Fuels, Proc. 191st. Meeting of the American Chemical Society, New York, 1986*, American Chemical Society, Washington, DC, 1987, pp. 40–67.
9 W. W. Howe, *Anal. Chem.*, 33 (1961) 255–260.
10 E. W. Baker, T. F. Yen, J. P. Dickie, R. E. Rhodes and L. F. Clark, *J. Am. Chem. Soc.*, 89 (1967) 3631–3639.
11 A. J. G. Barwise and E. V. Whitehead, in A. G. Douglas and J. R. Maxwell (Editors), *Advances in Organic Geochemistry 1979*, Pergamon Press, Oxford, 1980, pp. 181–192.
12 E. W. Baker and J. W. Louda, *Org. Geochem.*, 10 (1986) 905–914.
13 J. M. E. Quirke, T. Dale, E. D. Britton, R. A. Yost, J. Trichet and H. Belayouni, in R. H. Filby and J. F. Brauthaver (Editors), *Metal Complexes in Fossil Fuels, Proc. 191st. Meeting of The American Chemical Society, New York, 1986*, American Chemical Society, Washington, DC, 1987, pp. 308–331.
14 S. Kaur, M. I. Chicarelli and J. R. Maxwell, *J. Am. Chem. Soc.*, 108 (1986) 1347–1348.
15 H. Belayouni and J. Trichet, in M. Bjoroy *et al.* (Editors), *Advances in Organic Geochemistry 1981*, Wiley, Chichester, 1982, pp. 328–335.
16 R. V. Tyson, R. C. L. Wilson and C. Downie, *Nature (London)*, 277 (1979) 377–380.

17 H. Rieber in G. Einsele and A. Seilacher (Editors), *Cyclic and Event Stratification*, Springer Berlin, Heidelberg, New York, 1982, pp. 527–541.
18 N. R. Sherwood and A. C. Cook, in D. I. Gravestock *et al.* (Editors), *Contributions to Geology and Hydrocarbon Potential of Eromanga Basin, Spec. Publ. Geol. Soc. Aust.*, 12 (1982) 255–266.
19 M. Gibbons, *Ph.D. Thesis*, University of Newcastle, Newcastle, 1978.
20 A. M. Abed and S. A. Bilal, *J. Petrol. Geol.*, 513 (1983) 261–272.
21 K. G. Bell and J. M. Hunt, in I. Breger (Editor), *Organic Geochemistry*, Pergamon, London, 1963, pp. 333–366.
22 S. Talukdar, O. Gallango and M. Chin-A-Lien, *Org. Geochem.*, 10 (1986) 261–279.
23 J. G. Erdman, *U.S. Pat.*, 3 190 829 (1965).
24 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 43 (1978) 2923–2925.
25 S. Kaur, *Ph.D. Thesis*, University of Bristol, Bristol, 1987.
26 C. S. Hein, J. P. Gill, R. P. Evershed and G. Eglinton, *Anal. Chem.*, 57 (1985) 1872–1879.
27 M. I. Chicarelli, G. A. Wolff, M. Murray and J. R. Maxwell, *Tetrahedron*, 40 (1984) 4033–4039.
28 D. W. Thomas and M. Blumer, *Geochim. Cosmochim. Acta*, 28 (1964) 1147–1154.
29 M. I. Chicarelli, *Ph.D. Thesis*, University of Bristol, Bristol, 1985.
30 M. I. Chicarelli and J. R. Maxwell, *Tetrahedron Lett.*, 27 (1986) 4653–4654.
31 P. Farrimond, P. Comet, G. Eglinton, R. P. Evershed, M. A. Hall, D. W. Park and A. M. K. Wardroper, *Mar. Pet. Geol.*, 1 (1984) 340–354.
32 E. W. Baker and S. E. Palmer, in D. Dolphin (Editor), *The Porphyrins*, Vol. I, Academic Press, New York, 1978, pp. 485–551.
33 A. J. G. Barwise and I. Roberts, *Org. Geochem.*, 6 (1984) 167–176.

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GAS–LIQUID CHROMATOGRAPHIC ANALYSES

XLIX[a]. POLYCHLORINATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS ON LOW-POLARITY NB-54 AND NB-1701 CAPILLARY COLUMNS

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SUMMARY

The gas chromatographic retention behaviour of complex mixtures of sixteen polychlorinated dibenzo-*p*-dioxins and fourteen polychlorinated dibenzofurans, containing in addition to the most toxic 2,3,7,8-chloro isomers the so-called "window" isomers, was studied on low-polarity NB-54 and NB-1701 capillary columns under suitable temperature-programmed conditions. The retention data for the components are given and their separation is discussed. The results are compared with those of the related isomers reported previously on low-polarity and polar stationary phases.

INTRODUCTION

Many papers have been published in the last 10 years on analytical methods for polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in various biological and environmental samples[1–8]. After clean-up procedures the final analyses were performed by gas chromatography (GC) with electron-capture detection (ECD) and frequently with mass-selective detection. Packed and capillary columns with low-polarity or polar stationary phases as listed in Table I[9–58] have been used separating the individual components from complex mixtures. Isomers, their concentration levels, total amounts and relative recoveries, etc., have been widely reported, but relatively few publications[9,11–17,20,25,31–33,38] seem to give the exact retention data for compounds, however, although the chromatograms were shown.

As a continuation of our research on harmful organochlorine compounds in the environment[59], this paper reports the GC retention behaviour of several PCDDs and PCDFs on capillary columns coated with low-polarity NB-54 and NB-1701 stationary phases. The mixtures were analysed using a double-column system with suitable temperature programming. The relative retention data are given, the results being compared with those reported previously for related isomers.

[a] For Part XLVIII, see I. O. O. Korhonen, *J. Chromatogr.*, 363 (1986) 277.

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TABLE I

STATIONARY PHASES USED FOR GC SEPARATION OF PCDD AND/OR PCDF COMPOUNDS

This is not a complete list, but it contains the most important phases.

Stationary phase	*Refs.*	*Stationary phase*	*Refs.*
C-87	25	OV-101	2, 10, 27, 34
Carbowax 20M	2, 9	OV-105	2
CPS-2	10	OV-210	2
Cyanopropyltolylallylsiloxane	11	OV-225	2, 27
DB-5	10, 12–24	OV-240-OH	35
DB-17	25, 26	OV-1701	28, 33
DB-1701	25	SB smectic	36, 37
Dexil 300	2	SE-54	2, 10, 15, 33, 38–42
Dexil 410	27	Sil-88	21, 37, 43
Methylsilicone	2, 28, 29	Silar 10C	2, 25, 31, 44–47
OV-1	2, 27, 28, 30–33	SP-2100	2, 48
OV-3	2	SP-2330	10, 15, 19, 20, 25, 28, 30, 32, 49–54
OV-7	2	SP-2331	6, 16, 26, 50, 55–57
OV-17	2, 12, 34	SP-2340	10, 58
OV-17/Poly S-179	2	SP-2350	58
OV-61	2	XE-60	2

EXPERIMENTAL

Materials

All 2,3,7,8-substituted PCDDs (2, 6, 10–12, 15 and 16) and PCDFs (18, 21, 22, 25–27, 29 and 30) were obtained from Wellington Labs. (Ontario, Canada) and the other isomers (1, 3–5, 7–9, 13, 14, 17, 19, 20, 23, 24 and 28) from CIL (Cambridge Isotope Labs., MA, U.S.A.). The mixtures analysed contained suitable amounts of the individual components for the sensitivity of the electron-capture detector.

Methods

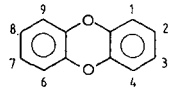
GC analyses were carried out on a Nordion Micromat HRGC 412 gas chromatograph under the following operating conditions: injection and electron-capture detector temperatures, 275 and 320°C, respectively; helium carrier gas velocity, 32 cm min^{-1}; and chart speed, 10 mm min^{-1}. A double-column system was used with the following low-polarity columns: a fused-silica NB-54 (5% phenyl-, 1% vinylmethylsilicone, similar to SE-54) wall-coated open-tubular (WCOT) column (25 m × 0.32 mm I.D., film thickness 25 μm) and a fused-silica NB-1701 (7% phenyl-, 7% cyanopropylmethylsilicone, similar to OV-1701) WCOT column (25 m × 0.32 mm I.D., film thickness 25 μm), both supplied by HNU-Nordion (Helsinki, Finland). The oven temperature was held at 100°C for 1 min, then programmed to 180°C at 20°C min^{-1} and from 180 to 280°C at 5°C min^{-1}, and held at the final temperature until elution of peaks had ceased. Both columns were operated simultaneously.

The chromatographic data were recorded with a Trendcom Synnyvale Model 200 integrator using standard programs, the retention times being measured from the time of sample injection.

TABLE I.

RETENTION DATA FOR POLYCHLORINATED DIBENZO-*p*-DIOXINS OBTAINED ON NB-54 AND NB-1701 CAPILLARY COLUMNS

Conditions as in Fig. 1.



Peak No.	*Systematic No.*[a]	*Compound*[b]	*Column*				
			NB-54		*NB-1701*		
			ART[c]	*RRT*[d]	*ART*[c]	*RRT*[d]	*RRT*[e]
1	42	1,3,6,8	19.50	0.919	20.62	0.911	1.057
2	48	2,3,7,8	21.21	1.000	22.63	1.000	1.067
3	41	1,2,8,9	21.94	1.034	24.11	1.065	1.099
4	58	1,2,4,6,8	23.21	1.094	24.61	1.087	1.060
5	61	1,2,4,7,9	23.21	1.094	24.61	1.087	1.060
6	54	1,2,3,7,8	24.80	1.169	26.40	1.167	1.065
7	56	1,2,3,8,9	25.22	1.189	27.39	1.210	1.086
8	71	1,2,4,6,7,9	26.89	1.268	29.02	1.282	1.079
9	72	1,2,4,6,8,9	26.89	1.268	29.02	1.282	1.079
10	66	1,2,3,4,7,8	28.48	1.343	30.77	1.360	1.080
11	67	1,2,3,6,7,8	28.60	1.348	30.92	1.366	1.081
12	70	1,2,3,7,8,9	29.00	1.367	31.72	1.402	1.094
13	63	1,2,3,4,6,7	29.05	1.370	31.90	1.410	1.098
14	74	1,2,3,4,6,7,9	32.53	1.534	36.20	1.600	1.113
15	73	1,2,3,4,6,7,8	34.03	1.604	37.91	1.675	1.114
16	75	1,2,3,4,6,7,8,9	41.44	1.954	48.02	2.122	1.159

[a] Taken from ref. 60.
[b] Numbers indicate the chlorinated positions.
[c] Absolute retention times (min) were measured from sample injection (Fig. 1).
[d] Relative retention time for 2 taken as 1.000.
[e] Relative retention time for the corresponding isomer on NB-54 taken as 1.000.

RESULTS AND DISCUSSION

PCDDs

Chromatograms of a mixture of PCDDs (1–16) are illustrated in Fig. 1 and Table II gives the corresponding retention data.

As would be expected, the individual components are eluted on the low-polarity columns used in the order corresponding to their degree of chlorination, *i.e.*, tetra- < penta- < hexa- < hepta- < octachloro isomers. The so-called "window" isomers for low-polarity phases, *viz.*, the first and last isomer eluting in each group, are then 1 and 3 for tetra-, 4 + 5 and 7 for penta- and 8 + 9 and 13 for hexachloro PCDDs, respectively (Fig. 1). Based on earlier observations[14,15,17,31,32], the deficient isomers not investigated in this work elute between these "window" isomers.

On NB-54, a mixture of sixteen components shows 13 resolved peaks, so that 4 and 5, 8 and 9, and 12 and 13 overlapped and 10 and 11 are partially separated from

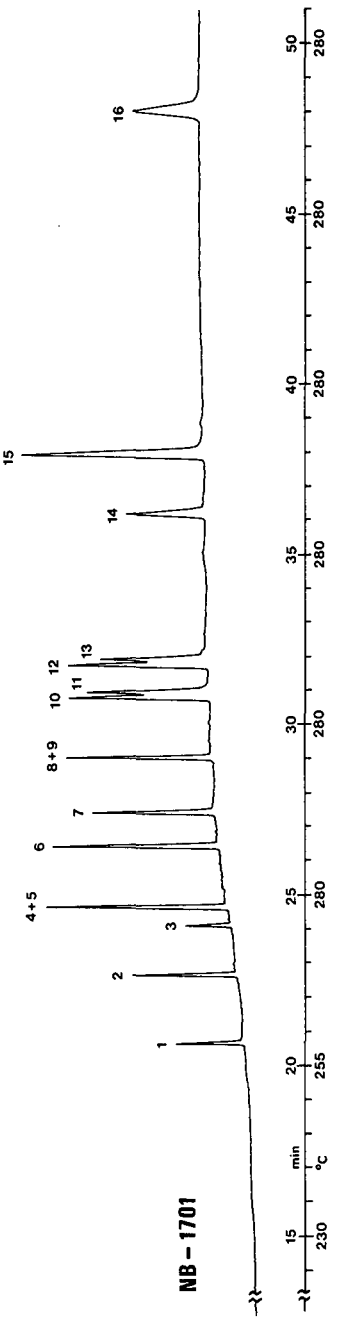


Fig. 1. Chromatogram of a mixture of polychlorinated dibenzo-*p*-dioxins (PCDDs), separated on low-polarity NB-54 and NB-1701 capillary columns with the following temperature programme: initial 1 min at 100°C, 100 to 180°C at 20°C min^{-1}, 180 to 280°C at 5°C min^{-1}, held at 280°C until elution of peaks had ceased. Peaks 1–16 are identified in Table II.

each other. With increasing column polarity, *i.e.*, on NB-1701 the retention of the isomers increases (Table II), as expected, the mixture giving 14 resolved peaks (Fig. 1). The retention order between the isomers remains unchanged. Compounds 4 and 5, and 8 and 9 are again coincident, whereas 12 and 13 show partially separated peaks like 10 and 11. The separation of the latter pair would have been expected to be better with a higher column polarity. However, earlier results with polar Silar 10C[46,47] and SP-2330[6] capillary columns showed only a slightly better separation between 10 and 11. Isomers 4 and 5, and 8 and 9 are also coincident on these phases, whereas 12 and 13 are completely separated.

The results obtained are in good agreement with the calculated and measured values reported previously on a low-polarity DB-5[17] capillary column and in contradiction to the elution order of the pair 10 and 11, obtained earlier on low-polarity SE-54 and OV-1701 capillary columns[33]. Hence, it seems evident that the retention order of isomers on these columns would also be the same as in the present investigation, particularly owing to the almost identical stationary phases and the fact that retention order of PCDDs is shown to be apparently constant with different low-polarity and slightly polar phases[17].

PCDFs

Generally the same trends as above are found also with the mixture of PCDFs (17–30) (Fig. 2 and Table III). The separation is nearly complete on both stationary

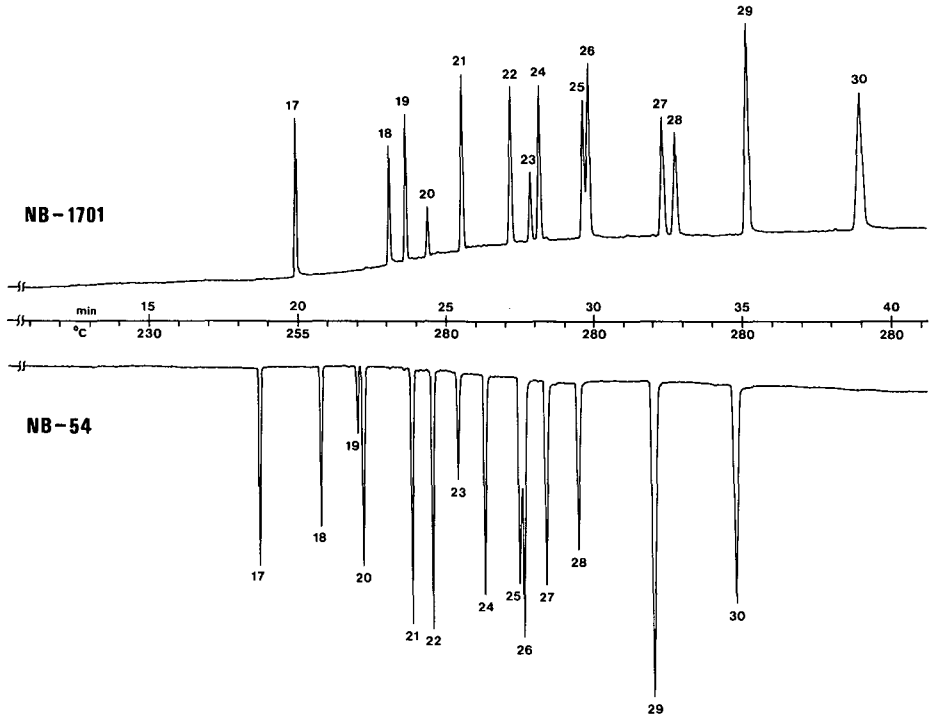
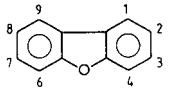


Fig. 2. Chromatogram of a mixture of polychlorinated dibenzofurans (PCDFs), separated on NB-54 and NB-1701 capillary columns under conditions as in Fig. 1. Peaks 17–30 are identified in Table III.

TABLE III

RETENTION DATA FOR POLYCHLORINATED DIBENZOFURANS OBTAINED ON NB-54 AND NB-1701 CAPILLARY COLUMNS

Conditions as in Fig. 2.



Peak No.	*Systematic No.*[a]	*Compound*[b]	*Column*						
			NB-54			*NB-1701*			
			ART[c]	*RRT*[d]	*RRT*[e]	*ART*[c]	*RRT*[d]	*RRT*[e]	*RRT*[f]
17	69	1,3,6,8	18.77	0.902	0.963	19.86	0.863	1.058	0.963
18	83	2,3,7,8	20.81	1.000	0.981	23.01	1.000	1.106	1.017
19	63	1,2,8,9	22.01	1.058	1.003	23.59	1.025	1.072	0.978
20	106	1,3,4,6,8	22.23	1.068	–	24.34	1.058	1.095	–
21	94	1,2,3,7,8	23.90	1.148	0.964	25.47	1.107	1.066	0.965
22	114	2,3,4,7,8	24.59	1.181	–	27.11	1.178	1.102	–
23	96	1,2,3,8,9	25.40	1.220	1.007	27.79	1.208	1.094	1.015
24	116	1,2,3,4,6,8	26.37	1.267	–	28.10	1.221	1.066	–
25	118	1,2,3,4,7,8	27.52	1.322	0.966	29.57	1.285	1.074	0.961
26	121	1,2,3,6,7,8	27.69	1.331	0.968	29.76	1.293	1.075	0.962
27	130	2,3,4,6,7,8	28.41	1.365	–	32.22	1.400	1.134	–
28	120	1,2,3,4,8,9	29.50	1.418	–	32.69	1.421	1.108	–
29	131	1,2,3,4,6,7,8	32.08	1.542	0.943	35.09	1.525	1.094	0.926
30	134	1,2,3,4,7,8,9	34.81	1.673	–	38.86	1.689	1.116	–

[a] Taken from ref. 60.
[b] Numbers indicate the chlorinated positions.
[c] Absolute retention times (min) were measured from sample injection (Fig. 2).
[d] Relative retention time for 18 taken as 1.000.
[e] Relative retention time for the corresponding PCDD isomer taken as 1.000 (Table II).
[f] Relative retention time for the corresponding isomer on NB-54 taken as 1.000.

phases, only compounds 25 and 26 partially overlapping. It should be noted that the predicted retention indices for these two isomers are close together[17], the components being nearly coincident also on polar stationary phases such as Silar 10C[25,44,47] and SP-2330[6]. Owing to the relatively small difference between the polarities of NB-54 and NB-1701 (McReynolds constants *ca.* 320 and 780, respectively), the retention order of the isomers is unaltered. However, if analysed on a polar column, the alterations would be evident based on earlier observations with tetrachlorodibenzofurans on DB-5 (low-polarity) and SP-2330 (polar) stationary phases[15]. This different behaviour of PCDFs is due to their unsymmetrical molecular structure compared with symmetrical PCDDs, shown also by comparison of the retention behaviours of the same PCDD and PCDF isomers (Table III).

CONCLUSIONS

The results show that the mixture of all 30 compounds investigated can be separated on NB-1701, but on NB-54 two additional overlappings are evident, *viz.*, 3 with 9 and 10 with 27. The lower thermal stability of NB-1701 limits the final oven temperature to its maximum of 280°C on NB-54, owing to the double-column system used, resulting in relatively higher retention times for the octachloro isomer (16) particularly on OV-1701 (Fig. 1). However, the use of a column as polar as possible is recommended to achieve the maximum separation, but with highly polar columns the elution order of certain isomers shifts significantly[17], even to the extent that components are not eluted in order of their degrees of chlorination. The latter fact is shown particularly with some PCDF isomers on Silar $10C^{25}$, and also with our preliminary results on a highly polar NB-9C capillary column[61].

ACKNOWLEDGEMENT

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REFERENCES

1 J. W. A. Lustenhouwer, K. Olie and O. Hutzinger, *Chemosphere*, 9 (1980) 501; and references cited therein.
2 W. B. Crummett, *Chemosphere*, 12 (1983) 429; and references cited therein.
3 W. B. Crummett and D. I. Townsend, *Chemosphere*, 13 (1984) 777; and references cited therein.
4 O. Hutzinger, M. J. Blumich, M. van den Berg and K. Olie, *Chemosphere*, 14 (1985) 581; and references cited therein.
5 M. J. Boddington, P. Barrette, D. Grant, R. J. Norstrom, J. J. Ryan and L. Whitby (Guest Editors), *Proceedings of the 4th International Symposium on Chlorinated Dioxins and Related Compounds, Ottawa, October 16–18, 1984*; *Chemosphere*, 14 (1985) 575–989.
6 C. Rappe, L.-O. Kjeller, P. Bruckmann and K.-H. Hackhe, *Chemosphere*, 17 (1988) 3; and references cited therein.
7 O. Hutzinger, W. Crummett, F. W. Karasek, E. Merian, G. Reggiani, M. Reissinger and S. Safe (Guest Editors), *Proceedings of the 5th International Symposium on Chlorinated Dioxins and Related Compounds, Bayreuth, September 16–19, 1985*; *Chemosphere*, 15 (1986) 1085–2132.
8 Y. Masuda, O. Hutzinger, F. W. Karasek, J. Nagayama, C. Rappe, S. Safe and H. Yoshimura (Guest Editors), *Proceedings of the 6th International Symposium on Chlorinated Dioxins and Related Compounds, Fukuoka, September 16–19, 1986*; *Chemosphere*, 16 (1987) 1603–2210.
9 G. A. Eiceman, R. E. Clement and F. W. Karasek, *Anal. Chem.*, 51 (1979) 2343.
10 P. W. Albro, W. B. Crummett, A. E. Dupuy, Jr., M. L. Gross, M. Hanson, R. L. Harless, F. D. Hileman, D. Hilker, C. Jason, J. L. Johnson, L. L. Lamparski, B. P.-Y. Lau, D. D. McDaniel, J. L. Meehan, T. J. Nestrick, M. Nygren, P. O'Keefe, T. L. Peters, C. Rappe, J. J. Ryan, L. M. Smith, D. L. Stalling, N. C. A. Weerasinghe and J. M. Wendling, *Anal. Chem.*, 57 (1985) 2717.
11 F. I. Onuska, R. J. Wilkinson and K. Terry, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 9.
12 J. J. Ryan, R. Lizotte and W. H. Newsome, *J. Chromatogr.*, 303 (1984) 351.
13 F. D. Hileman, M. D. Hale, T. Mazer and R. W. Noble, *Chemosphere*, 14 (1985) 601.
14 M. D. Hale, F. D. Hileman, T. Mazer, T. L. Shell, R. W. Noble and J. J. Brooks, *Anal. Chem.*, 57 (1985) 640; and references cited therein.
15 W. J. Dunn III, M. Koehler, D. L. Stalling and T. R. Schwartz, *Anal. Chem.*, 58 (1986) 1835; and references cited therein.
16 D. S. Waddell, R. K. Boyd, H. S. McKinnon, L. Safe, S. Safe and B. G. Chittim, *Chemosphere*, 16 (1987) 1655; and references cited therein.

17 J. R. Donnely, W. D. Munslow, R. K. Mitchum and G. W. Sovocool, *J. Chromatogr.*, 392 (1987) 51; and references cited therein.
18 H. Y. Tong, D. L. Shore, F. W. Karasek, P. Helland and E. Jellum, *J. Chromatogr.*, 285 (1984) 423.
19 G. Lindström and C. Rappe, *Chemosphere*, 17 (1988) 921.
20 E. R. Barnhart, D. G. Patterson, Jr., N. Tanaka and M. Araki, *J. Chromatogr.*, 445 (1988) 145.
21 H. Beck, K. Eckart, W. Mathar and R. Wittkowski, *Chemosphere*, 17 (1988) 51.
22 G. Eklund, J. R. Pedersen and B. Strömberg, *Chemosphere*, 17 (1988) 575.
23 K. Ballschmiter, I. Braunmiller, R. Niemczyk and M. Swerev, *Chemosphere*, 17 (1988) 995.
24 M. Oehme, P. Fürst, Chr. Krüger, H. A. Meemken and W. Groebel, *Chemosphere*, 17 (1988) 1291.
25 W. V. Ligon, Jr. and R. J. May, *J. Chromatogr.*, 294 (1984) 87.
26 M. Swerev and K. Ballschmiter, *Anal. Chem.*, 59 (1987) 2536.
27 C. E. Parker, P. W. Albro, M.-J. Bobenrieth, T. W. Cochran and J. D. Robinson, *J. Chromatogr.*, 278 (1983) 1.
28 K. Scheidl, F. Wurst and R.-P. Kuna, *Chemosphere*, 15 (1986) 2089.
29 K. Norén, *Chemosphere*, 17 (1988) 39.
30 M. Morita, A. Yasuhara and H. Ito, *Chemosphere*, 16 (1987) 1959.
31 J. Lawrence, F. Onuska, R. Wilkinson and B. K. Afghan, *Chemosphere*, 15 (1986) 1085.
32 M. Oehme and P. Kirschmer, *Anal. Chem.*, 56 (1984) 2754.
33 T. Humppi and K. Heinola, *J. Chromatogr.*, 331 (1985) 410.
34 H.-R. Buser, *Anal. Chem.*, 49 (1977) 918.
35 P. Schmid and M. D. Müller, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 548.
36 M. Swerev and K. Ballschmiter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 544.
37 U. Riehle, J. Ehmann, M. Swerev and K. Ballschmiter, *Fresenius Z. Anal. Chem.*, 331 (1988) 821.
38 J. Paasivirta, J. Tarhanen, B. Juvonen and P. Vuorinen, *Chemosphere*, 16 (1987) 1787.
39 H.-R. Buser, *Chemosphere*, 16 (1987) 713.
40 H. Thoma, O. Hutzinger, M. Höhn and U. Rapp, *Chemosphere*, 15 (1986) 2115.
41 H.-R. Buser, *Chemosphere*, 17 (1988) 889.
42 S. Herve, P. Heinonen, R. Paukku, M. Knuutila, J. Koistinen and J. Paasivirta, *Chemosphere*, 17 (1988) 1945.
43 A. Munder, H. Buchert, R. Niemczyk, M. Swerev and K. Ballschmiter, *Fresenius Z. Anal. Chem.*, 328 (1987) 639.
44 C. Rappe, M. Nygren, G. Lindström, H.-R. Buser, O. Blaser and C. Wüthrich, *Environ. Sci. Technol.*, 21 (1987) 964.
45 H. Hagenmaier, M. Kraft, H. Brunner and R. Haag, *Environ. Sci. Technol.*, 21 (1987) 1080.
46 H. Hagenmaier, H. Brunner, R. Haag and M. Kraft, *Environ. Sci. Technol.*, 21 (1987) 1085.
47 H. Hagenmaier and H. Brunner, *Chemosphere*, 16 (1987) 1759.
48 W. A. Korfmacher, R. K. Mitchum, F. D. Hileman and T. Mazer, *Chemosphere*, 12 (1983) 1243.
49 S. Marklund, C. Rappe, M. Tysklind and K.-E. Egebäck, *Chemosphere*, 16 (1987) 29.
50 C. Rappe and L.-O. Kjeller, *Chemosphere*, 16 (1987) 1775.
51 S. E. Swanson, C. Rappe, J. Malmström and K. P. Kringstad, *Chemosphere*, 17 (1988) 681.
52 M. Oehme, S. Manø, A. Mikalsen and P. Kirschmer, *Chemosphere*, 15 (1986) 607.
53 M. Oehme, S. Manø and A. Mikalsen, *Chemosphere*, 16 (1987) 143.
54 A. Yasuhara, H. Ito and M. Morita, *Environ. Sci. Technol.*, 21 (1987) 971.
55 O. M. van Berkel, K. Olie and M. van den Berg, *Int. J. Environ. Anal. Chem.*, 34 (1988) 51.
56 T. Wakimoto, N. Kannan, M. Ono, R. Tatsukawa and Y. Masuda, *Chemosphere*, 17 (1988) 743.
57 H. Hagenmaier, M. Kraft, W. Jäger, U. Mayer, K. Lützke and D. Siegel, *Chemosphere*, 15 (1986) 1187.
58 E. S. Lahaniatis, E. Clausen, D. Bieniek and F. Korte, *Chemosphere*, 14 (1985) 233.
59 J. Paasivirta, *Harmful Compounds in the Environment*, Department of Chemistry, University of Jyväskylä, 1988, pp. 1–34.
60 K. Ballschmiter, H. Buchert, R. Niemzyk, A. Munder and M. Swerev, *Chemosphere*, 15 (1986) 901.
61 I. O. O. Korhonen and K. M. Mäntykoski, Unpublished results.

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CAPILLARY GAS CHROMATOGRAPHY–FOURIER TRANSFORM INFRARED SPECTROSCOPY OF PYRROLIZIDINE ALKALOIDS OF *SENECIO INAEQUIDENS* DC.

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SUMMARY

The pyrrolizidine alkaloid (PA) fraction of *Senecio inaequidens* DC. was studied by capillary gas chromatography–Fourier transform infrared spectroscopy. The vapour-phase IR spectra of PAs and the advantages and disadvantages of this combined technique for their structure elucidation in complex mixtures are discussed. Examples of the distinction between necine bases (retronecine and otonecine) and geometric isomers (senecionine and integerrimine) are given.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are well known for their hepatotoxic properties and, to a lesser extent, as inducers of pulmonary arterial hypertension. PAs are present in species belonging to plant families throughout the world, in particular Boraginaceae, Compositae (Senecioneae and Eupatorieae) and Leguminosae (genus *Crotalaria*). Comprehensive reviews and textbooks describing this class of compounds and their chemotaxonomic significance and toxicity are available[1–7].

Senecio inaequidens DC. (Compositae) is a species native to South Africa, naturalized in Italy after the Second World War and now so widely diffused in Eastern Italy as to be considered potentially dangerous, both as a food contaminant and directly for cattle. The composition of the PA fraction of *S. inaequidens* was investigated by Wiedenfeld *et al.*[8], who identified senecionine and retrorsine, and by Bicchi and co-workers[9,10]. In the latter studies, one of the most complex PA fractions ever studied was isolated during an on-going ontogenic study at the beginning of the vegetative period. Nineteen PAs were characterized and 16 identified by capillary gas chromatography (GC) and mass spectrometry (MS) in different ionization modes: electron impact (EI), positive ion chemical ionization (PICI) with ammonia and negative ion chemical ionization (NICI) with ammonia and hydroxyl ions as reagent species[10]. The identified PAs were macrocyclic diesters derived from two necine bases (retronecine and otonecine); in particular senecivernine (**1**) (M.W. 335), senecionine (**2**) (M.W. 335), seneciphylline (**3**) (M.W. 333), spartioidine (**4**) (M.W. 333), integerrimine (**5**) (M.W. 335), retrorsine (**7**) (M.W. 351), usaramine (**10**) (M.W. 351), senkirkine (**6**) (M.W. 365), neosenkirkine (**9**) (M.W. 365), otosenine (**11**) (M.W. 381),

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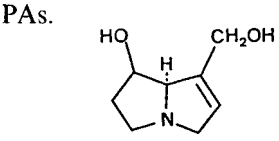
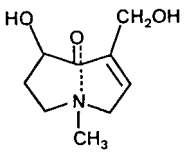
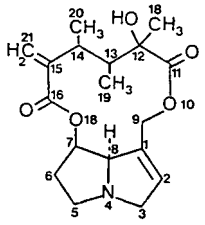
O-acetylsenkirkine (**13**) (M.W. 407), desacetyldoronine (**14**) (M.W. 417), florosenine (**16**) (M.W. 423), floridanine (**17**) (M.W. 441), doronine (**18**) (M.W. 449) and floricaline (**19**) (M.W. 483) were identified. Fig. 1 shows the structures of the identified PAs.



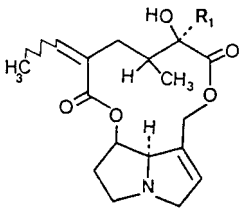
Retronecine



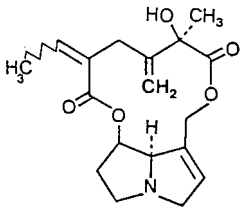
Otonecine



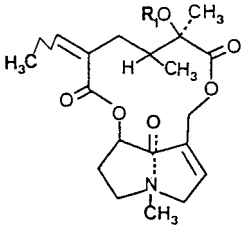
Senecivernine (1)



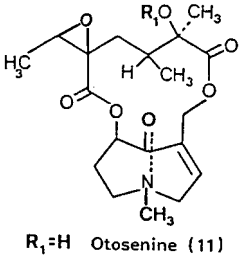
R_1=CH_3 Z Senecionine (2)
E Integerrimine (5)
R_1=CH_2OH Z Retrorsine (7)
E Usaramine (10)



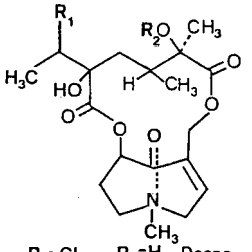
Z Seneciphylline (3)
E Spartioidine (4)



R_1=H Z Senkirkine (6)
E neo-Senkirkine (9)
R_1=Ac O-ac. Senkirkine (13)



R_1=H Otosenine (11)
R_1=Ac Florosenine (16)



R_1=Cl R_2=H Desac. doron. (14)
R_1=Cl R_2=Ac Doronine (18)

Fig. 1. Structures of PAs identified in *S. inaequidens* DC.

The structure elucidation of the components of such a complex mixture through their isolation is, of course, difficult and time consuming; better results can be obtained through the combined techniques [*i.e.*, capillary GC–MS and capillary GC–Fourier transform (FT)-IR].

GC–MS is the most widely used combined technique to identify PAs when a total plant extract is analysed. Several workers[8–16] have applied GC–MS in different ionization modes to identify PAs both as such and as their trimethylsilyl derivatives.

Even if MS is the pre-eminent technique for identifying a component of a complex mixture after a chromatographic separation, it sometimes has drawbacks, such as in the differentiation of structural isomers and in some doubtful or erroneous identifications produced by a library search. In such cases, FT-IR is at present the most useful complementary or alternative technique for characterizing a chromatographic peak. The use of capillary GC–FT-IR can be very helpful in the identification of PAs, considering the complexity of their structure and the number of structural isomers present in this class of compounds. IR spectra of PAs were studied by, among others, Culvenor and co-workers[17,18] and Gupta *et al.*[19], who discussed the IR spectra in the solid and liquid phase with respect to the absorptions of both the necine ring and the ester functions.

This paper describes the results obtained by applying capillary GC–FT-IR to the study of the PA fraction of *S. inaequidens*.

EXPERIMENTAL

Plant material

Plant material was collected in March 1986 from a roadside on the outskirts of Padua (Italy).

Reagents

All chemicals were of analytical-reagent grade (E. Merck, Darmstadt, F.R.G.). PS 264 and PS 122 are commercially available from Petrarch Systems (Bristol, PA, U.S.A.). Authentic samples of senecionine, seneciphylline, integerrimine and retrorsine were kindly provided by Dr. C. C. J. Culvenor (Parkville, Australia).

Sample preparation

A 25-g amount of air-dried plant material was extracted in a Soxhlet apparatus with methanol for 4 h. The extract was evaporated to dryness under vacuum and the residue suspended in 2.5% hydrochloric acid and washed with diethyl ether and chloroform. Half of the aqueous phase was basified with 25% ammonia solution and extracted with dichloromethane. The organic layer was again treated with 2.5% hydrochloric acid then 25% ammonia solution and again extracted with dichloromethane. The resulting solution was dried over anhydrous sodium sulphate and evaporated to dryness. To investigate the presence of PA N-oxides, the second half of the solution (resulting after washing with diethyl ether and chloroform) was reduced with zinc dust overnight, filtered and subsequently treated as described previously. The dried residues were weighed and dissolved in appropriate amounts of dichloromethane to produce suitable concentrations for capillary GC and capillary GC–FT-IR analysis. The sample under investigation contained 69.7 mg of PAs as free bases and 22.6 mg in the form of N-oxides.

Capillary GC analysis

Capillary GC analyses were carried out by introducing 1 μl of the PA extract dissolved in dichloromethane (1:250) into a Carlo Erba Mega 5360 instrument. The following conditions were used: carrier gas, hydrogen; flow-rate, 3 ml/min; injection system, split, with a splitting ratio of 1/30; injector temperature, 300°C; detection, flame ionization (FID); detector temperature, 300°C; column temperature, programmed from 120°C (1 min) to 280°C (20 min) at 3°C/min.

The column was a 30 m × 0.32 mm I.D. fused-silica capillary coated with 0.3 μm of PS 264 (polydimethylsiloxane, 7% diphenyl, 1% vinyl). To deactivate the column prior to coating with the stationary phase, the capillary was persilylated at 320°C for 4 h with a solution of polymethylhydrosiloxane (PS 122) in dichloromethane[20].

Capillary GC–FT-IR analysis

A Hewlett-Packard 5965 capillary GC–IR system was used. A 1-μl volume of PA extract solution in dichloromethane (1:100) was injected in the capillary GC–IR system. Capillary GC analysis was carried out on the fused-silica open-tubular column and under the chromatographic conditions given above.

FT-IR spectroscopy was carried out as follows: capillary GC–FT-IR interface, 100-μl volume light-pipe (10 cm × 1.2 mm I.D.); temperature, 280°C; make-up gas, helium at a flow-rate of 0.2 ml/min; time resolution (repetition rate), three scans per second at 8 cm^{-1} resolution; in most instances five interferograms were added in real time, resulting in an effective time slice of about 2 s; FT-IR detector, HgCdTe of narrow band width (4000–800 cm^{-1}).

RESULTS AND DISCUSSION

Fig. 2 shows the capillary GC–FID pattern of the PA extract of *S. inaequidens* and Fig. 3 that section of the capillary GC–FT-IR pattern in which the PAs eluted. As can be seen, capillary GC–FT-IR, is a very powerful tool for PA structure elucidation, despite the FT-IR detection sensitivity being lower than that of either FID of (MS) total ion current detection. The differences between the two chromatographic patterns can mainly be attributed to the detection limit of the FT-IR HgCdTe detector, which can be as much as one order of magnitude lower than that of the FID response; the high IR detector operating temperature (280°C), which involves a decrease in sensitivity; in the authors' experience, sensitivity decreases above 220°C; the method used to reconstruct the IR chromatogram; in fact, the Gram–Schmidt method employed here reconstructs each chromatographic peak through the contribution of each absorption band of the IR spectrum and the intensity of each band depends on its molar absorptivity, which is characteristic of each individual structure; and the chromatographic resolution, which can be lower when using capillary GC–FT-IR and it is not always possible to obtain spectra from all the peaks resolved by capillary GC analysis.

Some authors have suggested installing a flame ionization detector in series with the light-pipe to obtain comparable chromatographic results[21].

A short discussion of the absorption bands characterizing the PA vapour-phase FT-IR spectra present in the *S. inaequidens* extract is given below. Table I reports the characteristic IR bands of the significant vapour-phase FT-IR spectra of PAs.

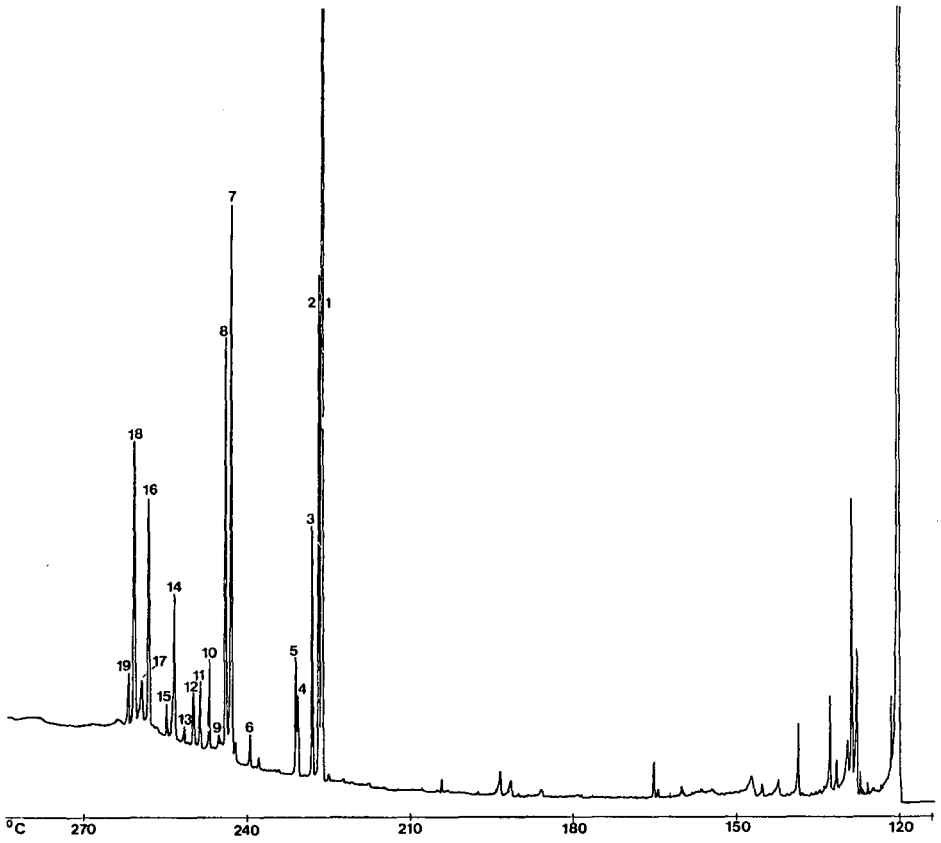


Fig. 2. Capillary GC–FID pattern of PA extract of *S. inaequidens* DC.

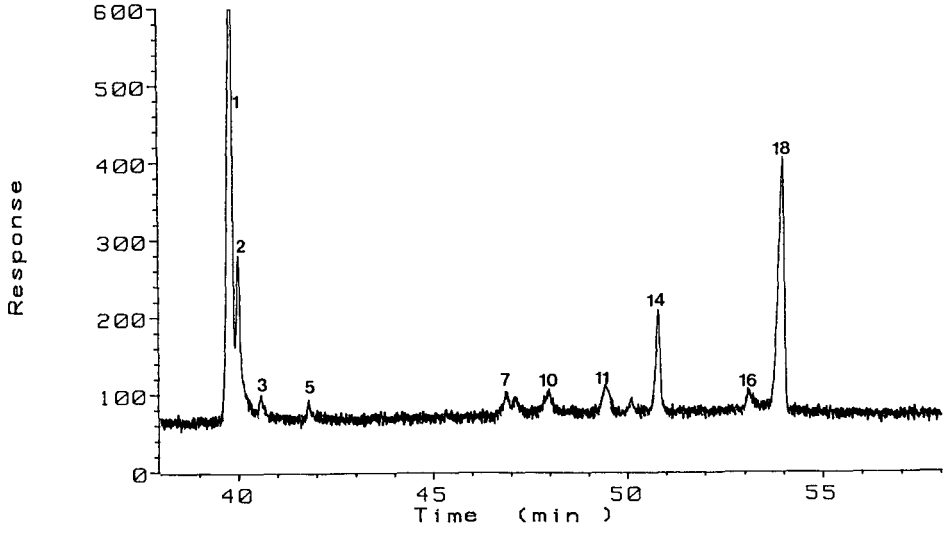


Fig. 3. Section of capillary GC–FT-IR pattern in which PAs eluted.

TABLE I
CHARACTERISTIC IR BANDS OF THE PA COMPOUNDS IDENTIFIED IN *S. INAEQUIDENS* DC.

No.	*Compound*	*IR band positions (cm^{-1})*[a]
1	Senecivernine	3557, 2981(m), 2945 (m), 2910(m), 2876(m), 2849(m), 1736(s), 1631, 1453, 1420, 1376, 1358, 1253(s), 1165(s), 1132(s), 945(m), 820
2	Senecionine	3563, 2982(m), 2941(m), 2915(m), 2875(m), 2843(m), 1735(s), 1645, 1450, 1373, 1325, 1224(s), 1176(s), 1156(s), 955, 820
3	Seneciphylline	3550, 2981(m), 2942(m), 2918(m), 2873(m), 2838(m), 1736(s), 1640, 1447, 1364, 1229(s), 1145(s), 955, 820
5	Integerrimine	3561, 2981(m), 2941(m), 2915(m), 2875(m), 2840(m), 1735(s), 1654, 1452, 1380, 1268(s), 1211(s), 1178(s), 1163(s), 1110(m), 1084(m), 1025, 950, 822
7	Retrorsine	3630, 3541, 2980(m), 2960(m), 2942(m), 2875(m), 2830(m), 1735(s), 1640, 1450, 1380, 1348, 1221(s), 1154(s), 1079(m), 1004, 948, 821
10	Usaramine	3636, 3543, 2979(m), 2962(m), 2940(m), 2870(m), 2830(m), 1735(s), 1640, 1444, 1375, 1260(s), 1218(s), 1155(s), 1059, 950
11	Otosenine	2988(m), 2961(m), 2940(m), 2870(m), 2840(m), 2814, 1745(s), 1679(m), 1450, 1370(m), 1231(s), 1185(s), 1150(s), 1111(s), 950
14	Desacetyldoronine	3560(m), 2990(m), 2969(m), 2945(m), 2887(m), 2855(m), 2813, 1747(s), 1674(s), 1454, 1379, 1330, 1210(s), 1178(s), 1150(s), 1090, 941
16	Florosenine	2991(m), 2966(m), 2930(m), 2890(m), 2850(m), 2814, 1766(s), 1678(m), 1454, 1371, 1232(s), 1185(m), 1145(s), 1105(s), 1060, 954
18	Doronine	3557, 2990(m), 2958(m), 2885(m), 2865(m), 2914, 1753(s), 1676(m), 1449, 1371(m), 1231(s), 1188(s), 1147(s), 1105(s), 1015(m), 952

[a] m = Medium; s = strong.

The absorption band in the 3630–3540 cm^{-1} range, weak where present, is due to OH stretching. The lack of hydrogen bonds in the vapour phase produces a lower intensity band than in the spectra in the solid and liquid phases.

The necine bases are characterized by a group of absorptions in the 3000–2800 cm^{-1} range which correspond to CH_2 symmetric and asymmetric stretching. In particular, retronecine derivatives show a series of absorptions near 2950 cm^{-1} (characteristic of all the macrocyclic esters), near 2975 and 2915 cm^{-1} (CH_2 asymmetric stretching), near 2870 and 2850 cm^{-1} (CH_2 symmetric stretching) and near 2825 cm^{-1} (symmetric stretching of CH_2 bound to the nitrogen atom in the heterocyclic ring). These data are in agreement with those reported by Gupta *et al.*[19]. The otonecine derivatives exhibit absorptions of the same intensity, normally falling at a slightly higher frequency (*ca.* 10 cm^{-1}), together with a characteristic absorption at 2815 cm^{-1} which can be attributed to the symmetric stretching of CH_3 on the nitrogen atom of the heterocyclic ring. The macrocyclic PA ester functions give rise to a medium to strong absorption band in the 1770–1730 cm^{-1} range corresponding to C=O stretching. The α,β-unsaturated esters also show a medium to weak absorption in the 1640 cm^{-1} range, due to C=C, which is stronger for the asymmetrically substituted compounds [*i.e.*, senecivernine (**1**), seneciphylline (**3**) and integerrimine (**5**)]. In this range of frequency, otonecine derivatives exhibit an important and distinctive medium-intensity band near 1675 cm^{-1}, attributed to the C=O group of the otonecine ring. As already reported for the IR spectra in the solid and liquid phases[22], the unusually low-frequency absorption can be correlated to the strong transanular interaction between the C=O group and the nitrogen atom[23,24].

A great deal of information can be drawn from the absorptions in the 1500–900 cm^{-1} range. The medium–weak absorptions near 1450 and 1360 cm^{-1} can be attributed to the CH_2 bending and twisting, respectively. The medium–strong absorptions, in contrast, in the 1265–1245 and 1230–1210 cm^{-1} ranges are related to C–O stretching of the ester group and to CH_2 wagging, respectively. All the PAs with a tertiary hydroxyl group in their structure give rise to a strong absorption near 1150 cm^{-1} corresponding to the C–O stretching. Finally, an absorption related to the ring deformation modes in the 960–940 cm^{-1} range is present for both the retronecine and otonecine derivatives in all instances.

Fig. 4 reports the vapour-phase FT-IR spectra of senecivernine (**1**) and desacetyldoronine (**14**), which is a member of the PA class identified for the first time in

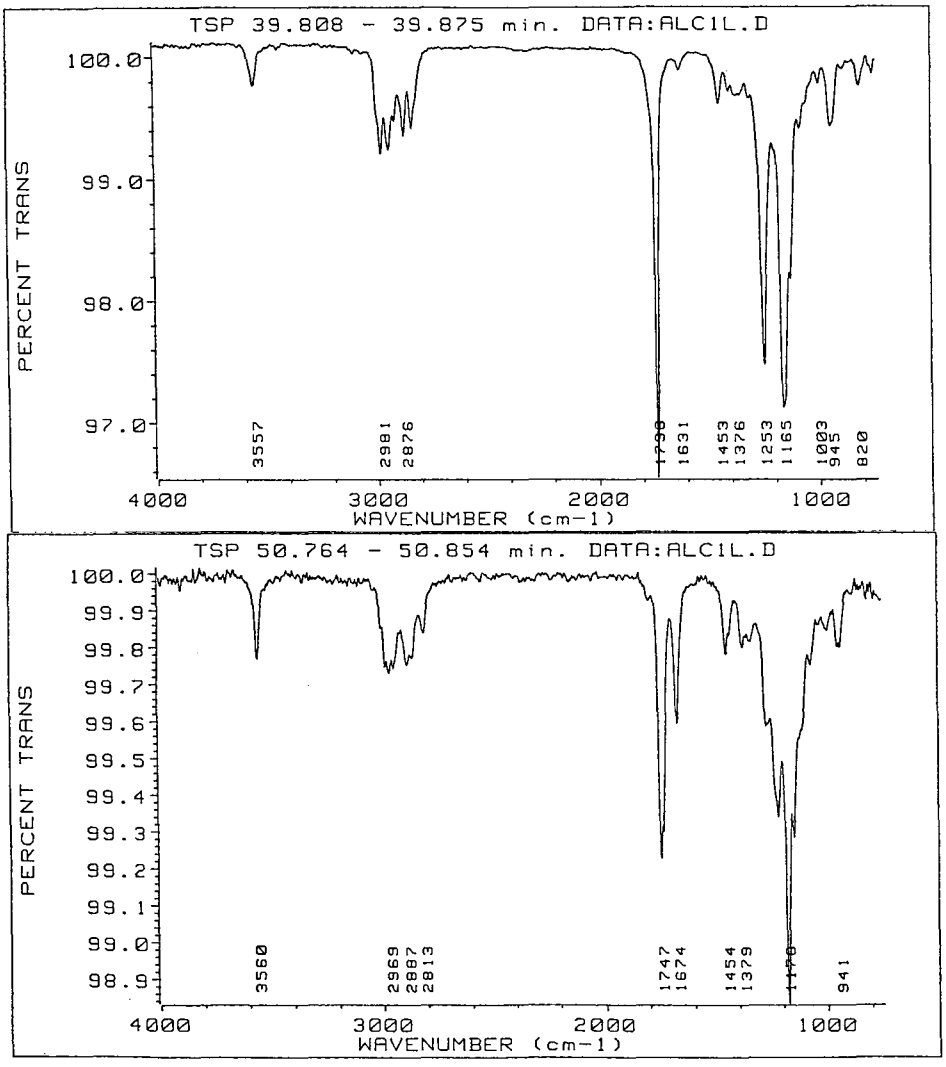


Fig. 4. Vapour-phase FT-IR spectra of senecivernine (**1**) and desacetyldoronine (**14**).

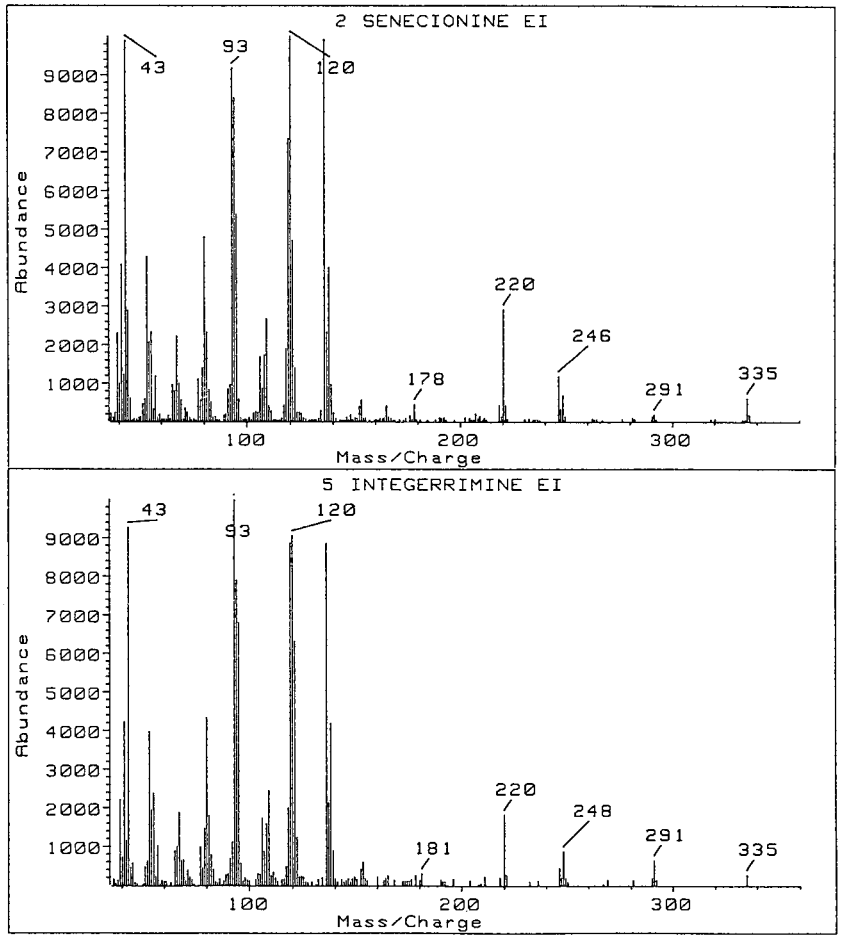


Fig. 5. EI mass spectra of senecionine (**2**) and integerrimine (**5**).

S. inaequidens[10]. Fig. 4 clearly demonstrates that vapour-phase FT-IR spectra can be very helpful in distinguishing the PA necine base through the different absorptions of a retronecine derivative, senecivernine, at 2981, 2945, 2910, 2876, 2849 and 2820 cm^{-1}, and an otonecine derivative, desacetyldoronine, at 2990, 2969, 2945, 2886, 2855, 2815 and, significantly, 1674 cm^{-1}.

Vapour-phase FT-IR spectra of PAs can also provide an unambiguous distinction between geometric isomers, which is not always directly possible through their mass spectra. The sample of *S. inaequidens* under analysis is characterized by the presence of four pairs of geometric isomers: senecionine–integerrimine, seneciphylline–spartioidine, retrorsine–usaramine and senkirkine–neosenkirkine. The EI mass spectra of senecionine and integerrimine (Fig. 5) clearly demonstrate how difficult it is to distinguish between them unequivocally, even when standard samples are available. The small differences in the intensities of the peaks at *m/z* 220, 246, 248, 291 and 335 are not sufficiently pronounced as to allow a correct identification, as they could also be

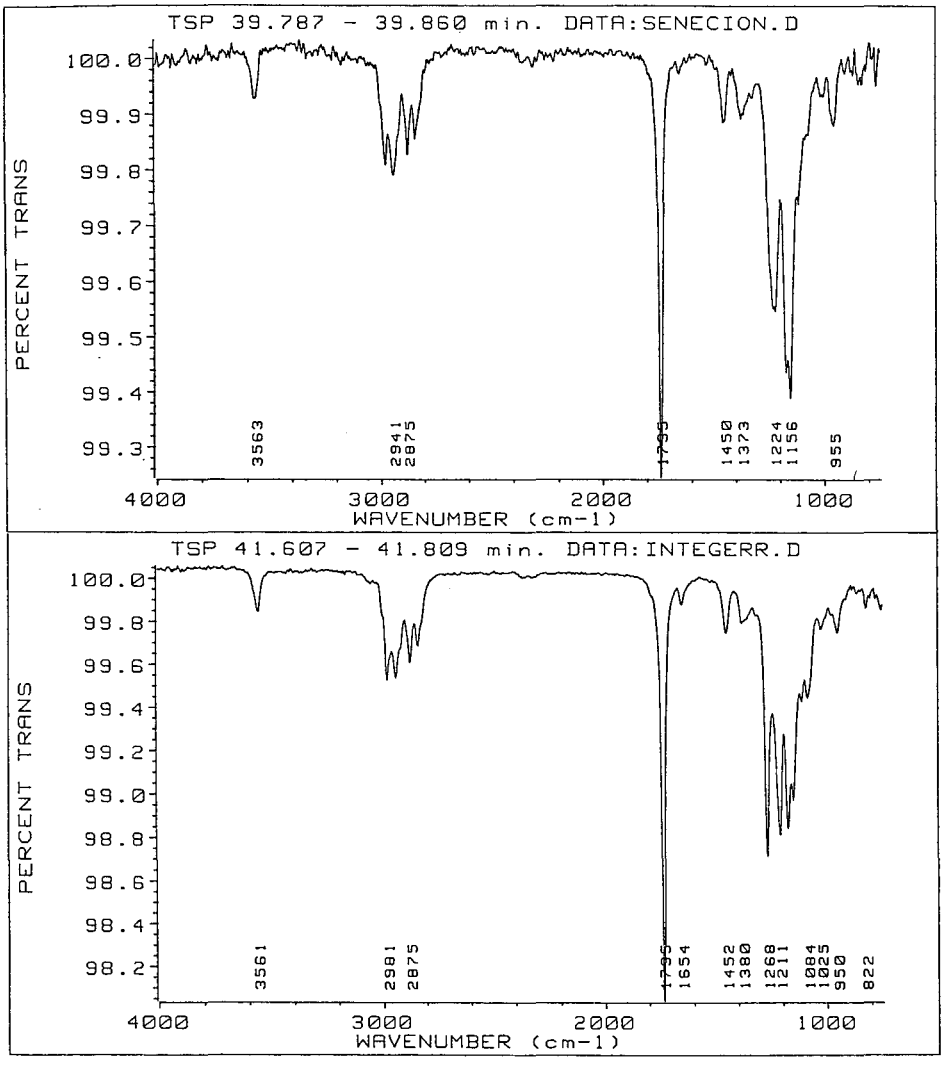


Fig. 6. Vapour-phase FT-IR spectra of senecionine (**2**) and integerrimine (**5**).

influenced by the operating conditions of the mass spectrometer ion source, which can vary slightly from instrument to instrument. Vapour-phase FT-IR spectra (Fig. 6), in contrast, afford a clear distinction between the two isomers in the 1300–1100 cm^{-1} range. In fact, whereas integerrimine absorbs at 1268, 1211, 1178 and 1163 cm^{-1}, senecionine absorbs at 1247, 1224, 1176 and 1156 cm^{-1}, at different intensities. These results were also confirmed with the retrorsine–usaramine pair.

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REFERENCES

1 L. B. Bull, C. C. J. Culvenor and A. T. Dick, *The Pyrrolizidine Alkaloids*, North-Holland, Amsterdam, 1968, Ch. 8 and 9.
2 F. L. Warren, in R. H. F. Manske (Editor), *The Alkaloids*, Vol. XII, Academic Press, New York, 1978, p. 246.
3 C. C. J. Culvenor, *Bot. Not.*, 131 (1978) 473.
4 L. W. Smith and C. C. J. Culvenor, *J. Nat. Prod.*, 44 (1981) 129.
5 D. J. Robins, *Fortschr. Chem. Org. Naturst.*, 42 (1981) 116.
6 A. R. Mattock, *Chemistry and Toxicology of Pyrrolizidine Alkaloids*, Academic Press, London, 1987, Ch. 6 and 7.
7 J. Robins, *Nat. Prod. Rep.*, 4 (1987) 577.
8 H. Wiedenfeld, U. Pastewska, P. Stengl and E. Roder, *Planta Med.*, 41 (1981) 124.
9 C. Bicchi, A. D'Amato and E. Cappelletti, *J. Chromatogr.*, 349 (1985) 23.
10 C. Bicchi, R. Caniato, R. Tabacchi and G. Tsoupras, *J. Nat. Prod.*, 52 (1989) 32
11 M. L. Deinzer, B. L. Arbogast, D. R. Buhler and P. R. Cheeke, *Anal. Chem.*, 54 (1982) 1811.
12 J. Luethy, U. Zweifel, B. Karlhuber and C. Schlatter, *J. Agric. Food Chem.*, 29 (1981) 302.
13 H. J. Huizing, F. de Boer, H. Hendriks, W. Balraadjsing and A. P. Bruins, *Biomed. Environ. Mass Spectrom*, 13 (1986) 293.
14 H. Hendriks, W. Balraadjsing, H. J. Huizing and A. P. Bruins, *Planta Med.*, 53 (1987) 456.
15 H. J. Segall, C. K. Winter and A. D. Jones, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 265.
16 H. Hendriks, H. J. Huizing and A. P. Bruins, *J. Chromatogr.*, 428 (1988) 352.
17 C. C. J. Culvenor and R. Dal Bon, *Aust. J. Chem.*, 17 (1964) 1296.
18 C. C. J. Culvenor and L. W. Smith, *Aust. J. Chem.*, 12 (1959) 255.
19 V. P. Gupta, S. K. Handoo and R. S. Sawhney, *Curr. Sci.*, 44 (1975) 451.
20 M. L. Lee, C. L. Wooley, R. C. Kong and B. E. Richter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 329.
21 P. R. Griffith, J. A. de Haseth and L. V. Azarraga, *Anal. Chem.*, 55 (1983) 1361A.
22 G. I. Birnbaum, *J. Am. Chem. Soc.*, 96 (1974) 6165.
23 L. H. Briggs, R. C. Cambie, B. J. Candy, G. M. O'Donovan, R. H. Russel and R. N. Seelye, *J. Chem. Soc.*, (1965) 2492.
24 B. H. G. Crout, *J. Chem. Soc., Perkin Trans. 1*, (1972) 1602.

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AUTOMATED DUAL COLUMN COUPLED SYSTEM FOR SIMULTANEOUS DETERMINATION OF CARBOXYLIC ACIDS AND INORGANIC ANIONS

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(First received November 22nd, 1988; revised manuscript received March 6th, 1989)

SUMMARY

Isocratic anion-exchange separations have often been found inadequate for mixtures of carboxylic acids with weakly and strongly retained inorganic anions. In the coupled column approach, separating performance is improved by the simultaneous use of two separation modes. After an injection onto an ion-exclusion column all anions of strong acids elute in a narrow zone close to the dead volume and are easily diverted to an anion-exchange system for an interference free separation. Carboxylic and other relatively weak organic or inorganic acids are separated by ion exclusion and are thus removed as interferences in the separation of anions by anion exchange. A simple and reliable preconcentration technique has also been developed, allowing a simultaneous trace enrichment of weak and strong anions prior to their injection on the coupled system.

INTRODUCTION

In highly purified water as it is used in power plants and in semiconductor manufacturing, trace concentrations of carboxylic acids and inorganic anions are usually narrowly specified[1]. If the acceptable levels are exceeded, different removal techniques have to be employed for inorganic anions and for organic acids. Since both types of contamination frequently occur in the same time, it becomes important for the operating personnel to be able to distinguish between the organic and inorganic contamination levels. Ion chromatography has gained a broad acceptance as a tool for analyzing low levels of inorganic ions in water samples[2–4].

Yet, a simultaneous determination of short-chain carboxylic acids in mixtures with the monovalent inorganic anions such as fluoride, chloride, carbonate and bromide still represents a difficult separation problem even with the most recently introduced high-efficiency anion-exchange columns[4].

Such inherent limitations of (mono-dimensional) anion-exchange chromatography are explainable with the help of a theoretical framework developed by Giddings[5–7]. This author has derived a fundamental relationship between the maximum number of peaks that can be resolved (peak capacity n) and the column

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efficiency as measured by the number of theoretical plates (N):

$$n = 1 + \tfrac{1}{4}[N^{1/2}\ln(V_x/V_0)] \quad (1)$$

where V_0 and V_x signify the initial and final volume of an available retention range on any given chromatographic column. Due to the characteristic peak grouping peculiar to anion-exchange separations a typical elution range ratio for the seven anions in question (fluoride, chloride, carbonate, bromide, formate, acetate and propionate) is $V_x/V_0 = 3$. Assuming $N = 3000$, the corresponding maximum peak capacity can be calculated by eqn. 1 as $n = 16$. Theory shows[7] that the number of fully resolved, randomly distributed single component peaks n_r never exceedes 18% of n, resulting in only about three fully resolvable peaks in an area "crowded" by seven or more anionic species. Under isocratic conditions attempts to extend the elution range lead to an excessively large or permanent retention for phosphate and sulfate. The resulting loss of information is considered unacceptable by the analysts in the power and semiconductor industries[4]. A promising approach to the problem appears to be offered by the newly introduced gradient techniques for single column[8] and suppressed ion chromatography[9]. In this report we describe yet another approach —an increase in resolving power that can be achieved by the coupling of two separation modes (ion exclusion and anion exchange)— in a single chromatographic system.

EXPERIMENTAL

Instrumentation

The liquid chromatographic system consisted of two Waters (Milford, MA, U.S.A.) Model 590 programmable pumps and two Model 430 conductivity detectors. Both chromatographic pumps were equipped with the high sensitivity accessories (pulse damping) and the event boxes (interfaces between the pumps and controlled external devices such as switching valves or preconcentration pumps) obtainable from the same supplier.

Four of Waters automatic column switching valves were used for changing the configuration of the system and for manual or automatic injection as determined by the program stored in one of the two programmable pumps. A trace enrichment module (Waters PN 07448) containing a single piston pump and a holder for preconcentration cartridges (Guard Pak Assembly ILC, Waters PN 33100) was also controlled by the same program through the event box interface. The anion-exchange and ion-exclusion columns were as described in our previous publication[10]. IC Pak anion concentrator cartridges (5 × 8 mm, Waters PN 07358) containing polyacrylate based anion-exchange resin of approximately same mequiv./g capacity as in the anion-exchange column were utilized for preconcentration of samples. Data acquisition was carried out with the help of a Model 840 data station and two SIM interfaces. Collection of chromatographic data was initiated by a signal connection via the event box.

Eluent preparation for system one (ion exclusion)

A 10 mM concentrate of 1-octanesulfonic acid was prepared first. An amount of 2.163 g of the sodium salt of octanesulfonic acid (98%, Aldrich 22, 156-2) purified by

crystallization was placed into a 250-ml beaker and dissolved in 100 ml of Milli-Q (Millipore, Bedford, MA, U.S.A.) water using a magnetic stirrer. A 100-ml volume of a precleaned cation-exchange resin was than added to this solution and the resulting slurry was stirred for *ca.* 10 min. In the next step the cation-exchange resin was removed from the solution by filtration through a prewashed (10 ml water) 0.45-μm filter (Type HA, Millipore). Proper care had to be taken that the resin inside the filter funnel was wet throughout the whole filtration. This ensured that the octanesulfonic acid was quantitatively transferred into the filtrate. In order to keep the resin covered with solution and to wash out the sulfonic acid as completely as possible *ca.* 800 ml of Milli-Q water had to be added from a clean container and prepared before the actual filtration began. The resulting *ca.* 900 ml of filtrate were transferred into a volumetric flask and filled up to 1000 ml. This 10 m*M* octanesulfonic acid solution was stable for up to one month.

The 1 m*M* eluent was than prepared freshly by diluting aliquots of the concentrate and by filtration through a Millipore HA 0.45-μm filter. The observed background conductance of such clean ion-exclusion eluent was in the range of 320 to 330 μS. The pH of the eluent was *ca.* 3.0 which led to an useful retention behavior for all acids with pK_a values of 3 or greater.

Eluent preparation for system two (anion exchange)

A 0.649-g amount of 1-octanesulfonic acid, sodium salt was dissolved in 1 l of Milli-Q water in a volumetric flask. Filtration and degassing with the help of 0.45-μm Millipore HA filters followed. Resulting solution was 3 m*M* in sodium octanesulfonate. It was found that interferences by chromatographic peaks stemming from impurities could be kept at a minimum, if both the sodium salt and the prepared sulfonic acid were from the identical batch and from the same manufacturer.

Sample preparation

Samples were collected in 200-ml polystyrene tissue culture flasks (Corning Glass Works, Corning, NY, U.S.A.) (PN 25115). These particular flasks were chosen after a long term evaluation of possible sample containers from several different manufacturers. It was found that all other containers except the ones recommended here, contributed traces of anions at concentrations above the detection limits of our method. Prior to sample collection the tissue culture flasks were rinsed five times to overflowing with Milli-Q water as well as soaked for 24 h filled with the same ultrapure water. The samples were drawn into the system through a PTFE tubing immersed in the flask and connected to a trace enrichment pump (TEP in Fig. 1) at the other end. Additional steps that are discussed in the Results and Discussion section of this article were required for highly alkaline samples and for samples with high concentration of boric acid.

Standard solutions

All standard mixtures were prepared by a dilution of 1000-ppm stock solutions containing a single anion. Weighed amounts of salts rather than acids were used for the preparation of stock solutions. Concentrates of 1000 ppm of anions of carboxylic acids were found stable for at least six weeks. No measurable changes of concentrations of stock solutions of inorganic acids were found during a time period lasting twelve

months. The Milli-Q water and polypropylene containers (Nalge, Rochester, NY, U.S.A.) (PN 4000) were utilized for all standards in the ppm range of concentrations. The tissue culture flasks were used for standards containing less than 500 ppb[a] of a single anion. All standards containing less than 50 ppm of any anion were prepared freshly for each of the experiments.

System operation

The system schematics illustrated in Fig. 1 could be used for both, manual injection of mixtures at higher concentrations (*ca.* 1 ppm and more) and for automatic preconcentration at sub-ppm levels. Each of the four six-port high-pressure switching valves (TEV, 1TV, 2TV and MIV in Fig. 1) fulfills a different function within the coupled system. The trace enrichment valve (TEV) switches the trace enrichment cartridge (CON) from the sample line to the flow of the eluent coming either from pump 1 or from pump 2 as determined by the position of the first transfer valve (1TV). The second transfer valve (2TV) provides the necessary link between the two systems

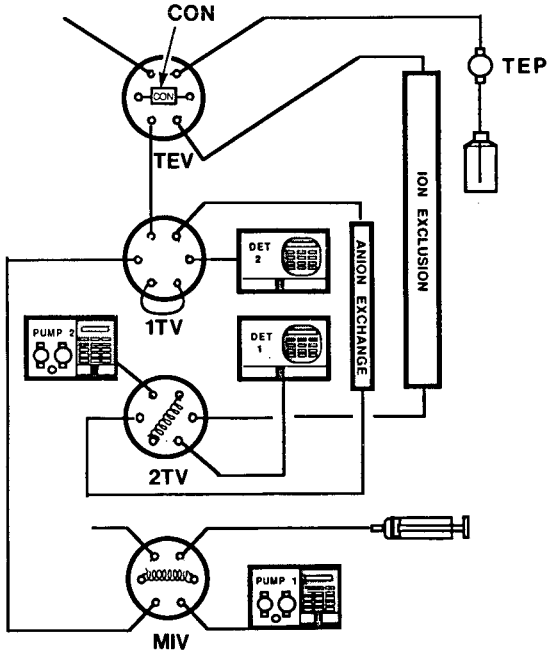


Fig. 1. General configuration of the coupled ion-exclusion–anion-exchange system. Pump 1 and detector 1 (DET 1) are used in conjunction with the ion-exclusion column. Pump 2 and detector 2 are parts of the anion-exchange system. TEP = Single piston trace enrichment pump; CON = precolumn used for trace enrichment; TEV = high-pressure switching valve utilized for connecting the precolumn into the sample stream for a known period of time; 1TV = first transfer valve connecting both pumps to the precolumn; 2TV = second transfer valve which makes it possible to transfer fractions of the ion-exclusion column eluate to the anion-exchange column; MIV = manual injection valve used for sample introduction in cases that do not require preconcentration (ppm range).

[a] Throughout this article the American billion (10^9) is meant.

TABLE I

PROGRAM FOR THE FRACTION TRANSFER BETWEEN TWO SYSTEMS (MANUAL INJECTION)

Refer to Fig. 2 for the positions of the second transfer valve (2TV) controlled by the events 5 and 6. Events 1–4 and 7 have been assigned to various functions of the sample preconcentration procedure explained in Table II and in Fig.3.

Step No.	*Time (min)*	*Events*			*Description*
		5	*6*	*8*	
1	–	N	F	F	Loading the sample into the sample loop of the manual injection valve. N = on, F = off signifying two different levels of voltage on one of the eight pairs of contacts (8 events) in the M590 event box
2	0	N	F	F	Manual injection onto the ion-exclusion column. Data acquisition by system 1 is activated by a direct signal connection between the manual injector and the system interface (SIM)
3	4.5	F	N	P	Transfer of 500 µl from the ion-exclusion separation to the anion-exchange column. A signal pulse P (event 8) starts the data acquisition on system 2
4	25.0	N	F	F	2TV returns to its original position separating the two systems

for the transfer of a fraction of the void volume from the ion-exclusion separation onto the anion-exchange column. The appropriate time sequence of column switching steps by valves 1TV, 2TV and TEV is determined by a program written and stored in the microprocessor of one of the two programmable chromatographic pumps. Detailed descriptions of such programs are given in Tables I and II. The only column switching valve that is actuated manually outside the control of an automatic program is the

TABLE II

PROGRAM FOR THE FRACTION TRANSFER BETWEEN TWO SYSTEMS (WITH SAMPLE PRECONCENTRATION)

Refer to Fig. 3 for the positions of the valves controlled by the events 1–6. TEV, events 1 and 2; 1TV, events 3 and 4; 2TV, events 5 and 6; TEP, event 7. Data acquisition for both separations, event 8. N = on, F = off.

Step No.	*Time (min)*	*Events*								*Description*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
1	0.0	F	N	N	F	N	F	F	F	Equilibrate precolumn
2	2.0	N	F	N	F	N	F	N	F	Load sample
3	12.0	F	N	F	N	N	F	N	P	Inject part 1
4	12.5	F	N	N	F	N	F	F	F	Inject part 2
5	13.0	N	F	N	F	N	F	F	F	End injection
6	16.5	N	F	N	F	F	N	F	F	Transfer
7	36.5	N	F	N	F	N	F	F	F	Reset valves

manual injection valve (MIV). A signal connection between the MIV and the system interface module (SIM) of the system 1 starts the recording of the ion-exclusion separation in the same instant as the MIV is turned from the load position to the inject position (from step 1 to step 2 in Fig. 2). Another signal connection (MIV to M590 event box) starts the timer for the automatic program sequence (steps 2, 3 and 4). The data acquisition for the anion-exchange separation is then initated simultaneously with the actuation of the 2TV (step 3 in Table I and in Fig. 2).

The positions of the valves TEV and 1TV remain unchanged during the entire manual injection procedure (see Fig. 2) and are therefore not listed in Table I.

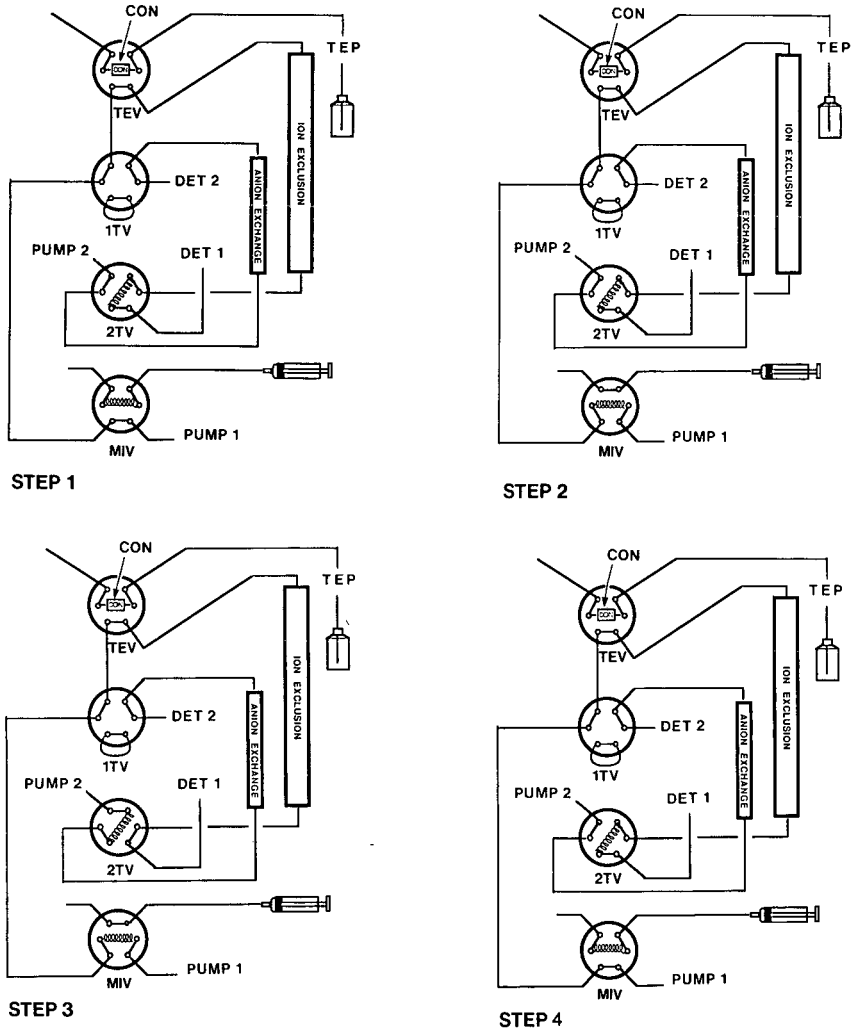


Fig. 2. Column coupling at relatively high concentrations (ppm range). Refer to the description presented in the Experimental section and to the program given in Table I. step 1 = Manually load sample into loop via syringe; step 2 = manual injection into system 1; step 3 = transfer void volume from system 1 to system 2; step 4 = transfer valve returns to the original position, the two systems are separated again.

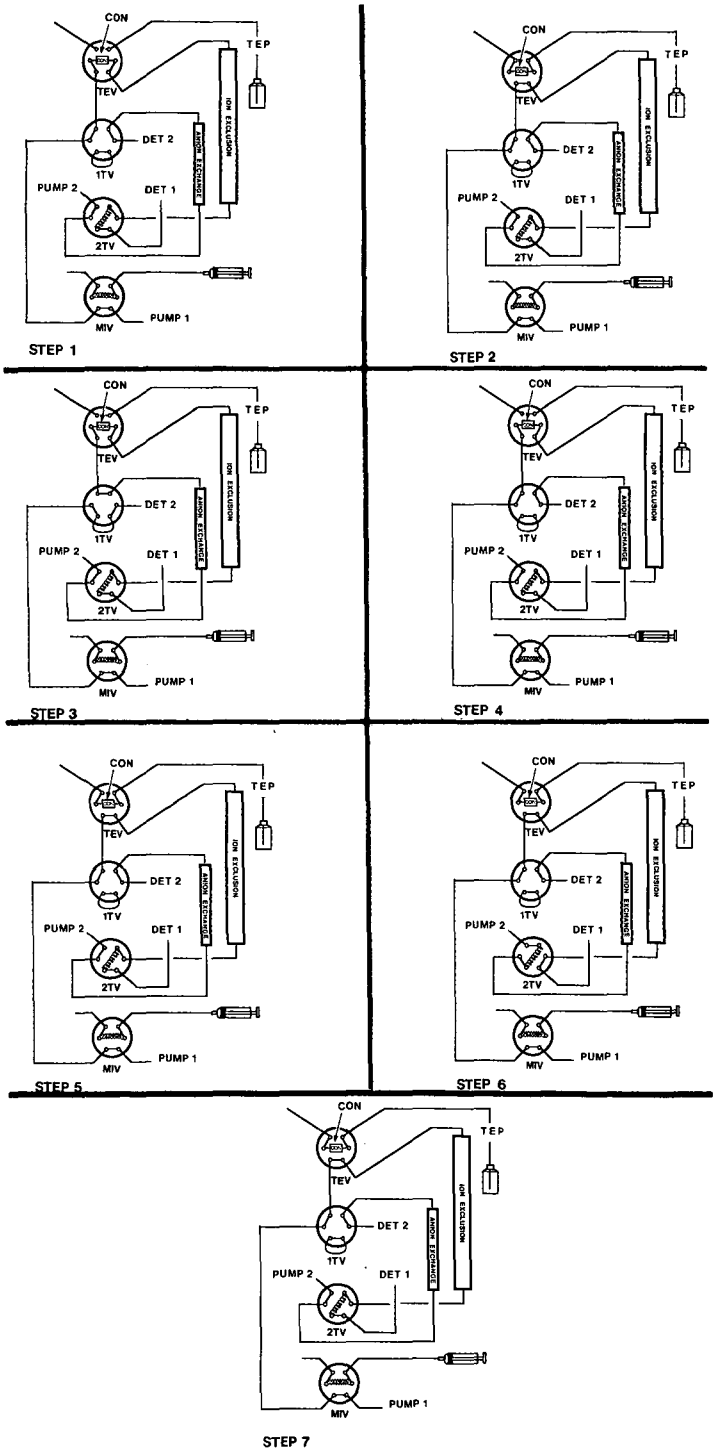


Fig. 3. Simultaneous trace enrichment of the weak and strong anions followed by concurrent ion-exclusion and anion-exchange separations. Table II describes the details of the column switching program. Additional description is offered in the Experimental section. Step 1 = Purge sample line/equilibrate concentrator/TEP on; step 2 = load sample onto concentrator/TEP on; step 3 = inject sample with eluent 2/TEP off; step 4 = inject sample with eluent 1; step 5 = end of injection; step 6 = transfer void volume to system 2; step 7 = reset valves to the initial position.

During a procedure utilizing trace enrichment on a precolumn mounted on the TEV it is the position of the MIV that remains unchanged (see Fig. 3). Injection of a sample is achieved by positioning the precolumn (CON) into the eluent stream. Such an injection is then a part of a fully automatic sequence an example of which is given by Table II. In the automatic procedure the data acquisition is initiated simultaneously for both chromatographic systems by step 4 in Table II. The real retention time for the second system (anion exchange) is than calculated by subtracting the time difference between steps 7 and 4 in Table II from the apparent retention time obtained in the recording. As an alternative, a synchronous program with the one in pump 1 (see Table II) can be run inside pump 2 containing only one segment providing a pulsed output at the time of step 7. By connecting SIM 1 to the event box of pump 1 and with a connection between SIM 2 and the event box 2, real retention times can be recorded for both systems.

RESULTS AND DISCUSSION

Limitations of anion-exchange separations

As predicted by theory (see Introduction), the anion-exchange separation mode is inherently inadequate whenever mixtures of carboxylic acids and early eluting inorganic anions are to be separated. To illustrate the phenomena we have attempted a separation of thirteen organic and inorganic anions (Fig. 4). Note that while the chosen anion-exchange column efficiently separates the six late eluting anions (peaks 8–13 in Fig. 4), a lack of selectivity and separation efficiency is observed for the initial seven peaks of some of the inorganic anions and of all organic acids present in the sample. Furthermore, the initial peak of the seven coeluting anions could easily be mistaken for a signal corresponding to a larger concentration of just a single anion (*i.e.*, fluoride).

Coupling of ion exclusion and anion exchange

In an earlier work[10] we have successfully applied column coupling to simultaneous separations of traces of inorganic anions in the presence of large concentra-

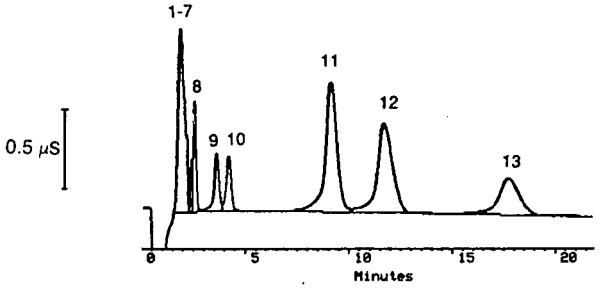


Fig. 4. Attempted anion-exchange separation of a mixture of thirteen organic and inorganic anions. Conditions: column, Waters IC PAK Anion (5 × 0.46 cm I.D.); eluent, 3 m*M* sodium octanesulfonate; flow-rate, 1 ml/min; sample, 100 μl of a mixture of thirteen anions; detection, Waters M430 conductivity detector. Anions: 1 = fluoride (0.5 ppm); 2 = glycolate (2.0 ppm); 3 = formate (1.0 ppm); 4 = acetate (10.0 ppm); 5 = propionate (20.0 ppm); 6 = butyrate (20.0 ppm); 7 = iodate (50.0 ppm); 8 = chloride (1.0 ppm); 9 = bromide (2.0 ppm); 10 = nitrate (2.0 ppm); 11 = iodide (20.0 ppm); 12 = sulfate (2.0 ppm); 13 = thiocyanate (5.0 ppm).

tions of boric acid. Use was made of the fact that on an ion-exclusion column all strongly acidic anions elute within the dead volume, while only the weak acids, as for example the boric and carbonic acid, are retained and separated. In order to broaden the scope of the technique and to make it applicable to a larger variety of weak and strong acids we have now carried out a more detailed investigation of the dead volume zone of an ion-exclusion separation.

The mixture of anions for our experiments with fraction transfer from ion exclusion to anion exchange consisted of two anions of weak acids (fluoride and phosphate) and of three anions belonging to strong acids (chloride, bromide and nitrate). The ion-exclusion chromatogram resulting from an injection of such a mixture is shown in Fig. 5. In this separation four of the chosen anions coelute with a strongly negative signal caused by water from the injected sample and do not appear in the form of distinct chromatographic peaks if conductivity is utilized as a detection mode. The width of the fluoride peak was used to estimate the optimum size of the fraction cuts from the ion-exclusion separation for the transfer to the anion-exchange column. To prevent fraction splitting it is recommended[7] not to exceed the size of about 4σ of an average peak for any given separation. Based on these considerations 500 μl were chosen for a fraction volume. The perceived end point of the negative deflection marking the dead volume was chosen as a center with the experimental fractionation range reaching an equal distance in both directions (see Fig. 5).

As seen from Figs. 6 and 7, anions of strong acids are concentrated in fraction 1. Their recoveries from that cut after the transfer to the anion-exchange column are in excess of 90%. On the other hand, the investigated fractionation procedure fails for the anions of weak acids. The recoveries for fluoride remain low throughout the whole range between 4 and 6 min. Because of such strong retention, ion exclusion becomes clearly the preferred separation mode for fluoride in the coupled systems. The bulk of the phosphate concentration was found to be located at the boundary between fractions 1 and 2. The recoveries for phosphate were split almost equally between these two fractions. To improve the results for this anion fraction cut 1 could be shifted by about 250 μl toward the larger retention volumes. However, such change would sharply decrease the recoveries for the other inorganic anions in that fraction. The indicated location of fraction 1 thus remains the best possible compromise for the determination of phosphate simultaneously with other anions under the conditions of

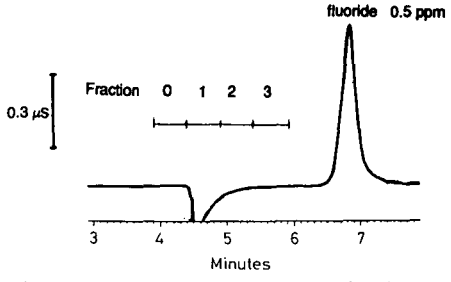


Fig. 5. Ion-exclusion chromatogram of six inorganic anions. The scale indicates the intervals during which the four fractions (0–3, see Fig. 6) were collected. Conditions: columns, two Waters Ion Exclusion (15 × 0.78 cm I.D.) in series; eluent, 1 m*M* octanesulfonic acid; flow-rate, 1.2 ml/min; sample, 100 μl of 0.5 ppm fluoride, 2 ppm chloride, 4 ppm bromide, 4 ppm nitrate, 6 ppm phosphate and 4 ppm sulfate; detection, Waters M430 conductivity detector.

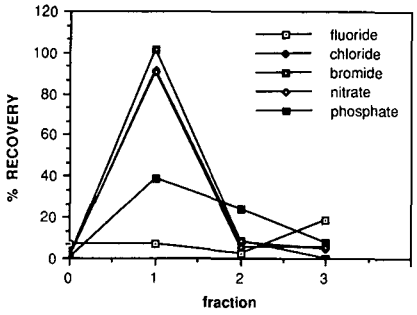


Fig. 6. Recoveries of selected inorganic anions after their transfer from the ion-exclusion to the anion-exchange column. Fractions were taken at intervals indicated in Fig. 5. Note: the distribution curves for chloride and nitrate are overlapped.

coupled dual column system. Since the sulfate was not one of the original components of the mixture injected onto the ion-exclusion column, the corresponding peak in anion-exchange separation in Fig. 7 indicates that traces of sulfate are present in the ion-exclusion eluent and transferred along with the anions from the injected sample. Such contamination by sulfate makes of course any accurate determination of that anion impossible on a coupled system. In the subsequent experiments we could prevent such sulfate contamination by using a higher purity sodium octanesulfonate obtained from a different source (Millipore Corp.). It should be noted that the percentage recoveries for chloride and for nitrate in Fig. 6 are somewhat higher than those listed for the same two anions by direct injection in Table III. These variations of recovery rates are explainable by the fact that the two sets of recovery results were obtained by two different operators on two non-identical coupled systems assembled from the scratch. In both cases the delay volume as represented by the length of the tubing connecting the ion-exclusion and the anion-exchange system were considered to be negligible.

Having thus determined the most suitable section of the ion-exclusion eluate for the transfer to the anion-exchange column we have reinjected the mixture from Fig. 4. The chromatogram in Fig. 8 documenting the resolving power of a coupled system (compare Figs. 4 and 8) was obtained with the help of the fully automated transfer procedure given in Table I and illustrated in Figs. 1 and 2.

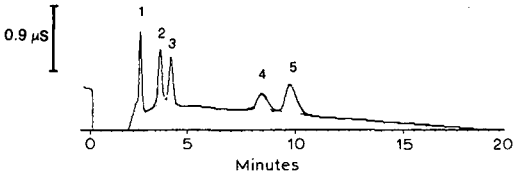


Fig. 7. Anion-exchange separation of fraction 1 from the ion-exclusion chromatogram in Fig. 5. Conditions: flow-rate, 1.2 ml/min. The 3 m*M* sodium octanesulfonate eluent was adjusted to pH = 6.0 using a 1 mg/l LiOH solution. Other conditions as in Fig. 4. Anions: 1 = chloride (2 ppm); 2 = bromide (4 ppm); 3 = nitrate (4 ppm); 4 = phosphate (6 ppm); 5 = sulfate.

TABLE III

RECOVERIES AT ppm AND ppb LEVELS

For acceptable recoveries of weak acids the pH of the preconcentrated samples has to be sufficiently high to ensure a full dissociation of analytes.

Anion	*Concentration (ppm)*	*Injection/preconcentration volume (ml)*	*Recovery (%)*
Chloride	2	0.1	74.1
Nitrate	4	0.1	80.3
Fluoride	0.0033	35	84.4
Formate	0.0067	35	91.2
Acetate	0.0167	35	95.4
Propionate	0.0333	35	87.1
Chloride	0.0067	35	59.7
Nitrate	0.0133	35	60.2

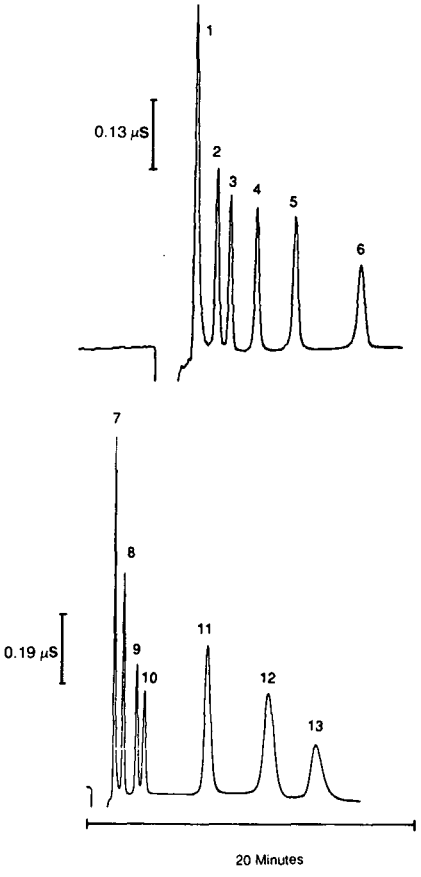


Fig. 8. Simultaneous separation of weakly and strongly acidic anions on a coupled system. Conditions: flow-rate, 1 ml/min for both separation systems; sample, 100 μl of a mixture of strong and weak acid anions. Other conditions were identical with those in Fig. 4 (anion exchange) and in Fig. 5 (ion exclusion). Peak identification and concentrations as in Fig. 4.

Sample preconcentration for the coupled ion-exclusion and anion-exchange separations

Simultaneous preconcentration of weakly and strongly acidic anions is a problem requiring some consideration. For optimum recoveries of weakly ionized anions on anion-exchange concentrator columns one has to make sure that such anions do not have to compete for ion-exchange sites with either more strongly attracted anions from the same sample or with a much greater mass of other anions of comparable strength. Under practical conditions one attempts to fulfill these requirements by providing a sufficiently high anion-exchange capacity on the concentrator and by conditioning the concentrator precolumn with a solution of the lowest possible eluting strength. In the coupled system under discussion 1 m*M* octanesulfonic acid coming from pump 1 is the weaker anion-exchange eluent of the two employed mobile phases. For this reason the automatic sequence depicted in Fig. 3 employs an initial step during which a controlled volume of the ion-exclusion eluent is pumped through the concentrator to condition it for the next sample enrichment (see step 1 in Fig. 3). Duration of step 1 and of all the other steps of the automatic trace enrichment and column coupling procedure is given in Table II. The actual loading of the anions from the sample onto the concentrator is carried out during step 2. In step 3 the preconcentrated anions are eluted from the concentrator and onto the ion-exclusion column by a 0.5-ml segment of the anion-exchange eluent. This is followed by an equal volume of the ion-exclusion eluent from pump 1 (step 4). During step 4 the remaining interstitially held volume of pure water stemming from the sample is replaced by the ion-exclusion eluent. The concentrator column is taken out of the eluent stream in step 5. Fraction one (see Fig. 5) of the ion-exclusion separation is being transferred to the anion-exchange column during step 6. Both, the ion-exclusion and anion-exchange separation run simultaneously and are completed within step 7. Recoveries of several anions with and without the preconcentration procedure just described are given in Table III. The percentage values for chloride and nitrate at relatively high concentrations (2 and 4 ppm) were obtained by comparing peak areas generated using the procedure described in Table I with those measured after a direct injection on the single-column anion-exchange system. These values reflect the efficiency of transfer of strong anions from the ion-exclusion to the anion-exchange system. The remaining data in Table III were collected at concentration levels requiring sample preconcentration. In the case of weak acids (fluoride through propionate) the listed values relate only to the performance of the preconcentration procedure (step 2, Table II) (*i.e.*, at 100% preconcentration efficiency 35 ml of 3.3 ppb fluoride should generate the same peak area as 100 μl of 1.155 ppm of the same anion, etc.). The values of recoveries for sub-ppm levels of chloride and nitrate represent the combined efficiencies of both the sample preconcentration and transfer. With the known recoveries for the fraction transfer determined at ppm levels (chloride 74.1% and nitrate 80%) and the sub-ppm values listed in Table III, the preconcentration efficiencies for chloride and nitrate can now be calculated as 80.5% and 75% respectively. As can be seen in the next section of this article, the relatively low recoveries given in Table III for chloride and nitrate, which result from the two steps just discussed, remain without any negative influence on the reproducibility of the method. A chromatogram showing simultaneous separation of ppb levels of carboxylic acids in a mixture with inorganic anions is presented in Fig. 9.

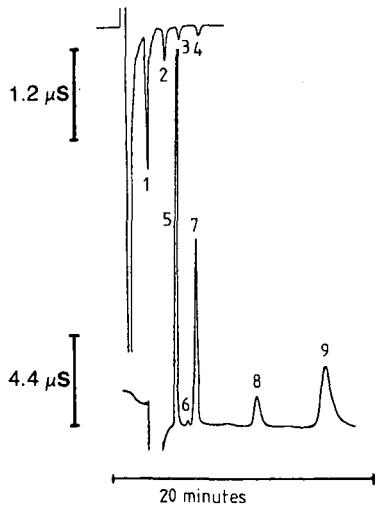


Fig. 9. Chromatogram of ppb concentrations of carboxylic acids and inorganic anions. Conditions: anion-exchange eluent, 2.1 m*M* sodium octanesulfonate; sample, 33 ml of the low-ppb mixture of anions were preconcentrated on a precolumn (CON, Fig. 1). All other conditions were as given in Fig. 8. It should be noted that because of the delayed start of data acquisition for the upper chromatogram (ion exclusion) 2.8 min should be added to the retention times for peaks 1–4. Anions: 1 = fluoride (5 ppb); 2 = formate (5 ppb); 3 = acetate (10 ppb); 4 = propionate (25 ppb); 5 = chloride (25 ppb); 6 = bromide; 7 = nitrate (25 ppb); 8 = iodide (25 ppb); 9 = sulfate (25 ppb).

Reproducibility of separations on the coupled system

Precision of the results on the investigated coupled column system was evaluated at two different concentration levels. In the first series of experiments three repetitive injections were made at five different concentrations not requiring any sample preconcentration (first concentration level: 1–45 ppm, see Table IV). The main purpose of these measurements was to evaluate the reproducibility of the transfer of strongly acidic anions from the dead volume zone of the ion-exclusion column to the anion-exchange separation system. The reproducibility of retention times for each of the peaks of the selected anion standards was calculated from the total of fifteen injections. In all instances the values of the relative standard deviations (R.S.D.) of retention times were found to be less than 0.9%. The relative standard deviations of peak areas were estimated from three analyses performed at each of the five concentrations and are presented in Table IV. The reproducibility of the chloride results was found to be acceptable in the entire investigated range from 1 to 45 ppm. The precision of the results for nitrate and for iodide deteriorated below 15 and 35 ppm, respectively. Given such a kind of concentration dependency of the standard deviations, the increased imprecision for nitrate and for iodide was attributed to the relatively high values of detection limits under the chosen experimental conditions. UV detection is known to provide a better sensitivity for nitrate[11] and amperometry has been shown to enable a more sensitive detection for iodide[12] than the conductivity detection utilized in our work on the coupled systems. Overall, the data in Table IV give an indication that the analysis of strongly acidic anions can be achieved with an acceptable reproducibility with the investigated configuration of the coupled system.

TABLE IV

REPRODUCIBILITY OF PEAK AREAS (% R.S.D.) AFTER THE TRANSFER FROM ION EXCLUSION TO ANION EXCHANGE AT FIVE DIFFERENT CONCENTRATIONS

Concentration 1: 1 ppm chloride, 1 ppm nitrate and 5 ppm iodide; concentration 2: 15 ppm all three anions; concentration 3: 25 ppm all three anions; concentration 4: 35 ppm all three anions; concentration 5: 45 ppm all three anions.

Anion	*Concentration*				
	1	*2*	*3*	*4*	*5*
Chloride	0.8	0.4	0.3	0.4	0.2
Nitrate	3.1	0.2	0.4	0.5	0.1
Iodide	10.5	24.0	9.8	1.2	0.9

Additional evidence is provided by the data in Table V (second concentration level 0.5–45 ppb) generated using the automatic preconcentration and fraction transfer outlined in Table II and illustrated in Fig. 3 (steps 1–7). As in Table IV each value in Table V represents an estimate of the relative standard deviations of peak areas based on three independent measurements. Precision of the results was found to be satisfactory with the exception of that for the peak areas of iodide. As in the previous case it was concluded that the relative imprecision of the results for this anion was caused by the relatively lower sensitivity of conductometric detection. The reproducibility evaluation of retention times was based on twelve data points for each of the anions in Table V. The precision of the retention times at the second concentration level was found to be comparable to that determined at the first concentration level. All corresponding relative standard deviations were equal to or lower than 0.9.

TABLE V

REPRODUCIBILITY OF PEAK AREAS (%R.S.D.) AT FOUR DIFFERENT ppb CONCENTRATIONS (PRECONCENTRATION AND TRANSFER FROM ION EXCLUSION TO ANION EXCHANGE)

Concentration 1: fluoride 0.5 ppb, all other anions at 15 ppb; concentration 2: fluoride 1.0 ppb, all other anions at 25 ppb; concentration 3: fluoride 5.0 ppb, all other anions at 35 ppb; concentration 4: fluoride 10.0 ppb, all other anions at 45 ppb.

Anion	*Concentration*			
	1	*2*	*3*	*4*
Fluoride	6.5	2.2	0.6	1.2
Acetate	2.7	4.2	3.8	6.2
Propionate	13.2	2.6	3.5	1.0
Chloride	3.3	0.6	1.1	1.0
Nitrate	0.9	0.8	1.6	0.9
Iodide	22.0	6.7	12.0	10.0

Extreme sample matrices

At concentration levels not requiring preconcentration the discussed method of column coupling offers considerable advantages in dealing with various difficult matrices without the need for an extensive sample preparation. As has been shown[10] ppm levels of anions are separated without interferences after an injection of samples containing elevated concentrations of boric acid. In a similar manner coupled systems represent a convenient approach to the determination of trace anions in concentrated solutions of for example sodium hydroxide or sodium carbonate[13].

In alkaline solutions the hydroxide molarity is usually two or more orders of magnitude greater than that of the analyte anions present at low-ppb concentrations. During the necessary preconcentration step hydroxide would thus elute most of the other anions from the concentrator column, causing low or near zero recoveries not only for the carboxylic acids but for the most inorganic anions as well. However, as we have found, such interference with the trace enrichment step can be prevented by connecting a column containing a cation-exchange resin in its hydrogen form between the trace enrichment pump and the concentrator (TEP and CON in Fig. 1). The required ion-exchange capacity of the cation-exchange resin can be calculated from the known pH value of the water samples. As long as there are protons available at the cation-exchange sites, alkaline cations are continuously removed from the sample stream and the excess of hydroxide anions disappears into the undissociated water molecules as determined by the ionic product of water. A typical chromatogram obtained from an alkaline water sample using the procedure just described is presented in Fig. 10.

Elevated levels of boric acid usually do not cause any decreases of recoveries during the preconcentration step. As determined by its acidic dissociation constant, boric acid does not compete for the anion-exchange sites below pH 9. On the other hand boric acid, employed as a neutron absorber in nuclear power generating stations,

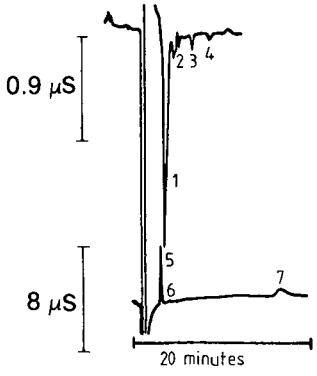


Fig. 10. Water sample from a pressurized water reactor steam generator at pH 9.5. During the preconcentration 33 ml of the sample were pretreated by contact with a strong cation exchanger in the hydrogen form. The cation-exchange resin was packed in a small precolumn connected to the sample line between the sample container and the preconcentrator cartridge, see text. Conditions: cation-exchange precolumn, Waters IC PAK Cation Guard (5.00 × 0.46 cm I.D.); anion-exchange eluent, 1.8 m*M* sodium octanesulfonate. Remaining conditions were as in Fig. 8. Anions: 1 = fluoride (5.5 ppb); 2 = formate (3.0 ppb); 3 = acetate (25.0 ppb); 4 = propionate (20.0 ppb); 5 = chloride (14 ppb); 6 = nitrate (1 ppb); 7 = sulfate (5 ppb).

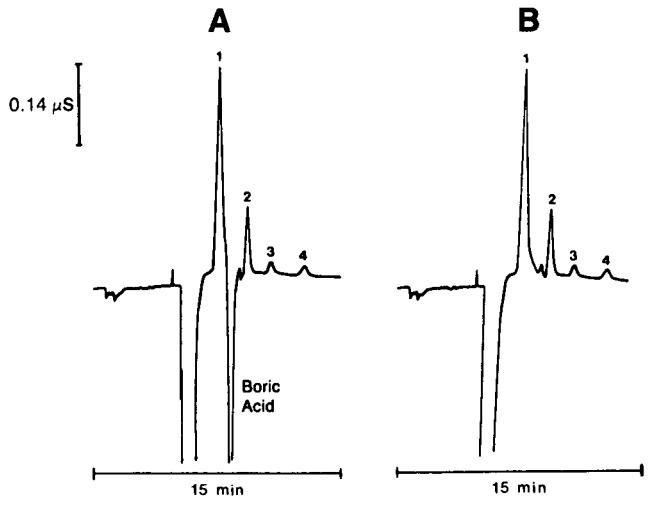
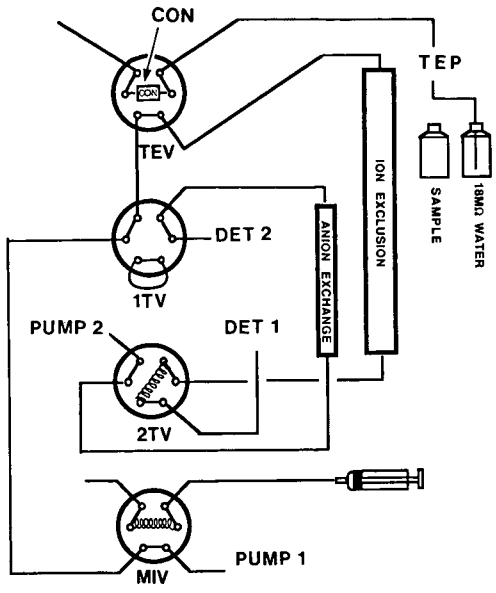


Fig. 11. Synthetic sample from a primary side of a pressurized water reactor prepared by an addition of 2000 ppm boron (as boric acid) to an aqueous solution containing 1 ppb fluoride (1), 2 ppb formate (2), 5 ppb acetate (3) and 10 ppb propionate (4). (A) Ion-exclusion separation using conditions from Fig. 4. A 33-ml volume of the sample was preconcentrated using the procedure outlined in Table II and in Fig. 2. (B) Ion-exclusion separation using water rinsing step for the precolumn. Further details are given in Fig. 12 and in the text.

frequently exceeds the concentrations of other inorganic and organic species in water by a factor of 10^5 and more. Without any additional precaution a small segment of a highly concentrated boric acid solution remains inside the concentrator even after the flow of the sample had been stopped. Under normal conditions (for example in Table II) the preconcentration is immediately followed by an elution step during which the retained anions are transferred to the chromatographic column. In the case of excessive boric acid concentrations the resulting ion-exclusion chromatograms then have the appearance of the one shown in Fig. 11A. Boric acid contained in the interstitial volume of the concentrator resin transfers to the ion-exclusion column along with the anionic species, and since it is also retained, causes a prominent negative conductivity peak disturbing the quantitation of fluoride and formate. Fig. 11B shows an improved separation achieved by a modification of the trace enrichment and fraction transfer procedure. During an additional step inserted between steps 2 and 3 in Fig. 3, the concentrator column is rinsed with approximately 3.5 ml of deionized water. In this fashion the interstitially held boric acid is completely removed while the recoveries of anionic analytes during the subsequent steps remains identical with those in Table III. Instrumental configuration during such rinsing is given in Fig. 12.

CONCLUSIONS

Using the methods described in this report it is possible to overcome the limitations of anion-exchange separations. With the help of eqn. 1 the total number of "resolvable peaks" on the anion-exchange column is calculated as $n_r = 3$ in an obvious agreement with the experiment represented by the chromatogram in Fig. 4. Estimating



STEP 3

Fig. 12. Coupled system configuration during the rinsing step. Removal of excess boric acid from the concentrator column. After completion of step 2 in Table II the automatic program is interrupted. The trace enrichment pump is stopped and the sample line is immersed in a precleaned container filled with ultra high purity water. TEP is turned on again and *ca.* 6 ml of clean water containing no more than low-ppt concentrations of anions of interest are pumped through the concentrator (CON). After the successful removal of boric acid from the concentrator column the automated program continues with step 3.

the available range of retention volumes on the ion-exclusion system as $V_R/V_0 = 14/4$ and the separation efficiency $N = 5000$ as well as taking into account eqn. 2[5]

$$n_r = 0.18(n_{c1} + n_{c2}) \quad (2)$$

where $n_{c1} = 16$ represents the peak capacity of the anion-exchange separation and $n_{c2} = 23$ the peak capacity of the ion-exclusion separation. The total number of resolvable peaks is calculated as $n_r = 7$. According to the definition of n_r and in agreement with the experimental data, the coupled ion-exclusion and anion-exchange system is shown to provide sufficient resolving power to be able to separate many of the known mixtures of early eluting anions and carboxylic acids. The simultaneous analysis of weak and strong anions using the described instrumental procedure allows a fast determination of weakly and strongly dissociated anions in a large variety of water samples. The analysis is largely independent of the typical sample matrices encountered in the power generating and semiconductor industries.

REFERENCES

1 *Guidelines for Pure Water*, Technical Document I-85, Semiconductor Equipment and Materials Institute, Mt. View, CA, 1985.
2 J. P. Denoncourt and Y. Egozy, *Ultrapure Water*, 3 (1986) 40.

3 M. M. Plechaty, *LC · GC, Mag. Liq. Gas Chromatogr.*, 2 (1984) 684.
4 G. D. Burns, R. C. Nolan, J. E. Crutchfield and T. O. Passel, *Proceedings 47th International Water Conference, Pittsburgh, October 27–29, 1986*, Engineer's Society of Western Pennsylvania, Pittsburgh, PA, Paper No. 12, pp. 105–119.
5 J. C. Giddings, *Anal. Chem.*, 56 (1984) 1259 A.
6 J. C. Giddings, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 319.
7 J. C. Giddings, in H. J. Cortes (Editor), *Multidimensional Chromatography: Techniques and Applications*, Marcel Dekker, New York, in press.
8 W. R. Jones, P. Jandik and A. L. Heckenberg, *Anal. Chem.*, 60 (1988) 1977.
9 R. D. Rocklin, C. A. Pohl and J. A. Schibler, *J. Chromatogr.*, 411 (1987) 107.
10 W. R. Jones, A. L. Heckenberg and P. Jandik, *J. Chromatogr.*, 366 (1986) 225.
11 P. R. Haddad and P. Jandik, in J. Tarter (Editor), *Ion Chromatography*, Marcel Dekker, New York, 1986, p. 128.
12 P. Jandik, D. Cox and D. Wong, *Am. Lab. (Fairfield, Conn.)*, 18 (1986) 114.
13 W. R. Jones, W. J. Wildman, A. L. Jagoe, A. L. Heckenberg and P. Jandik, presented at the *Pittsburgh Conference and Exposition, Atlantic City, NJ, March 10–14, 1986*, Abstract No. 074.

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APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE SEPARATION OF PEPTIDES FROM PHOSPHORYLATED AND DEPHOSPHORYLATED CASEIN HYDROLYSATES

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SUMMARY

Peptides from phosphorylated and dephosphorylated casein hydrolysates were fractionated on a TSK G2000SW size-exclusion column. The fractionated peptides were separated by reversed-phase high-performance liquid chromatography on a C_{18} column using aqueous trifluoroacetic acid as the mobile phase and acetonitrile as the mobile phase modifier in the linear gradient elution system. The separation of the dephosphorylated and phosphorylated hydrolysates gave 213 and 187 peptides, respectively, of which 116 and 99, respectively, were reported. A study of their composition and retention times verified that the peptide separation mechanism includes ionic interactions, hydrogen bonding and peptide characteristics, in addition to overall peptide hydrophobicity.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a method for resolving complex peptide mixtures from protein cleavage reaction, especially when only small amounts are available for amino acid sequencing studies[1]. Unlike size-exclusion and ion-exchange chromatographies, with RP-HPLC there is no obvious correlation between retention and molecular size and no apparent relationship between retention and acidity[2]. Retention of peptides on a reversed-phase column has been shown to be largely dependent on their amino acid composition but not on their sequence, and to be primarily determined by the ionization state, the location of charges and the hydrophobicity[3]. Many authors have predicted the retention times of different peptides with fewer than 29 amino acid residues, under particular sets of chromatographic conditions[4–6].

The work reported here aimed to separate and identify peptides from casein hydrolysates previously fractionated on a TSK G2000SW size-exclusion column. Separation was achieved by RP-HPLC and identification by Pico-Tag (Waters) amino acid analysis. N- and C-terminal amino acid analyses were also performed on a C_{18} Pico-Tag column. The peptide separation is discussed on the basis of their hydro-

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phobicity, conformation, amino acid composition, hydrogen bonding and ionic interactions with the stationary phase.

EXPERIMENTAL

Materials

Phosphorylated and dephosphorylated casein hydrolysates (CHPS and CHDS) were kindly provided by Laboratoire Sopharga (France). A controlled hydrolysis of whole casein by physiological enzymes (chymotrypsin and trypsin) was carried out in a continuous-flow membrane enzymic reactor, according to the procedure developed by Maubois and Brulé[7], and led to the following molecular weight distribution of peptides: mol.wt. > 5000 daltons, 3%; 1000 < mol.wt. < 5000 daltons, 28%; mol.wt. < 1000 daltons, 69% including 8% of free amino acids[8]. The resultant hydrolysate was ultrafiltered with a membrane, to retain the enzyme and to obtain a permeate that contained phosphorylated and dephosphorylated peptides. The permeate was acidified, and a divalent cation salt was added to aggregate the phosphopeptides. Thereafter, fractionation of the two groups of peptides was performed by ultrafiltration and diafiltration with water so as to remove the whole dephosphorylated peptide fraction (CHDS). The diafiltrated concentrate obtained corresponded to the phosphopeptidic fraction (CHPS). HPLC-grade water and methanol, and monobasic and dibasic sodium phosphate, were obtained from Fisher Scientific (Quebec, Canada). Trifluoroacetic acid (TFA) was supplied by Pierce (U.S.A.).

Triethylamine (TEA), sequanal grade, TFA, phenyl isothiocyanate (PITC) and amino acid standard mixture H were obtained from Pierce. Absolute ethanol, sodium acetate trihydrate and hydrochloric acid (R. P. Normapur) were supplied by Prolabo (Paris, France). Acetonitrile "Baker Analyzed" reagent for chromatography was purchased from J. T. Baker (Deventer, The Netherlands). HPLC-grade acetic acid, ammonia (Suprapur) and 2-propanol (LiChrosolv) were obtained from Merck (Socolab, France). Double-distilled water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Methods

Size-exclusion chromatography. High-performance size-exclusion chromatography (HPSEC) was carried out on a TSK G2000SW column (600 × 7.5 mm I.D.) with a guard column (60 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) using an LKB HPLC system equipped with a Model 2150 pump, a Model 2152 controller, a Model 2151 variable-wavelength monitor, a Rheodyne M7010 sample injection valve with a 20-μl loop, and a Hewlett-Packard 3390 A integrator. The mobile phase was 0.1% TFA, 0.05 *M* phosphate buffer (pH 5.0) and 35% methanol. The system was run isocratically at a flow-rate of 0.75 ml/min under constant temperature. Polypeptides were monitored at 214 nm with an absorbance scale of 0.05[9]. The mobile phase was filtered through a 0.45-μm filter (Millipore) and sonicated before use. Hydrolysate fractions were collected manually and pooled.

Reversed-phase chromatography. Peptides from each fraction obtained by HPSEC were separated on a Waters μBondapak C_{18} reversed-phase column (10 μm, 300 × 3.9 mm I.D.) with a Waters HPLC system equipped with two F-6000A pumps, an M720 solvent programmer, a WISP automated sample injector, an M441

fixed-wavelength detector (214 nm) and an M730 two-channel chart recorder. A water-bath was used to keep the column at 40°C. After equilibration of the column with 0.115% TFA (solvent A) at a flow-rate of 2 ml/min, peptides were eluted by linearly increasing the concentration of solvent B [60% (v/v) acetonitrile in 0.1% TFA] as follows: 0–24 min, 0–48% B; 24–25 min, 48–100% B; 25–25.5 min, 100–0% B. The time necessary from one run to the other, including the equilibration time, was 33 min. Prior to use, the mobile phases were degassed with helium. Peptides were monitored at 214 nm with an absorbance scale of 0.1. Portions of the RP-HPLC column fractions were evaporated in a Speed-Vac Concentrator (Savant, Hicksville, NY, U.S.A.).

Peptides that coeluted were isolated by utilization of a second solvent system: (A) 25 m*M* ammonium acetate (pH 6.0) and (B) 60% (v/v) acetonitrile in 50 m*M* ammonium acetate (pH 6.0); all other conditions were the same. The absorbance scale was increased to 0.2 and the flow-rate was decreased to 1 ml/min.

Peptide identification. Peptides were hydrolysed with 5.7 *M* triple-distilled hydrochloric acid in evacuated sealed tubes for 24 h at 110°C. The amino acid analyses were then performed on a Waters Pico-Tag amino acid analysis system according to the manufacturer's instructions. Prior to hydrolysis, Pyrex tubes were heated at 500°C for 16 h to eliminate any contamination.

The identity of each peptide was established by comparison of its amino acid composition with that of α_{s1}-, α_{s2}-, β- and κ-caseins according to the Petrilli's program[10], using an Apple IIE computer, and confirmed by N-terminal and C-terminal analysis according to the methods of Tarr[11] and Ribadeau-Dumas[12], respectively. The chromatography of phenylthiohydantoin (PTH) and phenylthiocarbamyl (PTC) derivatives was then carried out on a Pico-Tag column (Waters) using the LKB system. For PTC derivatives, 1 m*M* EDTA (Fisher Scientific) was added to solvent A, and the gradient was modified by increasing the solvent B concentration as follows: 0–4 min, 0–30% B; 4–12 min, 30–45% B; 12–12.5 min, 45–100% B; 14–15 min, 100–0% B. Injections could be performed every 21 min. The gradient for the identification of PTH derivatives was modified; solvents A and B were the same, and concentration of eluent B increased in the following manner: 0–3.5 min, 0–2% B; 3.5–6 min, 2–36% B; 6.0–7.0 min, 36–40% B; 10.5–10.7 min, 40–60% B; 11.0–11.5 min, 60–0% B. Between two injections, the time required for the analysis and equilibration was 20 min. In each case the flow-rate was kept constant at 1.0 ml/min, and norleucine was used as internal standard.

The hydrophobicity of peptides (kcal/mol) was calculated according to the values given by Cheftel *et al.*[13]. The total number of hydrophobic amino acid residues was determined by the presence of tryptophan, phenylalanine, leucine, isoleucine, tyrosine, valine, methionine and proline in the peptide.

RESULTS AND DISCUSSION

The elution profiles obtained for CHDS and CHPS on a TSK G2000SW HPSEC column are given in Fig. 1A and B. The retention times for each fraction collected were different for the two hydrolysates since the chromatograms showed few distinctive differences. Ten and eight fractions (as indicated by numbers) were obtained for CHDS and CHPS, respectively. Fractions 7 and 8 of CHDS were combined for further studies. HPSEC fractions 4 (550–840 daltons) and 7–8 (210–410 daltons), respectively,

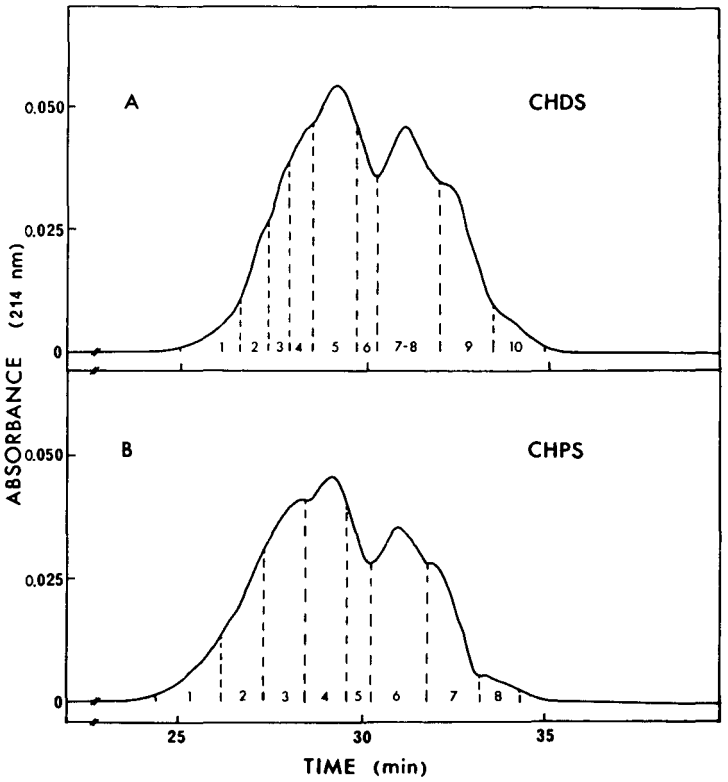

Fig. 1. Elution profiles of tryptic and chymotryptic digests of casein on a TSK-G2000 SW column: (A) CHDS, (B) CHPS. Fractions 1–10 were collected manually. Each hydrolysate (2 mg) was dissolved in 1 ml of mobile phase [0.1% TFA, 0.05 *M* phosphate buffer (pH 5.0) and 35% methanol]. The injection volume was 20 μl.

from CHPS and CHDS, contained more peptides that were identified in each hydrolysate. The total numbers of peptides that were isolated and identified in CHDS and CHPS were 213 and 187, respectively. This difference between the two hydrolysates could be explained by the irreversible binding of phosphopeptides at low concentration by the C_{18} column.

The chromatogram of fraction 5 of a CHPS hydrolysate obtained from HPSEC and injected on a μBondapak reversed-phase column is shown in Fig. 2 as an example. The straight line represents the gradient used. The first peak was eluted in the isocratic mode, as the gradient was effective at 2.5 min; it contained salts from the phosphate buffer used on the size-exclusion column. Free amino acids, including phenylalanine and tyrosine and some small peptides with polar amino acids, were present in the peak juxtaposed to the first peak.

The identified peptides, 116 for CHDS and 99 for CHPS, with their RP elution times, hydrophobicities (kcal/mol), percentages of eluent B in mobile phase for a specific elution time, numbers of hydrophobic residues[14] over total residues and sequences are given in Tables I and II. The elution times obtained with the first RP

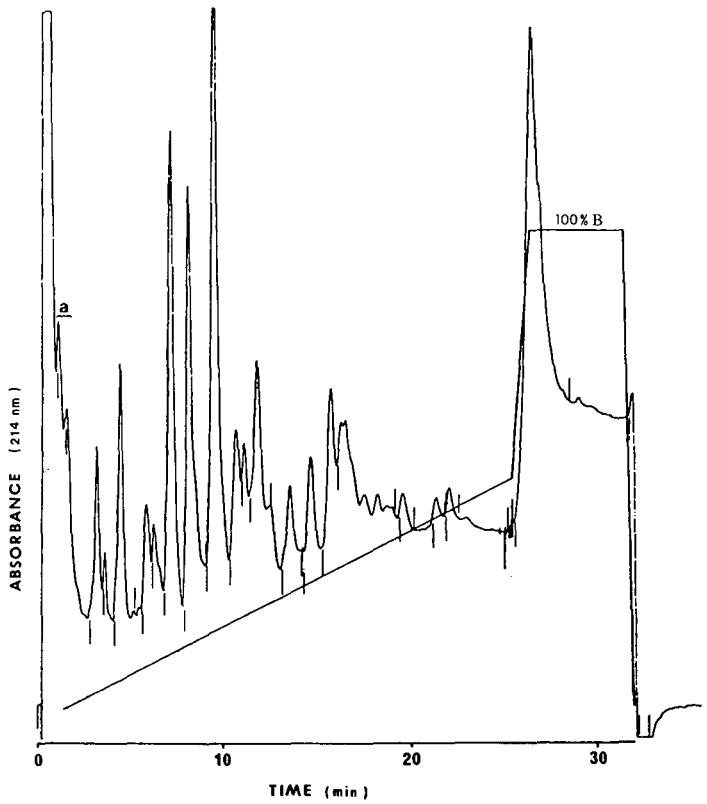

Fig. 2. Reversed-phase peptide mapping on a Waters μBondapak C_{18} column of fraction CHPS-5. Each fraction from HPSEC was diluted with 500 μl of solvent A, filtered through a VH 0.45 μm (Millipore). The injection volume was 100 μl. Chromatography was performed as described under Experimental.

system were used for the peptides that were rechromatographed in a second system for further purification. An example of such a chromatogram is shown on Fig. 3.

Comparison of the results from Tables I and II indicates that many peptides are common to both digests and that they elute at the same time, even though one protein hydrolysate is phosphorylated and the other one is not.

Two or more adjacent fractions obtained by HPSEC had the same peptide but in different concentrations. This may be due to the low resolution of the HPSEC column. The molar composition of the peptide could be used to predict in which HPSEC fraction the purified peptide should elute[15].

Generally peptides in the same HPSEC fraction appeared to separate by RP chromatography according to their hydrophobicity. However, peptides eluted during the first part of the gradient were more polar and less strongly adsorbed. Janssen *et al.*[16] reported that short hydrophilic peptides with basic character are poorly adsorbed owing to minor interaction with the support, and thus cannot be determined under the chromatographic conditions used for longer and more hydrophobic peptides. Addition of an anionic reagent such as TFA to the mobile phase was helpful to increase

TABLE I

IDENTIFICATION OF PEPTIDES FROM A CASEIN HYDROLYSATE (CHPS)

The single-letter code for amino acids is used.

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
CHPS-2	κ : 41–42	2.71	2.86	0.42	1/2	NY
	κ : 146–156	<21.64	16.51	<38.28	7/11	QPHQPLPPTVM
	κ : 144–163	21.64–22.18	30.20	38.28–39.36	13/20	MHQPHQPLPPTVMFPPQSVL
CHPS-3	α_{s1}: 125–132	4.34	6.06	3.68	1/8	EGIHAQQK
	α_{s2}: 126–137[d]	5.95	7.50	6.90	1/12	EQLSTSEENSKK
	β : 89–97	5.95	9.25	6.90	4/9	QPEVMGVSK
	κ : 61–68	7.10	11.59	9.20	3/8	YAKPAAVR
	α_{s1}: 80–90	7.60	11.55	10.20	3/11	HIQKEDVPSER
	α_{s2}: 200–205	9.76	12.53	14.52	5/6	VIPYVR
	α_{s2}: 115–125	12.82	14.59	20.64	5/11	NAVPITPTLNR
	α_{s1}: 106–119[d]	14.42	17.00	23.84	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.52	21.34	28.04	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.20	16.05	31.40	6/15	NQGLPQEVLNENLLR
	β : 49–68	19.88	32.66	34.76	12/20	IHPFAQTQSLVYPFPGPIPN
	β : 144–163	21.42–22.08	30.20	37.84–39.16	13/20	MHQPHQPLPPTVMFPPQSVL
CHPS-4	α_{s1}: 1–3	1.70–2.76	4.82	0–0.52	1/3	RPK
	α_{s1}: 55–58		5.54		1/4	EDIK
	β : 29–32		6.50		1/4	KIEK
	κ : 62–65		5.56		1/4	AKPA
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 77–80		4.76		1/4	HYQK
	α_{s1}: 80–83		4.86		1/4	HIQK
	β : 100–105	4.20	7.42	3.40	2/6	EAMAPK
	κ : 80–86		7.52		2/7	SNTVPAK
	α_{s1}: 125–132		6.06		1/8	EGIHAQQK
	α_{s1}: 120–124		5.75		2/5	LHSMK
	α_{s1}: 37–42	4.60	6.17	4.20	2/6	VNELSK
	β : 89–97	5.47	9.25	5.94	4/9	QPEVMGVSK
	β : 177–183	8.14	11.12	11.28	4/7	AVPYPQR
	β : 170–176	9.51	13.01	14.02	5/7	VLPVPQK

	β : 33–48[d]	10.50	9.84	16.00	2/16	FQSEEQQQTEDELQDK
	α_{s2}: 138–150[d]		13.31		4/13	TVDMESTEVFTKK
	β : 194–202	11.02	11.51	17.04	5/9	QEPVLGPVR
	α_{s2}: 81–89		12.71		4/9	ALNEINQFY
	α_{s2}: 115–125	12.79	14.59	20.58	5/11	NAVPITPTLNR
	α_{s1}: 106–119[d]	14.44	17.00	23.88	6/14	VPQLEIVPNSAEER
	α_{s1}: 105–119[d]		18.48		6/15	KVPQLEIVPNSAEER
	α_{s1}: 104–119[d]		21.34		7/16	YKVPQLEIVPNSAEER
	β : 192–202		16.79		7/11	LYQEPVLGPVR
	α_{s1}: 174–193	16.50	21.34	28.00	6/20	TDAPSFSDIPNPIGSENSEK
	β : 33–52[d]	17.16–17.51	18.54	29.32–30.02	5/20	FQSEEQQQTEDELQDKIHPF
	α_{s1}: 170–193		24.55		7/24	GIQYTDAPSFSDIPNPIGSENSEK
	α_{s1}: 25–34		15.99		6/10	VAPFPQVFGK
CHPS-5	β : 98–99	1.55	3.18	0	1/2	VK
	α_{s2}: 204–205		2.42		1/2	VR
	α_{s2}: 171–173		4.26		1/3	YQK
	β : 94–97	2.27–2.72	3.22	0–0.44	1/4	GVSK
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 194–197		6.95		2/4	IQPK
	β : 100–105	4.32	7.42	3.64	2/6	EAMAPK
	α_{s1}: 37–42	4.75	6.17	4.50	2/6	VNELSK
	β : 89–97	5.56	9.25	6.12	4/9	QPEVMGVSK
	β : 177–183	8.26	11.12	11.52	4/7	AVPYPQR
	β : 49–52	9.24	8.70	13.48	3/4	IHPF
	β : 108–113	10.62	11.18	16.24	4/6	EMPFPK
	α_{s1}: 146–154	12.90	17.23	20.80	6/9	YPELFRQFY
	α_{s1}: 106–119[d]	14.56	17.00	24.32	6/14	VPELEIVPNSAEER
	α_{s2}: 100–109	15.64	18.00	26.28	7/10	YQGPIVLNPW
	β : 134–139	16.75	12.92	28.50	5/6	HLPLPL
	α_{s1}: 25–34	17.54	15.99	30.08	6/10	VAPFPEVFGK
CHPS-6	α_{s2}: 171–173	1.86	4.26	0	1/3	YQK
	β : 126–127	4.04	2.85	3.08	1/2	TL
	α_{s2}: 183–184		4.55		2/2	VY
	α_{s1}: 91–92	5.00	5.28	5.00	2/2	YL
	κ : 17–21	6.95	7.37	8.90	2/5	FFSDK
	β : 193–198		9.36		4/6	YQEPVL
	β : 177–183	7.47	11.12	9.94	2/7	AVPYPQR

(Continued on p. 196)

TABLE I *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
	α_{s2}: 200–207	8.26–9.19	17.81	11.52–13.38	7/8	VIPYVRYL
	β : 191–193		7.69		3/3	LLY
	β : 49–52		8.70		3/4	IHPF
	κ : 44–50	9.98–10.58	8.73	14.96–16.16	3/7	QQKPVAL
	α_{s1}: 146–150		11.07		3/5	YPELF
	α_{s2}: 142–147		5.92		2/6	ESTEVF
	α_{s2}: 100–106		12.42		4/7	YQGPIVL
	β : 184–190	11.26–12.24	10.69	17.52–19.48	4/7	DMPIQAF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	β : 114–119	13.15	12.29	21.30	5/6	YPVEPF
	β : 184–190	14.44	10.69	23.88	4/7	DMPIQAF
	α_{s1}: 133–144	15.56	15.55	26.12	6/12	EPMIGVNQELAY
	α_{s1}: 152–164	16.75	18.25	28.50	6/13	QFYQLDAYPSGAW
	α_{s2}: 97–109	17.72	23.18	30.44	9/13	QYLYQGPIVLNPW
	α_{s1}: 25–32	18.51	14.50	32.02	6/8	VAPFPQVF
	β : 203–209	19.16	15.44	33.32	6/7	GPFPIIV
CHPS-7	α_{s1}: 23	1.83	2.65	0	1/1	F
	α_{s1}: 104		2.87		1/1	Y
	α_{s2}: 206–207	4.94	5.28	4.88	2/2	YL
	α_{s2}: 99–100	9.00	5.28	13.00	2/2	LY
	α_{s1}: 152–154		6.07		2/3	QFY
	β : 184–190	12.06–13.27	10.69	19.12–21.54	4/7	DMPIQAF
	β : 114–119		12.29		5/6	YPVEPF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	κ : 26–30		11.28		4/5	IPIQY
	β : 184–190	14.59	10.69	24.18	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	α_{s1}: 145–150	17.86	13.72	30.72	5/6	FYPELF
CHPS-8		3.02		1.04	0/1	N or D
	α_{s1}: 23–24	8.55	5.30	12.10	2/2	FF
	α_{s1}: 152–154	8.96	6.07	12.92	2/3	EFY

[a] From ref. 13.
[b] Eluent B: 60% (v/v) acetonitrile in 0.1% TFA.
[c] From ref. 14.
[d] Ser (P) peptides.

TABLE II

IDENTIFICATION OF PEPTIDES FROM A DEPHOSPHORYLATED CASEIN HYDROLYSATE (CHDS)

The single-letter code for amino acids is used.

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
CHDS-1		1.75		0	0/1	N or D
CHDS-2		1.66		0	0/1	N or D
	β : 53–68	21.08	23.97	37.16	9/16	AQTQSLVYPFPGPIPN
CHDS-3		1.63		0	0/1	N or D
	κ : 98–102	2.78	6.69	0.56	2/5	HPHPH
	α_{s1}: 125–132	4.11	6.06	3.22	1/8	EGIHAQQK
	α_{s1}: 106–119	14.46	17.00	23.92	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.62	21.34	28.24	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.18	15.51	31.36	6/15	HQGLPQEVLNENLLR
	β : 144–163	21.84	30.20	38.68	13/20	MHQPHQPLPPTVMFPPQSVL
CHDS-4		1.66	2.87	0	1/1	Y
			1.49		0/1	K
	α_{s1}: 35–36		2.04		0/2	EK
	α_{s1} 4–7	2.30	7.55	0	2/4	HPIK
	κ : 98–102	2.79	6.69	0.58	2/5	HPHPH
	α_{s1}: 125–132	4.12	6.06	3.24	1/8	EGIHAQQK
	β : 89–97	5.64–5.87	9.25	6.28–6.74	4/9	QPEVMGVSK
	α_{s1}: 106–119	13.68–14.51	17.00	22.36–24.02	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.63	21.34	28.26	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.22	15.51	31.44	6/15	HQGLPQEVLNENLLR
	β : 49–68	19.50–21.03	32.66	34.00–37.06	12/20	IHPFAQTQSLVYPFPGPIPN
CHDS-5	α_{s1}: 35–3€	<2.59	2.04	<0.18	0/2	EK
	β : 30–32		4.36		1/3	IQK
	α_{s1}:100–102		4.65		1/3	RLK
	β : 94–97		3.22		1/4	GVSK
	α_{s1}: 55–58		5.54		1/4	EDIK
	κ : 62–63		2.23		0/2	AK
	κ : 64–65		3.33		1/2	PA
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	β : 100–105	3.80	7.42	2.60	2/6	EAMAPK

(Continued on p. 198)

TABLE II *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
	α_{s1}: 37–42	4.16	6.17	3.32	2/6	VNELSK
	α_{s1}: 125–132		6.06		1/8	EGIHAQQK
	α_{s1}: 84–90	5.12	6.69	5.24	2/7	EDVPSER
	β : 108–113	7.75	11.18	10.50	4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 170–176	9.07	13.01	13.14	5/7	VLPVPQK
	β : 33–48	10.10	9.84	15.20	2/16	FQSEEQQQTEDELQDK
	α_{s2}: 138–150	10.55	13.31	16.10	4/13	TVLMESTEVFTKK
	α_{s2}: 81–89	11.23	12.71	17.46	4/9	ALNEINQFY
	β : 194–202	12.35	11.51	19.70	5/9	QEPVLGPVR
	β : 193–202		14.38		6/10	YQEPVLGPVR
	α_{s1}: 106–119	14.07	17.00	23.14	6/14	VPQLEIVPNSAEER
	β : 134–139	16.12	12.92	27.24	5/6	HLPLPL
CHDS-6	α_{s2}: 110–113	<2.56	3.62	<0.12	1/4	DQVK
	α_{s2}: 180–181		3.91		1/2	LK
	β : 30–32		4.36		1/3	IEK
	α_{s2}: 171–173		4.26		1/3	YQK
	κ : 62–63		2.23		0/2	AK
	κ : 64–65		3.33		1/2	PA
	α_{s2}: 42–45		4.66		2/4	EVVR
	α_{s2}: 167–170		3.65		1/4	ISQR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 194–197		6.95		2/4	IQPK
	β : 100–105	3.79	7.42	2.58	2/6	EAMAPK
	α_{s1}: 37–42	4.14	6.17	3.28	2/6	VNELSK
	α_{s1}: 84–90	5.08	6.69	5.16	2/7	EDVPSER
	κ : 1–10	6.26–6.86	7.65	7.52–8.72	2/10	EEQNQEQPIR
	κ : 17–21		7.37		2/5	FFSDK
	α_{s2}: 162–165		6.55		2/4	NFLK
	β : 108–113	6.86–7.70	11.18	8.72–10.40	4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 49–52	8.47	8.70	11.94	3/4	IHPF
	β : 170–176	9.38	13.01	13.76	5/7	VLPVPQK
	β : 33–48	10.04	9.84	15.08	2/16	FQSEEQQQTEDELQDK
	β : 114–123	12.28	13.22	19.56	5/10	YPVEPFTESQ
	β : 194–202		11.51		5/9	QEPVLGPVR

	β : 193–202		14.38		6/10	YQEPVLGPVR
	α_{s1}: 106–119	14.04	17.00	23.08	6/14	VPQLEIVPNSAEER
	β : 134–139	16.03	12.92	27.06	5/6	HLPLPL
CHDS-7-8	α_{s2}: 171–173	<3.79	4.26	<2.58	1/3	YQK
	α_{s1}: 170–173		3.21		1/4	GTQY
	α_{s2}: 182–188	3.79	6.80	2.58	2/7	TVYQHQK
	α_{s1}: 157–159	4.56–4.82	4.14	4.12–4.64	1/3	DAY
	β : 120–125		3.39		1/6	TESQSL
	β : 53–58		3.44		1/6	AQTQSL
	α_{s1}: 91–92		5.28		2/2	YL
	κ : 1–10	5.48–7.20	7.65	5.96–9.40	2/10	EEQNQEQPIR
	κ : 39–42		5.27		2/4	GLNY
	β : 193–198		9.36		4/6	YQEPVL
	α_{s2}: 174–181		15.05		5/8	FALPQYLK
	κ : 17–21		7.37		2/5	FFSDK
	κ : 35–38	7.20–8.42	8.36	9.40–11.84	3/4	YPSY
	β : 108–113		11.18		4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 191–193		7.69		3/3	LLY
	α_{s2}: 200–207	8.91	17.81	12.82	7/8	VIPYVRYL
	α_{s2}: 138–149	9.79	11.82	14.58	4/12	TVDMESTEVFTK
	α_{s2}: 101–109	10.42	15.13	15.84	6/9	QGPIVLNPW
	β : 184–190	11.44–11.74	10.69	17.88–18.48	4/7	DMPIQAF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	α_{s1}: 133–142	12.46–12.98	11.94	19.92–20.96	5/10	EPMIGVNQEL
	α_{s1}: 133–144		15.55		6/12	EPMIGVNQELAY
	β : 194–202		11.51		5/9	QEPVLGPVR
	β : 114–119		12.29		5/6	YPVEPF
	α_{s1}: 92–98		10.56		4/7	LGYLEQL
	α_{s1}: 154–159		9.32		3/6	YQLDAY
	κ : 25–30		14.15		5/6	YIPIQY
	β : 184–190	14.44	10.69	23.88	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	β : 59–68	17.58	20.52	30.16	8/10	VYPFPGPIPN
	α_{s1}: 25–32	18.40	14.50	31.80	6/8	VAPFPQVF
	β : 203–209	19.04	15.44	33.08	6/7	GPFPIIV

(Continued on p. 200)

TABLE II *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
CHDS-9		<3.82	2.87	<2.64	1/1	Y
			2.65		1/1	F
	α_{s1}: 93–94		2.87		1/2	GY
	α_{s2}: 206–207	4.63	5.28	4.26	2/2	YL
	α_{s1}: 92–94	5.31	5.28	5.62	2/3	LGY
	κ : 103–105	5.91	5.10	6.82	2/3	LSF
	β : 120–127	7.08	6.24	9.16	2/8	TESQSLTL
	α_{s1}: 146–149	7.82–8.78	8.42	10.64–12.56	3/4	YPEL
	α_{s1}: 152–154		5.42		2/3	QFY
	β : 191–193		7.69		3/3	LLY
	α_{s1}: 23–24		5.30		2/2	FF
	β : 184–191	11.56	13.10	18.12	5/8	DMPIQAFL
	α_{s2}: 100–106	12.34	12.42	19.68	5/7	YQGPIVL
	β : 114–119	13.08	12.29	21.16	5/6	YPVEPF
	β : 184–190	14.59–15.11	10.69	24.18–25.22	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	α_{s2}: 7–20		12.67		4/14	VSSSEESIISQETY
	α_{s1}: 145–150	17.68	13.72	30.36	5/6	FYPELF
CHDS-10		<2.86	2.87	<0.72	1/1	Y
			2.65		1/1	F
	α_{s1}: 152–154	7.88–8.75	5.42	10.76–12.50	2/3	QFY
	κ : 17–18		5.30		2/2	FF

[a] From ref. 13.
[b] Eluent B: 60% (v/v) acetonitrile in 0.1% TFA.
[c] From ref. 14.

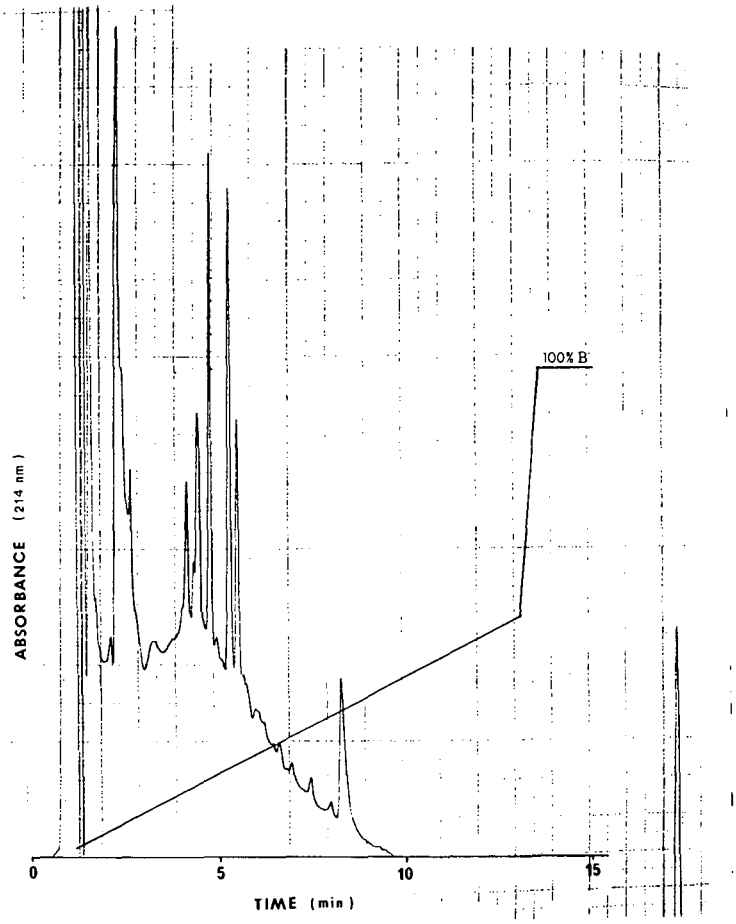

Fig. 3. Elution profile of peak a from Fig. 2 (CHPS-5), obtained as described under Experimental on a Waters μBondapak C_{18} reversed-phase column.

the elution time of small peptides such as α_{s2}: 204–205 (Val-Arg), which could be well separated and identified. The addition of perfluorinated organic acid, TFA, to the mobile phases has been justified due to its characteristics[17,18]. Elution conditions using TFA in the RP system had been previously studied with casein digests[19,20] and found to be suitable for peptide separation.

Changes in selectivity from the first mobile phase to the second could explain the variations in hydrophobicity between peptides eluted from the second RP chromatography. Solute–solute interactions in the mobile phase and at the surface of the stationary phase could be responsible for the elution of peptides with different hydrophobicities[21].

The elution times of peptides (α_{s1}: 106–119, α_{s2}: 126–137, α_{s2}: 138–150, and β: 33–48) with one or two phosphorylated or dephosphorylated serines are identical in both casein digests. This finding does not agree with previous results obtained by

Grego *et al.*[22], who observed higher retention times for corresponding dephosphorylated peptides. This result could be explained by the possible contamination of CHPS by dephosphorylated peptides[23]. The elution times of peptides with one or two dephosphorylated serines were shorter than that of the peptide with four dephosphorylated serines (α_{s2}: 7–20) and with lower hydrophobicity. Carles[24] has also reported that the retention times of peptides β: 1–25 and β: 2–25, which have four phosphorylated serines, increased with dephosphorylation.

The fraction CHPS-4 contained three peptides, α_{s1}: 106–119, α_{s1}: 105–119 and α_{s1}: 104–119, in the proportion 1.0, 0.25 and 0.10, respectively. The low molar concentration of these peptides could be explained by the low probability that the enzyme will hydrolyse the peptide bond of the first amino acid in sequences such as K-V-P-Q-L and Y-K-V-P-Q-L. Trypsin cleaves mainly peptide bonds on the carboxyl side of lysine and arginine, whereas chymotrypsin cleaves on the carboxyl side of aromatic amino acids, such as tyrosine, tryptophan and phenylalanine, and other peptide bonds more slowly[25]. Although the hydrophobicity of these three peptides is different, they eluted together in the first RP-HPLC solvent system and had to be chromatographed in a second solvent system to be separated for identification.

The peptides (α_{s1}: 25–32; α_{s1}: 166–173; α_{s2}: 92–96, α_{s2}: 142–147 and β: 203–209) with aromatic amino acids, such as tyrosine and phenylalanine, eluted later than expected. This observation indicates that as well as hydrophobic interactions, which make the dominant contribution to peptide chromatography, multiple retention processes could be involved in the binding of peptides to the stationary phase. Wehr and Correia[26] reported that endcapping the free silanols with trichloromethylsilane did not significantly decrease retention times of peptides. Specific hydrogen-bonding interactions, due to the low pH of the mobile phase, with silanol groups may therefore be suspected[20]. The longer elution time may be explained by bond-breaking at higher concentrations in acetonitrile. Since it is a weak dipolar base, it is more difficult for acetonitrile to manipulate the hydrogen-bonding characteristics of the stationary phase than 2-propanol[22].

The percentage of eluent B in the gradient is given in column 5 of Tables I and II; it was found that the best resolution was obtained between 15% and 40%[27] of eluent B, corresponding to 7.5 and 22.5 min. The amount of acetonitrile [60% (v/v) acetonitrile in 0.1% TFA] in eluent B was found to be right for casein digests.

Ionic interactions between ionized silanols and cationic solutes (containing arginine, lysine and histidine residues) were suspected to occur with peptides β: 134–139 and β: 194–202. Hearn and Grego[28] reported that the interaction of peptides with alkylsilica involves both a hydrophobic and a silanophilic component in the retention mechanism. Peptides can interact with accessible silanol groups at the surface of the stationary phase if complete ionization of the surface silanols on the column is not prevented by TFA. Dipeptide (β: 126–127) Thr-Leu eluted at a higher retention time than expected from its hydrophobicity. The presence of TFA in this system should not be neglected; being an anionic counter-ion it might pair with a positively charged peptide to form a complex and thus increase the retention time. Since TFA has amphiphilic properties, it can also serve as a hydrophilic pairing agent with hydrophobic residues[29]. Some peptides, such as α_{s1}: 25–34, α_{s2}: 162–165, β: 33–52, β: 49–52, β: 144–163 and β: 192–202, with one or many basic residues and aromatic amino acids (tyrosine or phenylalanine), elute later than expected from their

hydrophobicity. Their binding to the stationary phase may be due to multiple retention processes, including ionic interactions and hydrogen bonding.

Peptides with a number of aspartic and glutamic acid residues, such as α_{s1}: 8–22, α_{s1}: 133–144 and β: 33–48, were retained longer in the column. Similarly, peptides such as α_{s1}: 92–98; α_{s1}: 154–159, α_{s2}: 81–89 and β: 33–52 were delayed much longer owing to the presence of aspartic, glutamic and aromatic amino acid residues in their sequences. Although aspartic and glutamic acids are very polar and have low hydrophobicity, the peptides with these amino acids at pH 7.4 had higher retention in the column[30]. Carles[24] observed a positive contribution of aspartic and glutamic acids to the peptide retention of a β-casein trypsin digest with a slightly acidic mobile phase (pH 6.5).

Peptide β: 184–190 was found at two different elution times in the first RP chromatography; this can be explained by the oxidation of the methionine residue to the more polar sulphoxide or sulphone[31]; the two forms, which represent different oxidation states of sulphur in methionine, were identified from the PTC-amino acid analysis chromatograms: methionine sulphoxide was eluted between arginine and threonine; methionine sulphone was eluted after proline. The presence of an aspartic acid residue could also contribute to the delay of the peptide.

Addition of one hydrophobic residue, leucine, to the latter peptide (β: 184–191) made it elute sooner than expected. Other peptides, β: 59–68, β: 100–105, α_{s2}: 200–207, and α_{s1}: 133–144, were also eluted earlier from the column. The latter had twelve amino acid residues and the highest hydrophobicity (15.55 kcal/mol); such peptides may have conformations in which hydrophobic residues are located inside and hydrophilic residues outside[3].

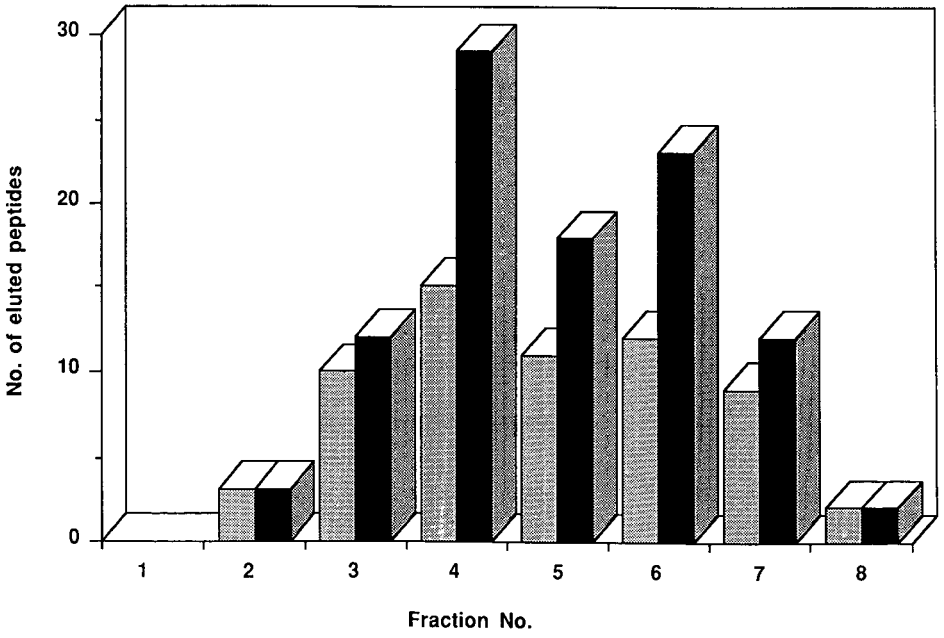

Fig. 4. Separation of CHPS peptides on a Waters μBondapak C_{18} reversed-phase column as described under Experimental. Peptides eluted according to their hydrophobicity (dotted areas) beside total number of peptides (black areas) eluted from each fraction obtained by HPSEC for CHPS.

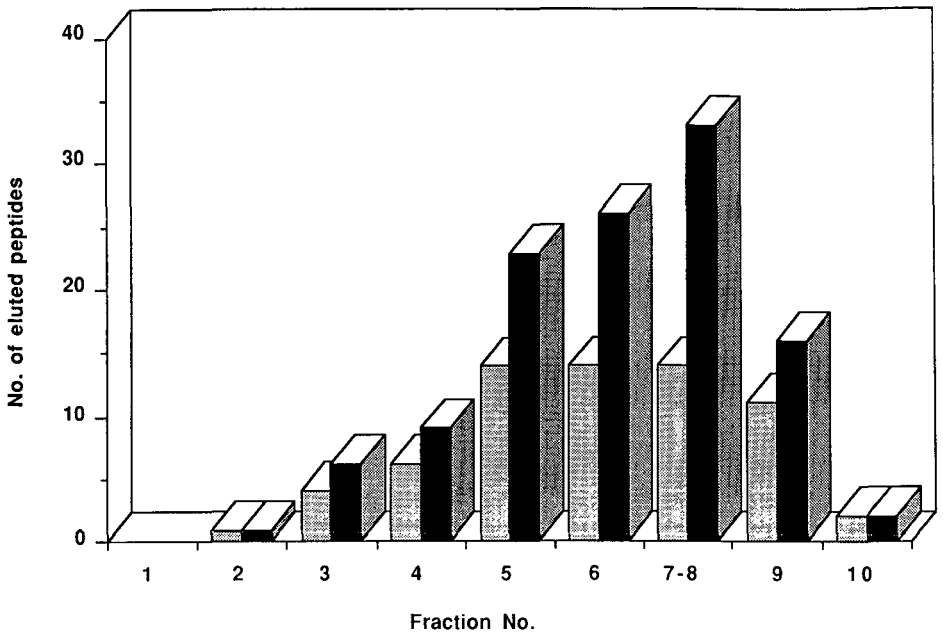

Fig. 5. Separation of CHDS peptides on a Waters μBondapak C_{18} reversed-phase column as described under Experimental. Peptides eluted according to their hydrophobicity (dotted areas) beside total number of peptides (black areas) eluted from each fraction obtained by HPSEC for CHDS.

Peptides obtained from CHDS (116) and CHPS (99) were eluted and identified for their amino acid sequences (Figs. 4 and 5). Out of these, the total numbers of peptides eluted according to their hydrophobicity were 65 and 62, respectively. This means that only 60% of the peptides were eluted from the RP-HPLC column according to their hydrophobicity. The separation of casein peptides was largely dependent on the overall peptide hydrophobicity, in addition to ionic interactions, hydrogen bonding and peptide characteristics.

The interactions occurring in RP-HPLC were taken into account in the methods recently developed for the prediction of retention data; however, some modifications to the predicted values might be needed[32,33], except when an internal peptide standard is chromatographed along with the peptides under investigation[34]. Taneja *et al.*[35] reported that since the peptides are retained much more tenaciously on a new column, the age of the column is important.

CONCLUSION

Being the most versatile and most widely used HPLC mode, RP chromatography has proved to be a reliable method with a high resolving power for the separation of peptides from casein hydrolysates. Peptides that elute together in the first mobile phase are easily separated by a second RP chromatography. RP-HPLC is considered to be a hydrophobic process in which the interaction between the peptide

and the stationary phase is regarded as being controlled by the net repulsions between the aqueous eluent and both the bonded phase and the non-polar part of the peptides. However, besides hydrophobicity, ionic interactions of the peptide side-chain with the free silanols of the stationary phase, hydrogen bonding with silica, and peptide characteristics such as the length of the peptide chain and the nature of the individual amino acid residues[36], might also contribute to peptide chromatography.

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REFERENCES

1 B. L. Jones and G. L. Lookhart, *Cereal Chem.*, 62 (1985) 89.
2 J. M. DiBussolo, in J. C. MacDonald (Consulting Editor), *HPLC: Instrumentation and Applications*, International Scientific Communications, Fairlfield, CT, 1986, p. 47.
3 T. Imoto and H. Yamada, *Molec. Cell. Biochem.*, 51 (1983) 111.
4 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
5 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
6 K. J. Wilson, A. Honegger, R. P. Stotzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31.
7 J. L. Maubois and G. Brulé, *Lait*, 62 (1982) 484.
8 Reabilan, Scientific Dossier, DS/FT, Sopharea Laboratories, Creuly, July, 1984.
9 M. A. Vijayalakshmi, L. Lemieux and J. Amiot, *J. Liq. Chromatogr.*, 9 (1986) 3559.
10 P. Petrilli, *Italian J. Biochem.*, 31 (1982) 391.
11 G. E. Tarr, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 223.
12 B. Ribadeau-Dumas, *Biochim. Biophys. Acta*, 168 (1968) 274.
13 J.-C. Cheftel, J. L. Cuq and D. Lorient, *Protéines Alimentaires Technique et Documentation*, Lavoisier, Paris, 1985.
14 J. M. Di Bussolo and J. R. Gant, *J. Chromatogr.*, 327 (1985) 67.
15 L. Lemieux and J. Amiot, *J. Chromatogr.*, submitted for publication.
16 P. S. L. Janssen, J. W. van Nispen, R. L. A. E. Hamelinck, P. A. T. A. Melgers and B. C. Goverde, *J. Chromatogr. Sci.*, 22 (1984) 234.
17 S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 129.
18 W. S. Hancock, *Chem. N.Z.*, 47 (1983) 145.
19 C. Carles and B. Ribadeau-Dumas, *J. Dairy Res.*, 53 (1986) 595.
20 M. Yvon, *Thèse de Docteur d'Université*, Université de Paris-VII, Paris, 1985.
21 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
22 B. Grego, G. S. Baldwin, J. A. Knessel, R. J. Simpson, F. J. Morgan and M. T. W. Hearn, *J. Chromatogr.*, 297 (1984) 21.
23 L. Roger, personal communication.
24 C. Carles, *Thèse de Docteur Ingénieur "Sciences Agronomiques"*, Institut National Agronomique de Paris-Grignon, Paris-Grignon, 1983.
25 Y. B. Keil, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 291.
26 C. T. Wehr and L. Correia, *Analytika (Johannesburg)*, Feb. (1982) 17.
27 M. Hermodson and W. C. Mahoney, *Methods Enzymol.*, 91 (1983) 352.
28 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 218 (1981) 497.
29 X. Geng and F. E. Regnier, *J. Chromatogr.*, 296 (1984) 15.
30 J. L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 1632.

31 P. M. Yuan, H. Pande, B. R. Clark and J. E. Shively, *Anal. Biochem.*, 120 (1982) 289.
32 C. A. Browne, H. P. J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
33 I. Molnar and M. Schoeneshoefer, in F. Lottspeich, A. Henschen, K.-P. Hupe (Editors), *High-Performance Liquid Chromatography in Protein and Peptide Chemistry; Proceedings International Symposium*, Walter de Gruyter, Berlin, New York, 1981, p. 97.
34 D. Guo, C. T. Mant, A. K. Taneja, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 499.
35 A. K. Taneja, S. Y. M. Lau and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 1.
36 D. Guo, C. T. Mant, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 519.

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC RETENTION BEHAVIOUR OF DIMETHYLPHENANTHRENE ISOMERS

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SUMMARY

The retention behaviour in reversed-phase liquid chromatography of all 25 dimethylphenanthrene (DMP) isomers on a polymeric C_{18} column was investigated and could be related to their molecular shape (length-to-breadth ratios and distorsion angles of the aromatic systems). These two descriptors of the aromatic molecular shape were generated by a molecular mechanics program and will be useful in the prediction of behaviour when reference standards are not available. An application of this concept to the identification of DMP isomers in a crude oil sample is presented.

INTRODUCTION

Complex mixtures of alkylated polycyclic aromatic hydrocarbons (PAHs) present in natural matrices contain numerous isomeric structures. Increasing attention has been paid to the separation and the quantification of individual alkylated PAHs both in organic geochemistry (alkylated PAH patterns are typical of the maturation of the sedimentary organic matter[1–3]) and in environmental chemistry (isomeric forms often have different carcinogenic or mutagenic activities).

One of the most commonly used techniques for the separation of PAHs is high-performance liquid chromatography (HPLC). Particularly reversed-phase liquid

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chromatography (RPLC) on chemically bonded C_{18} stationary phases has been successfully applied to the separation of methyl-substituted PAH isomers[4–9]. Recent studies have demonstrated enhanced selectivity of polymeric C_{18} phases compared with conventional and widely used monomeric C_{18} phases[7,8,10]. Moreover, Wise *et al.*[5] observed a relationship between the molecular shape of a PAH solute, defined as the length-to-breadth ratio (L/B), and its reversed-phase LC retention. Particularly when applied to three planar dimethylphenanthrene (DMP) isomers, the relationship correctly predicted their elution order on a monomeric and a polymeric C_{18} column[5].

In this study, the retention behaviour of all 25 DMPs in RPLC on a polymeric C_{18} phase was investigated. Special emphasis was placed on the effect or aromatic non-planarity described by the dihedral angle between the aromatic rings. A molecular mechanics method applied to each PAH molecule was used for the optimization of the geometry and the calculation of L/B values.

EXPERIMENTAL

Materials

The DMP and ethylphenanthrene (EtP) isomers listed in Table I were synthesized at the Institute of Petroleum and Organic Geochemistry, Jülich, F.R.G. The DMP and EtP isomers were prepared from dimethylstilbenes by photocyclization and dehydrogenation[3]. Some DMPs obtained as mixtures were separated by HPLC. Identification of DMP isomers was based on ^{1}H NMR data. The purity of the standards determined by capillary gas chromatography was generally greater than 97%.

The crude oil sample came from an Indonesian oil field. Sample preparation and PAH extraction have been described elsewhere[2].

Chromatography

Chromatographic separations were performed on columns (25 cm × 4.6 mm I.D.) containing 5-μm particle size Supelcosil LC-PAH polymeric C_{18} stationary phase (Supelco, Belfonte, PA, U.S.A.), equipped with guard columns (3 cm × 4.6 mm I.D.). Two LC systems were used. The first operated at room temperature and consisted of two LDC pumps (Constametric II and III), an LDC computerized solvent programmer (CCM), a Waters Assoc. injector (Wisp 710B) and an LDC Spectromonitor II UV-absorption detector. The second system operated at 35°C and was used only for the determination of the retention indices, and included a Spectra-Physics (Darmstadt, F.R.G.) pump, a Beckman (Berkeley, CA, U.S.A.) Model 210 injection valve, a Knauer (Berlin, F.R.G.) oven and a Knauer variable-wavelength detector. Acetonitrile–water (50:50, v/v) was used as the mobile phase at a flow-rate of 2 ml min^{-1}. The UV absorption was monitored at 254 nm.

The LC retention data were reported as log I, where I is the retention index as described by Popl *et al.*[11]:

$$\log I_x = \log I_n + \frac{\log R_x - \log R_n}{\log R_{n+1} - \log R_n}$$

where I_x is the retention index of compound x, I_n the retention index of the

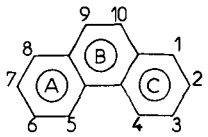

Fig. 1. Numbering of the phenanthrene molecule.

standard compound which elutes just prior to x, R_x the retention time of x, R_n the retention time of the standard eluting before x and R_{n+1} is retention time of the standard eluting after x. All retention times are corrected retention times (retention time of the solute minus that of the presumably unretained acetone)[12]. The following values (log I) were assigned to the PAH standards used for calibration: phenanthrene 3, benz[*a*]anthracene 4 and benzo[*b*]chrysene 5[7,11]. The log I values listed in Table I are mean values of two analyses.

Length-to-breadth ratios (L/B) and PAH non-planarity

The calculations were carried out for the DMP isomers with a VAX computer (installed at the CRPP, University of Bordeaux I) using the MM2MP2 empirical force field method[13]. The calculations yielded the geometry of the molecule at the energy minimum and also the dihedral angles between aromatic rings A and C (see Fig. 1). The L/B values were calculated based on the design of a rectangle fitted to the PAH molecule. Calculations for EtP isomers were carried out roughly without using the MM2MP2 program.

RESULTS AND DISCUSSION

A chromatogram of DMP standards used in the determination of retention indices is shown in Fig. 2.

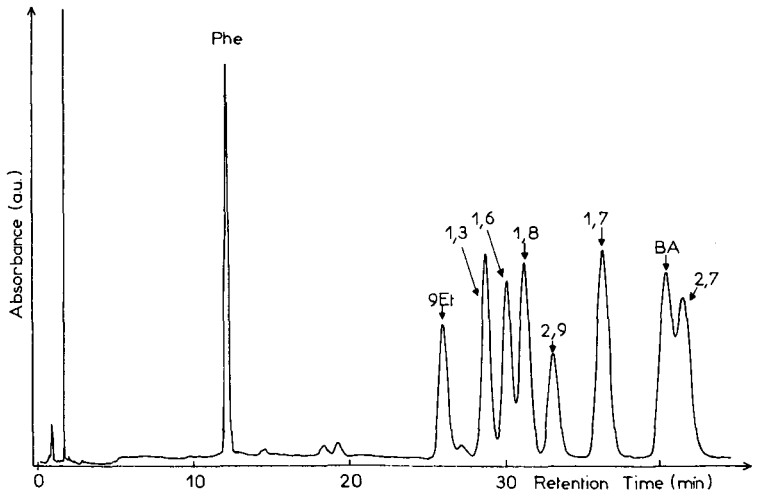

Fig. 2. Partial reversed-phase chromatogram of reference DMP standards used in the determination of LC retention indices (see text for experimental conditions). Phe = phenanthrene; 9-Et = 9-ethylphenanthrene; 1,3 = 1,3-dimethylphenanthrene, etc.; BA = benz[*a*]anthracene; benzo[*b*]chrysene (retention time 270.4 min) not shown.

TABLE I

RETENTION INDICES AND SHAPE PARAMETERS FOR DIMETHYL- AND ETHYLPHENANTHRENE ISOMERS

Log I values were determined by experiments whereas log I_c values were calculated through the correlation equation (see text).

Compound	*Log I*	*Log I_c*	*Deviation (%)*	*L/B*	*Dihedral angle (°)*[a]	*Length (Å)*	*Breadth (Å)*
1,2-DMP	3.80	3.85	+1.3	1.58	0.10	12.60	7.95
1,3-DMP	3.72	3.70	−0.5	1.28	0,05	11.80	9.20
1,4-DMP	3.67	3.73	+1.6	1.32	6.95	11.80	8.95
1,5-DMP	3.67	3.72	+1.3	1.31	6.40	11.80	9.00
1,6-DMP	3.76	3.75	−0.2	1.37	0.05	11.90	8.65
1,7-DMP	3.91	3.86	−1.2	1.59	0.05	12.60	7.90
1,8-DMP	3.79	3.84	+1.3	1.55	0.00	11.85	7.65
1,9-DMP	3.68	3.71	+0.8	1.28	0.05	11.80	9.20
1,10-DMP	3.65	3.71	+1.6	1.29	0.45	11.90	9.20
2,3-DMP	3.72	3.76	+1.1	1.38	0.00	12.70	9.20
2,4-DMP	3.75	3.76	−0.2	1.38	3.35	12.70	9.25
2,5-DMP	3.76	3.76	0.0	1.38	3.10	12.70	9.20
2,6-DMP	3.83	3.76	−1.6	1.40	0.00	12.45	8.90
2,7-DMP	4.01	3.91	−2.5	1.70	0.00	13.45	7.90
2,9-DMP	3.84	3.75	−2.3	1.37	0.00	12.60	9.20
2,10-DMP	3.78	3.74	−0.8	1.37	0.00	12.60	9.20
3,4-DMP	3.63	3.72	+2.5	1.31	11.90	11.95	9.10
3,5-DMP	3.67	3.71	+1.1	1.29	3.90	11.90	9.20
3,6-DMP	3.69	3.73	+1.1	1.33	3.73	11.85	8.90
3,9-DMP	3.75	3.63	−3.2	1.12	0.00	11.70	10.45
3,10-DMP	3.71	3.63	−2.1	1.12	0.00	11.70	10.45
4,5-DMP	3.42	3.71	+8.5	1.29	27.20	11.70	9.10
4,9-DMP	3.65	3.63	−0.5	1.29	5.70	11.80	10.45
4,10-DMP	3.64	3.63	−0.2	1.13	6.50	11.80	11.45
9,10-DMP	3.64	3.70	+1.6	1.28	1.28	11.70	9.15
1-EtP	3.70	n.d.[b]	n.d.	1.59	n.d.	12.64	7.95
2-EtP	3.78	n.d.	n.d.	1.77	n.d.	14.06	7.95
3-EtP	3.65	n.d.	n.d.	1.09	n.d.	10.69	9.81
4-EtP	3.55	n.d.	n.d.	1.09	n.d.	11.60	10.64
9-EtP	3.65	n.d.	n.d.	1.09	n.d.	11.60	10.64

[a] Angles between rings A and C (see Fig. 1).
[b] n.d. = Not determined.

The DMP isomers differ significantly in L/B values and also in dihedral angles (see Table I). DMP molecules with a methyl group in the bay region (position 4 and 5) exhibit strained molecular structures with large dihedral angles (more than 27° for 4,5-DMP and about 12° for 3,4-DMP). The theoretical calculations are in good agreement with X-ray crystallographic measurements[14–16]. The compounds with large dihedral angles generally elute before the unstrained DMP with low L/B and log I values. On the other hand, unstrained long or narrow DMP molecules are more strongly retained than those having a square-like shape. For example, 2,7- and 1,7-DMP exhibit high L/B and log I values.

The "slot model" previously proposed by Sander and Wise[4] mimics schematic-

ally the insertion of PAH molecules into the stationary phase. Planar molecules will be able to fit more easily than non-planar molecules into the slots of the bonded phase. The latter molecules interact weakly with the stationary phase and will be retained less. Differences in retention can be attributed to the thicknesses of the non-planar solute molecules, which are well described by the dihedral angles (D); the interaction of methyl groups will twist the aromatic molecule in certain cases instances, which would hinder penetration of the solute into the slots.

Some interesting features could be drawn from Fig. 3, which shows the correlation between LC retention (log I) and the L/B values for the 25 DMP isomers. The correlation equation obtained for 24 DMP (4,5-DMP was omitted) is $\log I_c = 0.366L/B + 3.234$ with a correlation coefficient $r = 0.713$. Predicted log I values were calculated and compared with the measured values. The average deviation was less than 2%, with high values for highly strained compounds (4,5-DMP and 3,4-DMP).

The correlation coefficient (r) between retention index (log I) and L/B values is 0.67 for the complete set of DMPs, increases to 0.79 with 23 DMPs when 3,4- and 4,5-DMP are neglected and reaches 0.85 with a set of 14 DMPs when all the compounds with dihedral angles greater than 3° are neglected. A correlation coefficient of 0.73 is obtained when retention indices are plotted as a function of the molecular length (L) and 0.46 for molecular breadth (B) (Table I). All this information indicates that (a) the retention behaviour of DMP isomers is mainly controlled by the length of the molecules and (b) when plotted against L/B, negative deviations of log I from the average linear trend are due to non-planarity of the molecules resulting from steric crowding of the methyl groups.

Most of the non-planar compounds with dihedral angles greater than 3° are eluted before the planar compounds (Fig. 3). Only 2,4- and the 2,5-DMP, despite having dihedral angles of 3.35° and 3.10°, respectively, are eluted together with the others DMP having average log I values. In this instance, the steric hindrance of the methyl group in position 4 or 5, which tends to reduce the retention of the molecule, is compensated for by the effect of the methyl group in position 2. This group has its greatest extension in the direction of the long axis of the molecule, and thus tends to increase the retention. Most of the DMP isomers bearing at least one methyl group in

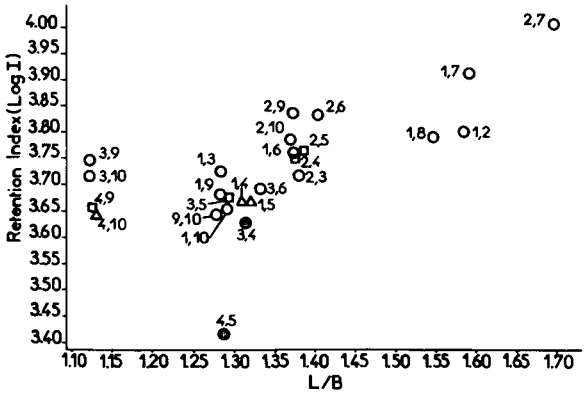

Fig. 3. Plot of LC retention indices *versus* L/B for all 25 DMP isomers with dihedral angles between 0° and 27.2° (○ = 0–3°; □ = 3–6°; △ = 6–10°; ● = >10°).

position 2 or 7 also exhibit high retention indices and high L/B values. The methyl group in this instance is in the same direction as the long axis of the molecule and tends to increase the molecular length (L) and consequently the retention on the stationary phase.

Analytical applications

Fig. 4 shows the partial HPLC trace obtained for the triaromatic fraction of a crude oil. True identifications of the DMP isomers were achieved by capillary gas chromatography–mass spectrometry and high-resolution Shpol'skii spectroscopy[3,17]. Despite the great selectivity of the column, some DMPs, are co-eluted as expected owing to their similar log I values. A difference of 0.03 log I units between isomeric compounds allows the separation of DMP isomers in the collected chromatographic fractions without any significant interferences. The non-planar DMPs and EtPs with strained structures (methyl groups in position 4 or 5, ethyl group in position 4) were not present in the natural sample studied, as reported in previous investigations for other sterically hindered aromatic compounds[2,3,6]. Such trends demonstrate the importance of theoretical information obtained by calculations for the prediction of retention behaviour and the presence of alkylated PAHs in natural samples.

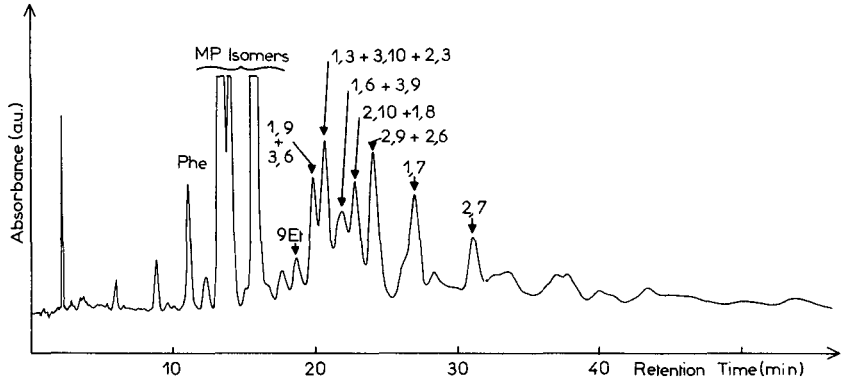

Fig. 4. Reversed-phase chromatogram of a triaromatic fraction from a crude oil. Identification of individual DMP isomers was done on collected fractions analysed by high-resolution Shpol'skii spectroscopy (see ref. 3 and text for experimental conditions). Phe = phenanthrene; MP = methylphenanthrenes; 9Et = 9-ethylphenanthrene; 1,9 = 1,9-dimethylphenanthrene, etc.

CONCLUSIONS

This study illustrates that the length-to-breadth ratio and the thickness described by dihedral angles provide a measure of the shape of aromatic isomers. These descriptors could well predict the elution order of DMPs on polymeric reversed-phase C_{18} stationary phases according to their molecular shape. The natural occurrence of specific DMP and EtP isomers could also be predicted according to their strained/non-strained conformation.

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REFERENCES

1 M. Radke, D. H. Welte and H. Willsch, *Geochim. Cosmochim. Acta*, 46 (1982) 1.
2 P. Garrigues, R. De Sury, M. L. Angelin, J. Bellocq, J. L. Oudin and M. Ewald, *Geochim. Cosmochim. Acta*, 52 (1988) 375.
3 P. Garrigues, E. Parlanti, M. Radke, J. Bellocq, H. Willsch and M. Ewald, *J. Chromatogr.*, 395 (1987) 217.
4 L. C. Sander and S. A. Wise, *Adv. Chromatogr.*, 25 (1986) 139.
5 S. A. Wise, W. J. Bonnett, F. R. Guenther and W. E. May, *J. Chromatogr. Sci.*, 19 (1981) 457.
6 P. Garrigues, M. P. Marniesse, S. A. Wise, J. Bellocq and M. Ewald, *Anal. Chem.*, 59 (1987) 1965.
7 S. A. Wise and L. C. Sander, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 244.
8 L. C. Sander and S. A. Wise, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 33.
9 R. H. Rohrbaugh and P. C. Jurs, *Anal. Chem.*, 59 (1987) 1048.
10 S. A. Wise, L. C. Sander, H. Ch. K. Chang, K. E. Markides and M. L. Lee, *Chromatographia*, 25 (1988) 473.
11 M. Popl, V. Dolanský and J. Mostecký, *J. Chromatogr.*, 117 (1976) 117.
12 S. A. Wise and W. E. May, *Anal. Chem.*, 55 (1983) 1479.
13 J. Kao and N. L. Allinger, *J. Am. Chem. Soc.*, 99 (1977) 975.
14 K. Takegoshi, F. Imashiro, T. Terao and A. Saika, *J. Chem. Phys.*, 80 (1983) 1089.
15 R. N. Armstrong, H. L. Ammon and J. N. Darnow, *J. Am. Chem. Soc.*, 109 (1987) 2077.
16 F. Imashiro, A. Saika and Z. Jaira, *J. Org. Chem.*, 52 (1987) 5727.
17 M. Radke, P. Garrigues and H. Willsch, *Org. Geochem.*, in press.

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SCREENING AND CONFIRMATION OF DRUGS IN HORSE URINE BY USING A SIMPLE COLUMN EXTRACTION PROCEDURE

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SUMMARY

A simple and reproducible column (Clean Screen-DAU, copolymeric bonded-phase silica column) extraction procedure has been described for the screening and confirmation of drugs in horse urine. The recovery of drugs by the column extraction was better than or comparable to the recovery by the liquid–liquid extraction, which is commonly used in the equine analytical laboratories. The column extraction provided broad coverage of drugs, separated extracts into three fractions (acidic/neutral, steroids, basic), produced a cleaner extract, and eliminated the need for special liquid–liquid extraction procedures for different drugs. The column extract was cleaner and did not contain impurities, whereas, the liquid–liquid extract was relatively impure and the extract required further thin-layer chromatographic cleanup. The column extraction procedure was used to confirm the presence of several potent drugs, such as fentanyl, etorphine, and mazindol.

INTRODUCTION

Medication is used in athletes (human and horses) for legitimate treatment as well as for illegal doping, where the intent is to achieve an artificial and unfair increase in performance in a competitive event. In order to discourage the illegal doping in horses, pre- or post-race urine samples from each racing horse is tested for the presence of drugs[1]. The methods which are commonly employed for testing the horse urine involves the extraction of urine by several liquid–liquid extraction procedures, qualitative screening of the extracted urine by thin-layer chromatography (TLC), and confirmation of the drug by gas chromatography–mass spectrometry (GC–MS)[1,2]. Although the liquid–liquid extraction procedure is commonly employed in urine analysis, there is a great deal of concern about the accuracy and reliability of the procedure[3,4]. The extraction procedure is time consuming, expensive, and require

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special procedures for the extraction of several important drugs such as clenbuterol, methylphenidate, opiates and ethacrynic acid[5–8]; and the procedure is not suitable for the confirmation of parent fentanyl, sufentanyl, and mazindol, which are present in urine in trace quantities[9]. Therefore, it is important to develop a sensitive, simple, and universal extraction procedure for the screening and confirmation of drugs in horse urine. Leferink *et al.*[2] have developed a solid-phase extraction procedure which, although provided a clean extract, gave poor recovery for many important drugs. The objectives of this investigation were (i) to develop a simple and reproducible extraction procedure by using a DAU (silica based mixed phase, bonded) chromatography column, and (ii) to compare the performances of the column and liquid–liquid extraction procedures for horse urine.

MATERIALS AND METHODS

The Clean Screen DAU chromatography columns (copolymeric bonded-phase silica columns, CSDAU-505), 2 cm high in a 5-ml disposable syringe, were obtained from Worldwide Monitoring (Horsham, PA, U.S.A.). The drug standards were obtained from Sigma, Aldrich, and the University of Minnesota Veterinary Hospital. Part of the drug-treated horses' urine samples were obtained from the Quality Assurance Program conducted by the Ohio State Racing Laboratories. The radioimmunoassay (RIA) kits for fentanyl and etorphine were obtained from the Diagnostic Products Corporation (DPC) (Los Angeles, CA, U.S.A.). FES spray was prepared by dissolving 500 mg of ferric chloride in 150 ml of ethanol and 40 ml of sulfuric acid (exothermic reaction). N-1-Napthylethylenediamine dihydrochloride (NED) spray was prepared by dissolving 1.0 mg of NED in 10 ml of absolute ethanol. β-D-Glucuronidase (β-D-glucuronide glucuronosohydrolase; E.C. 3.2.1.31) from Limpet (*Patella vulgata*) was obtained from Sigma.

Column extraction procedure (Fig. 1)

For the extraction of basic and glucuronide conjugated drugs, a 5-ml aliquot of urine samples containing drugs were mixed with 2 ml of acetate buffer (pH 5.0, 0.1 *M*) and 1 ml of β-D-glucuronidase (7000 units). The mixture was incubated at 60°C for 2 h. For the extraction of acidic and neutral drugs, a 2–4-ml aliquot of urine was mixed with 1.0 ml of NaOH, (0.1 *M*) and incubated at 25°C for 10 min. After incubations, the pH of the urine samples were adjusted to 6.0 with phosphate buffer (0.1 *M*) (Fig. 1). Both urine aliquots were extracted by using a DAU column as described below.

The DAU column was connected to a vacuum manifold and washed with methanol (5 ml), water (5 ml), and 1.0 *M* acetic acid (5 ml) as shown in Fig. 1. The two urine aliquots were poured into the treated column and the samples were pulled through the column at 2 ml/min. Thereafter, the column was washed with water (5 ml) and acetic acid (5 ml), dried under vacuum, and the drugs were eluted by the procedure described in Fig. 1. The fractions containing the acidic/neutral, steroid, and basic drugs were dried separately at 50°C under nitrogen. The dried residue was redissolved in 50 μl of dichloromethane (DCM) (for acidic drugs) or ethyl acetate (EA) (for basic or steroid drugs).

TLC screening of the column extract. The acidic/neutral extract was spotted on three TLC plates and the plates were developed in solvent 1: chloroform–cyclohex-

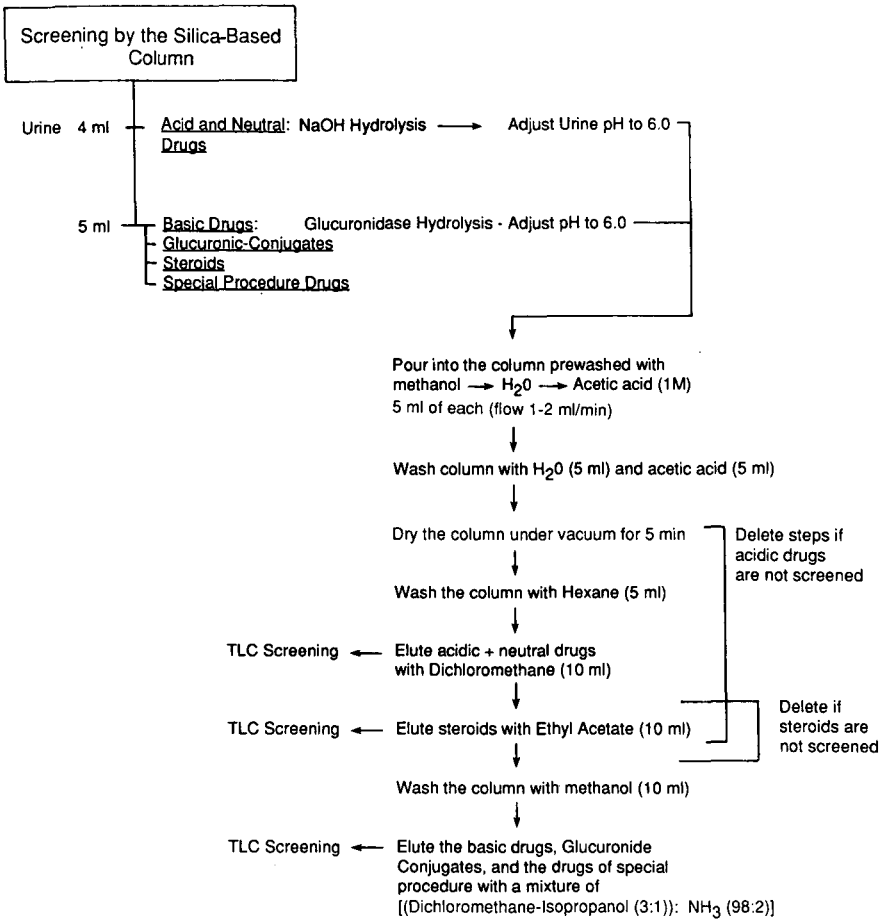

Fig. 1. Procedure for the extraction of drugs from horse urine by using a DAU column.

ane–acetic acid (4:4:2, v/v); solvent 2: chloroform–ethanol (9:1, v/v); and solvent 3: EA–methanol–NH_4OH (85:10:5, v/v/v)[10] respectively. The three plates were sprayed with Mandelin reagent[10] and heated at 60°C for 15 min. The acidic drugs developed a brown color. The plate developed in solvent 3 was further sprayed with the Dragendorff reagent[10] to screen theophylline which appeared as a dark orange spot. The steroid extract was spotted on one TLC plate and the plate was developed in solvent 4 [chloroform–EA–methanol (50:45:5, v/v/v)] followed by solvent 3. Steroids were detected by spraying the plate with H_2SO_4 + ethanol spray and heating it at 60°C until the standards appeared dark brown. The basic extract was spotted on three plates, and one plate was developed in solvent 3 and two plates were developed in solvent 5 [chloroform–methanol–propionic acid (72:18:10, v/v/v)]. The plates developed in solvent 3 were sprayed with Dragendorff reagent and sodium nitrite[10]. The plates developed in solvent 5 were sprayed with Dragendorff[10] or FES reagents. For detecting clenbuterol, plates were exposed to NO_2 gas and then sprayed with NED (Clenbuterol appeared as a pink spot).

Liquid–liquid extraction procedure (Fig. 2)

Acid drugs. A 2-ml aliquot of urine was mixed with 1.0 ml of NaOH (0.1 *M*) and the mixture was incubated at 25°C for 10 min (to hydrolyze the drug–amino acid conjugate). The pH of the mixture was adjusted to 3.0 with saturated phosphate buffer (pH 3.0) and the urine was extracted with 5 ml of DCM. The sample was centrifuged for 5 min at 1500 *g* and the DCM layer was dried at 50°C under nitrogen. For the screening of naproxen, flunixin, and indomethacin, part of the DCM extract was washed with lead acetate (0.1 *M*). We have observed that lead acetate wash also removed furosemide. The dried residue was redissolved in DCM (20 μl) for TLC screening.

Neutral drugs. A 5-ml aliquot of urine was mixed with 3 ml of phosphate buffer (pH 6.0). The mixture was extracted with 5.0 ml of EA. The EA extract was washed with 2.0 ml of $NaHCO_3$ (0.1 *M*) to remove the acidic drugs. The EA extract was dried and the dried residue was redissolved in 30 μl of EA for TLC screening.

Basic drugs. A 9-ml aliquot of urine was mixed with 2 ml of 0.5 *M* sodium carbonate buffer (pH 9.0). The mixture was extracted with DCM–isopropanol (3:1, v/v), and the organic layer was collected after centrifugation at 1500 *g* for 5 min. The organic layer was collected after centrifugation at 1500 *g* for 15 min. The organic layer was dried and the dried residue was redissolved in 30 μl of EA for TLC screening.

Steroids. A 9-ml aliquot of urine was mixed with 2 ml of saturated sodium borate solution and 5 ml of EA. After mixing, the sample was centrifuged for 5 min at

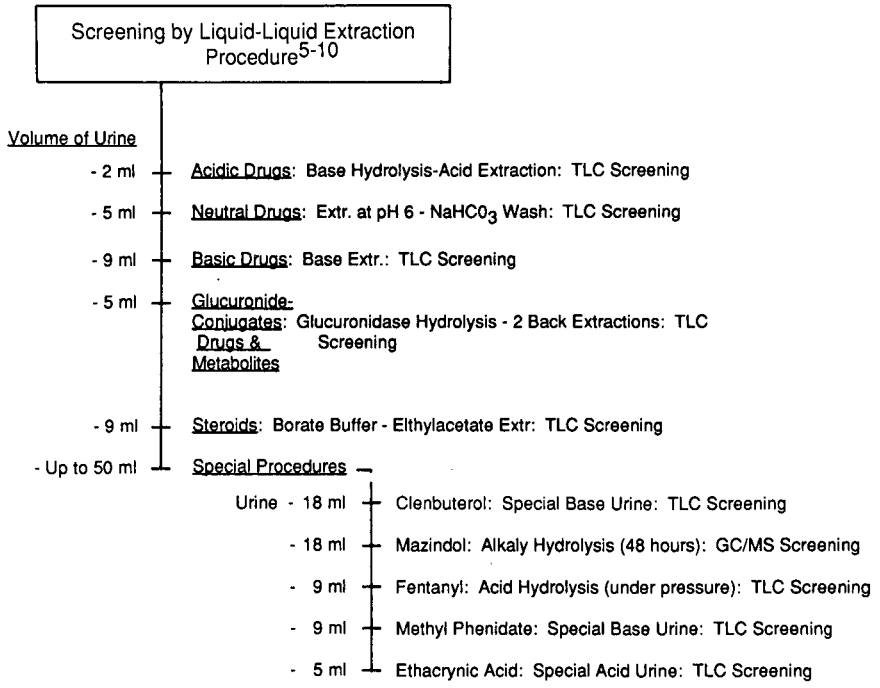

Fig. 2. Summary of the liquid–liquid extraction procedures which are commonly used for the screening and confirmation of drugs in horse urine.

1500 *g*, the organic layer was collected and washed with 1.0 ml of 15% sodium sulfate in 1.0 *M* NaOH. The EA layer was separated and dried with N_2 at 60°C. The dried residue was dissolved in 35 μl of EA.

Glucuronide-bound drugs/metabolites. A 5-ml aliquot of urine was mixed with acetate buffer (pH 5.0) and β-D-glucuronidase (7000 units) and the mixture was incubated at 60°C for 2 h. After incubation, the drugs were extracted from urine by a procedure described previously[11].

Clenbuterol extraction. An 18-ml aliquot of urine was mixed with NaOH (10 *M*) to adjust the pH of the urine to 12.0. The sample was extracted with 10 ml light petroleum (b.p. 34–36°C), the light petroleum layer was collected and dried at 50°C in N_2. The dried residue was redissolved in 20 μl of ethyl acetate for TLC screening.

TLC screening of the liquid–liquid extracts. The TLC screening of the acidic, neutral and steroid drugs were performed as described for the column extraction.

Comparison of the efficiency of column and liquid–liquid extraction procedures

The qualitative extraction efficiency was determined by extracting either the urine samples obtained from the drug treated horse or the urine samples mixed with known amounts of drug as shown in Table I. Each urine sample was extracted by the column and by the liquid–liquid extraction procedures as described earlier. Equivalent amounts of the dried residue were spotted on TLC plates along with the known quantity of each standard. The plates were developed in different solvents and sprayed with different reagents as described previously. The qualitative efficiency was determined by comparing the standard spot with that of the spot obtained from the two extraction procedures. The spots were scored from "+ + + +" to "−": when the spot appearing similar to the standard in intensity it was scored "+ + + +" and the absence of spot was scored "−".

The quantitative recovery of selected drugs was determined by using a GC–MS procedure. The column extracted or liquid–liquid extracted urine samples containing various drugs (Table II) were derivatized by N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and injected into the GC–MS system (HP-5990, DB-5 capillary column) by a procedure described previously[11]. The following GC–MS conditions were used: inlet temperature, 200°C; initial oven temperature 80°C; temperature program 20°C/min to 280°C; run time 35 min. The ion source temperature was 200°C, the electron energy was 70 eV, and the MS source pressure was $2.5 \cdot 10^{-6}$ Torr. The extraction efficiency was determined by comparing the area under the peak for each drug obtained from urine and from known standards.

The quantitative recovery of etorphine and fentanyl was determined by using a RIA kit. Urine samples (5 ml) containing these drugs were extracted by the two extraction procedures. The dried extract was diluted with the RIA buffer (100 μl) supplied by the manufacturer. Volumes of 50 μl of the buffer standards were mixed with [^{3}H]etorphine or [^{3}H]fentanyl and the antibodies. The mixture was incubated at room temperature for 2 h, the free isotope was removed by using dextran coated charcoal and the bound radioactivity was determined by using the scintillation counter. The concentration of the drug was determined by using a built-in RIA program in the counter.

TABLE I

QUALITATIVE TLC SCREENING OF VARIOUS DRUGS BY THE TWO EXTRACTION PROCEDURES

Abbreviations: BU = base urine extraction; AU = acid urine extraction; EH = glucuronide hydrolysis-base urine back extraction; Sp.BU = special base urine extraction; SU = steroid urine extraction; B = base urine column extract, A/N = acid urine column extract; and S = EA column extract. TLC systems; 1 = 4:4:2; 2 = 9:1; 3 = 85:10:5; 4 = 50:45:5; 5 = 72:18:10.

Drug	*Concentration or dose*	*TLC*	*Liquid–liquid extr.*		*Column extr.*	
			Extr.	*Qualitative efficiency*	*Fraction*	*Qualitative efficiency*
Acepromazine	1.0 μg/ml	3	BU	+ +	B	+ + +
Amphetamine	1.0 μg/ml	3	BU	+	B	+ + + +
Apomorphine	1.0 μg/ml	5	EH	+ + + +	B	+ + +
Buprenorphine	0.5 μg/ml	5	EH	+ +	B	+ + + +
Clenbuterol[a]	1.0 mg/horse, 4 h	3	Sp.BU	+ + +	B	+ + + +
Chlorpromazine	1.0 μg/ml	3	EH	+ + +	B	+ + +
Diazepam	1.0 μg/ml	5	EH	+ +	B	+ + + +
Dipyrone	2.0 μg/ml	3	BU	+ +	B	+ + +
Ephedrine	1.0 μg/ml	3	BU	+ +	B	+ + + +
Ethacrynic Acid	2.0 μg/ml	1,2	AU	+ +	A/N	+ +
Etorphine[a]	0.1 mg/horse, *i.m.* 4 h	–	EH	+ +	B	+ + +
Fentanyl[a]	1.0 mg/horse, *i.v.* 0, 2, 4, 6, 8, 24 h	–	EH	–	B	+ + + +
Flunixin	2.0 μg/ml	1,2	AU	+ + +	A/N	+ + +
Guaifenesin	2.0 μg/ml	3	BU	+ + + +	B	–
Hordenine	1.0 μg/ml	5	EH	+ + +	B	+ + + +
Hydrocortisone	2.0 μg/ml	3,4	SU	+ +	S	+ +
Hydromorphone	10 mg/horse, *i.m.* 4 h	5	EH	+ +	B	+ + + +
Hydromorphone	1.0 μg/ml	5	EH	+ +	B	+ + + +
4-Hydroxychlorpromazine	1.0 μg/ml	5	EH	+ +	B	+ + + +
Indomethacin	2.0 μg/ml	1,2	AU	+ + +	A/N	+ + +
Furosemide	2.0 μg/ml	1,2	AU	+ + + +	A/N	+ +
Lidocaine	1.0 μg/ml	5	EH	+ + +	B	+ + + +
Mazindol[b]	20 mg/horse, oral 4 h	–	EH	+ +	B	+ + + +
Meclizine	1.0 μg/ml	5	EH	+ +	B	+ + + +
Mefenamic Acid	2.0 μg/ml	1,2	AU	+ + +	A/N	+ + +
Meprobamate	1.0 μg/ml	3	BU	+ +	B	+ + + +
Methamphetamine	1.0 μg/ml	3	BU	+	B	+ + + +
Methadone	2.0 μg/ml	3	BU	+ +	B	+ +
Methyl phenidate	1.0 μg/ml	3	BU	+ +	B	+ + + +
Metolazone	1.0 μg/ml	3	NU	+ +	A/N	+ +
Nefopam	1.0 μg/ml	3	BU	+ + + +	B	+ + + +
Nalorphine	1.0 μg/ml	5	EH	+ + +	B	+ + +
Naloxane	2.0 μg/ml	3	BU	–	B	+ + + +
Naproxen	2.0 μg/ml	1,2	AU	+ + + +	A/N	+ + +
Nubain	1.0 μg/ml	5	EH	+ + +	B	+ + + +
N-N[1]-Diethyltryptamine	1.0 μg/ml	5	BU	+ + + +	B	+ + + +
Oxymorphone[a]	10 mg/horse, *i.m.* 4 h	5	EH	+ +	B	+ +
Pemoline	1.0 μg/ml	3	BU	+ + +	B	+ +
Phenolbutazone	2.0 μg/ml	1,2	AU	+ + +	B	+ + + +
Propionylpromazine[a]	10 mg/horse *i.m.* 4 h	3	BU	+ +	B	+ + + +
Propranolol	2.0 μg/ml	3	EH	+ + +	B	+ + + +
Quinine	1.0 μg/ml	3	BU	+ +	B	+ + + +
Xylazine	1.0 μg/ml	5	EH	+ +	B	+ + + +
Strychnine	0.5 μg/ml	3	BU	+ + +	B	–
Theophylline	1.0 μg/ml	3	NU	+	B	+ + +
Trimethoprime	1.0 μg/ml	3	BU	+ + + +	B	+ + + +
Tetracaine	1.0 μg/ml	5	EH	+ + +	B	+ + + +

[a] Ohio State University Sample. Dose, route and time of urine collection.

[b] Horse injected with mazindol at the University of Minnesota.

TABLE II

PERCENT RECOVERY OF DRUGS EXTRACTED BY USING THE COLUMN AND LIQUID–LIQUID EXTRACTION PROCEDURES

Values are mean ± SD, $n = 4$.

Drug	*Analytical procedures*	*Recovery (%)*	
		Liquid–liquid base extraction	*Column extraction*
Acepromazine	GC–MS	87 ± 10	79 ± 8
Amphetamine	GC–MS	15 ± 3[a]	87 ± 8
Canabinol	GC–MS	15 ± 5[a]	0.3 ± 0.2
Cocaine	GC–MS	8 ± 3[a]	77 ± 10
Diazepam	GC–MS	5 ± 2[a]	51 ± 8
Etorphine	RIA	79 ± 10	83 ± 13
Fentanyl	RIA	0.5 ± 0.2[a]	83 ± 8
Lidocaine	GC–MS	20 ± 6[a]	63 ± 5
Mazindol	GC–MS	50 ± 5	78 ± 5
Methamphetamine	GC–MS	5 ± 3[a]	88 ± 7
Naproxen	GC–MS	60 ± 10	61 ± 9
Phenylbutazone	GC–MS	69 ± 7	75 ± 8
Xylazine	GC–MS	5 ± 3[a]	71 ± 8

[a] $P < 0.05$ when compared with the column-extraction values.

Extraction and confirmation of fentanyl and mazindol in urine samples obtained from the drug-treated horses

For the confirmation of fentanyl, a horse was injected with fentanyl (1.0 mg, *i.v.*) and urine samples were collected at 0 (pre-injection) 2, 4, 6, 8 and 24 h after the injection. The 5-ml urine sample from each time interval was subjected to glucuronidase hydrolysis. At the end of hydrolysis, a 50-μl aliquot was removed from each sample for direct RIA analysis. The remaining samples were extracted by the column and by liquid–liquid extraction procedures. The direct urine, column extracted urine and the liquid–liquid extracted urine were screened for fentanyl by using the RIA kit. The amount of fentanyl present in each sample was calculated from the standard curve. For the GC–MS confirmation of parent fentanyl in horse urine, 10 ml aliquots from 0, 2, 4, and 6 h urine samples and a 30-ml aliquot from the 8-h urine sample were hydrolyzed with glucuronidase and extracted by the column as described previously. The extract was dried and the dried residue was redissolved in 10 μl of EA. Of the EA, 1 μl was injected into the GC–MS system. The oven temperature was programmed as follows: initial temperature, 150°C; final temperature, 280°C (rate, 20°C/min); run time 20 min. The ions monitored for the selected-ion screening were m/z 124, 146, 189 and 245. The 30-ml aliquot from the 8-h urine sample was also extracted by the liquid–liquid extraction procedure and analyzed by the GC–MS.

For the confirmation of Mazindol, a Standard Bred horse was fed with 20 mg mazindol and an urine sample was collected at 4 h after the drug feeding (by injecting furosemide, 0.5 mg/kg, *i.m.*). Duplicate 50-ml aliquots of the urine samples were hydrolyzed by glucuronidase. One set of urine sample was extracted by using ten

columns (one for 5 ml of urine). The final extract was pooled and dried. The dried residue was derivatized with BSTFA (10 μl) and 1 μl was injected into the GC–MS system. The GC–MS oven temperature was programmed from 150°C to 280°C with 10°C/min increments. The ions monitored for the quantitation of mazindol were at *m/z* 245, 267, 327, and 356. A 50-ml volume of urine was also extracted by the liquid–liquid extraction procedure and analyzed as described above.

RESULTS AND DISCUSSION

Overall performance of the column and liquid–liquid extraction procedures

This study indicated that the column extraction procedure provided a simple and efficient method for the screening and confirmation of drugs in horse urine. The column extraction separated extracts into three fractions (acidic/neutral, steroids and basics) and produced a clean extract which was suitable for direct GC–MS analysis without further cleanup. The liquid–liquid extraction normally required a TLC cleanup which significantly reduced the recovery of the drugs. For complete screening, the DAU column extraction required 10 ml of urine, whereas, the liquid–liquid extraction required 30–50 ml of urine. By using a vacuum manifold, 30 to 40 samples can be extracted in less than an hour by one technician.

Extraction and recovery of the acidic and neutral drugs

The acidic drugs commonly used in horses are phenylbutazone, fuorsemide, naproxen, flunixin, etc.; and the neutral drugs used in horses are theophylline, theobromine, metolazone, etc.[1,12]. This study indicated that the DAU column provided a relatively clean extraction of the acidic and neutral drugs. The qualitative and quantitative screening has shown that the extraction efficiencies of acidic drugs by the two procedures were similar, except for furosemide which is extracted better by the liquid–liquid extraction procedure (Tables I and II). Unlike the acidic drugs, theophylline exhibited better recovery by the column extraction procedure (Table I). Since the acidic and neutral drugs are used in large quantities, extraction by either procedure may be satisfactory for the screening of these drugs.

Extraction and recovery of the basic drugs and the drugs which required special liquid–liquid extraction procedure

Basic drugs include a broad range of compounds which have different chemical and pharmacological properties. Previous studies have shown that a single liquid–liquid extraction method did not cover all the basic drugs, and that special procedures were necessary to extract certain important drugs such as clenbuterol, methylphenidate and opiates[5–8]. This study indicated that the DAU column extracted a broad range of drugs and the recovery of drugs by the column extraction was better than or similar to the recovery of drugs by the liquid–liquid extraction (Tables I and II). Amphetamine and methamphetamine exhibited $< 25\%$ recovery by the liquid–liquid extraction, whereas, these drugs exhibited $> 85\%$ recovery by the column extraction (Tables I and II). Also the column extraction for these drugs was cleaner and did not contain impurities, whereas the liquid–liquid extraction was relatively impure and the samples required further TLC cleanup (Fig. 3). This study indicated that the column extraction provided an uniform extraction efficiency for a wide range of drugs in-

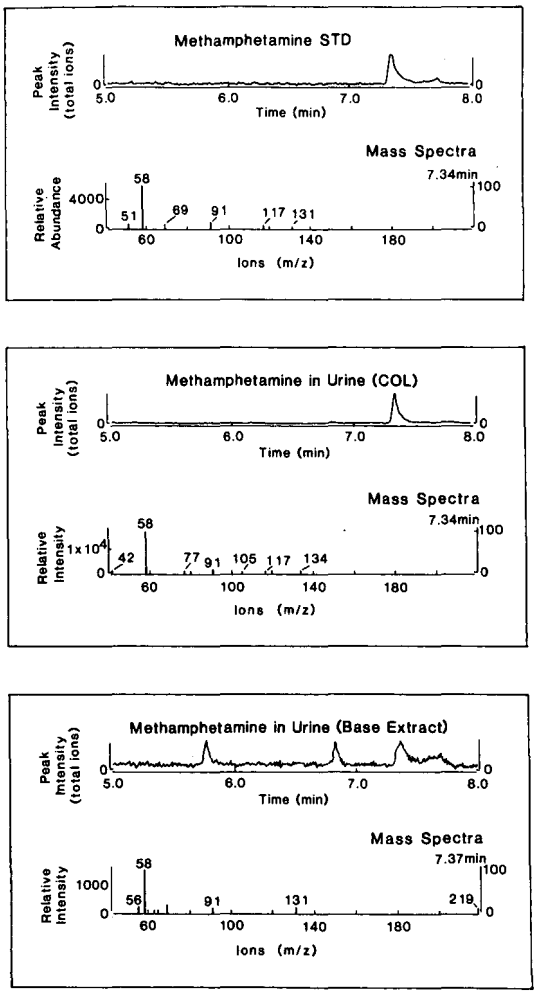

Fig. 3. Confirmation by GC–MS of methamphetamine extracted from horse urine by the column or liquid–liquid extraction procedure. Urine samples of 5 ml (containing 1.0 μg methamphetamine/ml urine) were extracted by the two extraction procedures. The extract was dissolved in 100 μl of ethyl acetate and 1.0 μl of the extract was analyzed by the GC–MS. Standard (STD) samples (containing 10.0 ng of the drug) were also analyzed.

cluding clenbuterol, methylphenidate, diazepam and opiates (Tables I and II). Cone *et al.*[5] have shown that the extraction efficiencies of opiates were significantly different for different solvents used for the liquid–liquid extraction of urine.

Extraction and recovery of steroids

Steroids were eluted from the column by using EA (Fig. 2). Since EA also eluted some non-specific compounds, the steroid extract was relatively dirty and appeared similar to the liquid–liquid extract. The recovery of steroids from the two procedures appeared similar (Table I).

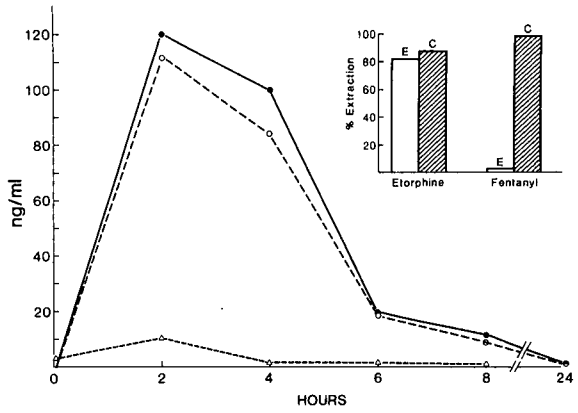

Fig. 4. Comparison of the recoveries of etorphine and fentanyl by the column and liquid–liquid extraction procedures. (●) Analysis of fentanyl by RIA in whole urine; (○) analysis of fentanyl by RIA in column extract; (△) analysis by RIA of liquid–liquid extract. The bar graphs shows the percent recoveries of the two drugs (hatched), column extraction and (open) liquid–liquid extraction.

Extraction, recovery and confirmation of potent drugs such as fentanyl, etorphine and mazindol

The potent drugs included in this study are known to stimulate the central nervous system at lower doses[13–18]. The short duration of action, low urinary concentration, and difficulty in detection have made these drugs attractive doping agents

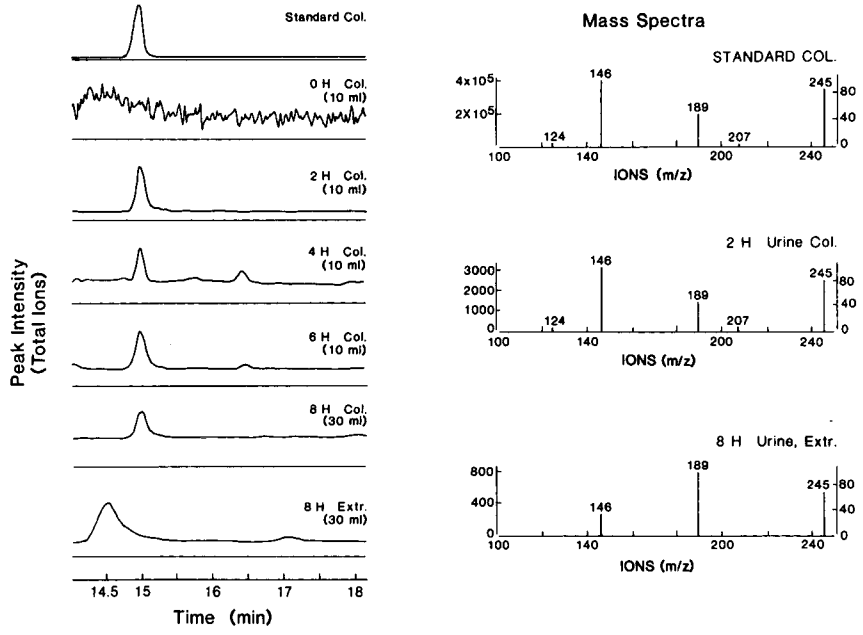

Fig. 5. Screening of fentanyl in urine obtained from fentanyl-injected horse and extracted by using the column (0, 2, 4, 6, 8 h urine) or liquid–liquid extraction (8 h urine) procedures. Parent fentanyl was confirmed by using the selected-ion monitoring GC–MS procedure.

at racetracks[19,20]. The identification of fentanyl and mazindol by the liquid–liquid extraction is particularly difficult because these drugs are first hydrolyzed to despropionyl fentanyl (DPF) and 2-(4-chlorobenzyl)benzoic acid (CBB) respectively[9,21], and then the hydrolyzed product is identified by GC–MS. Since DPF and CBB are not the natural metabolites of fentanyl or mazindol, confirmation of these drugs by confirming the presence of DPF or CBB can be easily challenged in court. This study had indicated that the DAU column selectively extracted etorphine, fentanyl, and mazindol with > 80% recovery (Table II, Fig. 4). The liquid–liquid extraction procedure was not suitable for the extraction of fentanyl and mazindol since the recovery was poor for both drugs (Table II, Fig. 4). Because of the selective and efficient extraction of fentanyl and mazindol by the column, it was possible to identify and confirm the unchanged parent drugs in horse urine. As shown in Figs. 4 and 5, the presence of parent fentanyl was identified by both RIA (Fig. 4) and GC–MS methods (Fig. 5) for up to 8 h after dosing the horse with fentanyl. Presence of parent mazindol was also confirmed by GC–MS of urine samples (Fig. 6) obtained from the mazindol treated horse. Based on these observations it is proposed that (i) the hydrolysis of fentanyl or mazindol was not necessary for the identification of these drugs when the column extraction procedure was used, and (ii) the poor recovery of fentanyl and mazindol by the liquid-liquid extraction procedure may be responsible for the difficul-

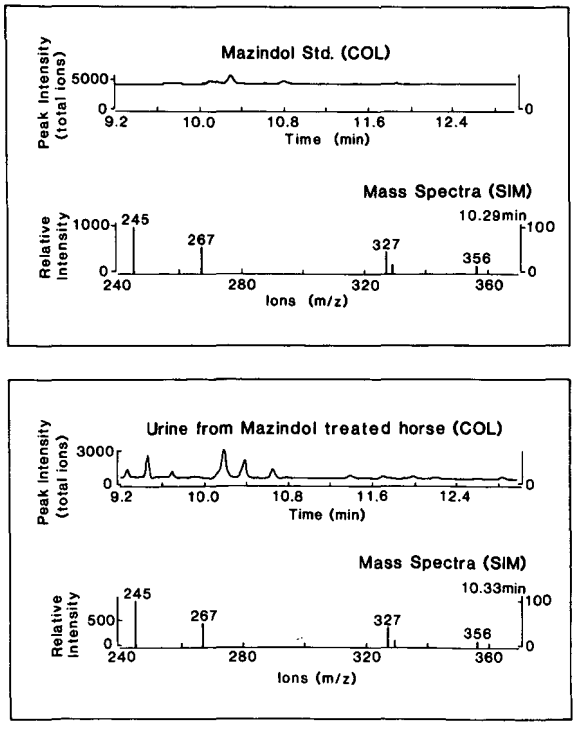

Fig. 6. Screening of mazindol in urine obtained from mazindol-fed horse and extracted by the column extraction procedure. The parent mazindol was confirmed by using a selected-ion monitoring (SIM) GC–MS procedure.

ties previous investigators encountered in the confirmation of parent drugs by GC–MS.

In conclusion, the silica-based DAU column provided a simple and efficient extraction of horse urine for the screening and confirmation of drugs. The recovery of drugs by the column extraction was better than or similar to the recovery by the liquid–liquid extraction procedure. The column extract was clean and could be subjected to direct GC–MS confirmation. The liquid–liquid extract normally required TLC cleanup which reduced the recovery to 25–30%. The column extraction also extracted the potent drugs, such as fentanyl and mazindol which was not possible by the common liquid–liquid extraction; and eliminated the need for special liquid–liquid extraction procedures.

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REFERENCES

1 T. Tobin, *Drugs and the Performance Horse,* Charles C. Thomas Publisher, Springfield, IL, 1981, pp. 5–20.
2 J. G. Leferink, J. Bankers and A. J. H. Schnotman, *Proceedings of the 6th International Conference of Racing Analyst and Veterinarians, Hong Kong, December 1–7, 1985,* Macmillan, New York, 1986, pp. 171–175.
3 H. J. Hansen, S. P. Caudill and J. Boone, *J. Am. Med. Assoc.*, 253 (1985) 2382–2387.
4 D. J. Hanson, *Chem. Eng. News,* 64 (1986) 7–14.
5 E. J. Cone, W. D. Darwin and W. F. Buchwald, *J. Chromatogr.*, 275 (1983) 307–318.
6 E. J. Cone, *J. Chromatogr.*, 129 (1976) 355–360.
7 B. R. Manno, J. E. Manno and C. A. Dempsey, *J. Anal. Toxicol.*, 10 (1986) 116–119.
8 K. S. Patrick and W. H. Elliott, *J. Chromatogr.*, 343 (1985) 329–338.
9 L. M. Young and V. Robillo, *Proceedings of the 6th International Conference of Racing Analyst and Veterinarians, Hong Kong, December 1–7, 1985*, Macmillan, New York, 1986, pp. 257–268.
10 B. Davidow, N. L. Petri and B. Quame, *Am. J. Clin. Pathol.*, 50 (1988) 714–717.
11 A. K. Singh, U. Mishra, M. Ashraf, E. H. Abdennebi, K. Granley, D. Dombrovskis, D. Hewetson and C. M. Stowe, *J. Chromatogr.*, 404 (1987) 223–232.
12 M. Donike, S. Horning, F. Mandel and W. Schanzer, *Proceedings of the 6th International Conference of Racing Analysts and Veterinarians, Hong Kong, December 1–7, 1985*, Macmillan, New York, 1986, pp. 187–193.
13 N. W. Shepard, *The application of Neurolepta-analgesia in anesthesia and other practices,* Pergamon Press, Oxford, 1965, pp. 1–25.
14 J. F. Gardocki and J. A. Yelnosky, *Toxicol. Appl. Pharmacol.*, 6 (1964) 48–53.
15 F. L. Grell, R. A. Koons and J. S. Denson, *Anesth. Analg. (Cleveland)*, 49 (1970) 523.
16 J. H. Gagerty and J. H. Trapold, *Triangle Engl. Ed.*, 15 (1976) 25–36.
17 J. H. Gagerty, C. Penberthy, L. C. Iorio and J. H. Trapold, *Arch. Int. Pharmacodyn.*, 214 (1975) 285–307.
18 W. F. M. Van Bever, C. J. E. Niemegeers, K. H. L. Schellekens and P. A. J. Janssen, *Arzeneim.-Forsch.*, 26 (1975) 1548.
19 J. M. Frincke and G. L. Henderson, *Drug Metab. Dispos.*, 8 (1980) 425–427.
20 G. L. Henderson, J. M. Frincke and R. J. Garber, *Proc. West. Pharmacol. Soc.*, 24 (1981) 137–140.
21 J. H. Gagerty and J. H. Trapold, *Triangle Engl. Ed.*, 15 (1976) 25–36.

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DESIGN AND APPLICATIONS OF BIOMIMETIC ANTHRAQUINONE DYES

PURIFICATION OF CALF INTESTINAL ALKALINE PHOSPHATASE WITH IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

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SUMMARY

A 330-fold one-step purification of alkaline phosphatase from a crude calf intestinal extract has been achieved using specific elution with inorganic phosphate (5 m*M*) from a purpose designed adsorbent comprising a terminal ring phosphonate analogue of C.I. Reactive Blue 2 coupled to Sepharose CL-6B-200. The resulting alkaline phosphatase preparation displayed a specific activity in excess of 1000 U/mg and was of equivalent purity to commercial "high purity" preparations as deduced by sodium dodecyl sulphate polyacrylamide gel electrophoresis and specific activity comparisons.

INTRODUCTION

Highly purified alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), E.C. 3.1.3.1] from calf intestinal mucosa is widely used in molecular biology and immunodiagnostics. Traditionally, the enzyme has been purified by a lengthy, multi-step procedure employing conventional chromatographic techniques[1,2]. However the reported yields are low and the final preparations are invariably contaminated with co-purifying enzymes such as phosphodiesterase (oligonucleate 5′-nucleotidohydrolase, E.C. 3.1.4.1)[3]. More recently, however, affinity chromatography has been successfully exploited for the purification of alkaline phosphatase from calf intestine[1,2] and other sources[4] using immobilised competitive inhibitors such as phosphonic and arsanilic acids as affinity ligands. Unfortunately, these derivatives tend to be difficult and expensive to synthesise, require relatively hazardous activation conditions to couple to the solid support and yield adsorbents with ill defined ligand concentrations and which display limited chemical and biological stability. These problems may well be ameliorated by the introduction of triazine dyes as affinity ligands[5]. For example, a number of commercially available

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triazine dyes have been screened for their ability to purify alkaline phosphatase from crude intestinal preparations[6]. Most of the immobilised triazine dyes bound the enzyme to some extent and a similarity between the sulphonated aromatic dyes and other alkaline phosphatase affinity ligands was noted. The use of selected dyes, when combined with specific elution, resulted in high purification factors. Immobilised dyes have also been used as components in a multiple-step calf intestine alkaline phosphatase purification procedure[7] and for the purification of alkaline phosphatases from other sources[8–10].

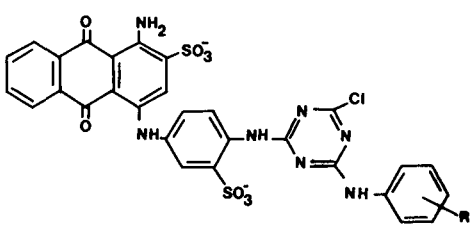
Specifically designed biomimetic dyes which display higher affinity for horse liver alcohol dehydrogenase than conventional commercially available dyes have recently been synthesised and mark the onset of a new era in the use of triazine dyes for protein purification[11,12]. This paper reports a similar rational approach to synthesise anthraquinone dyes based on C.I. Reactive Blue 2 bearing phosphonate and other terminal ring substituents for the purification of calf intestinal mucosa alkaline phosphatase.

EXPERIMENTAL

Materials

The commercial triazine dyes (Procion™ H and MX series) and the terminal ring bases, aniline, *p*-aminobenzoic acid, *m*- and *p*-aminobenzenesulphonic acids, *p*-aminobenzyl phosphonic acid and *m*-aminobenzeneboronic acid were a generous and much appreciated gift from Dr. C. V. Stead, ICI Organics Division, Manchester, U.K. The terminal ring analogues of C.I. Reactive Blue 2 (Fig. 1, I–V) were synthesised according to a previously published procedure[13].

Sepharose CL-6B-200, bovine serum albumin (fraction V powder, 98–99% albumin), calf intestinal alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), E.C. 3.1.3.1; Type I, 1–3 U/mg solid] and all other crude alkaline phosphatase preparations, phosphodiesterase I (oligonucleate 5′-nucleotide hydrolase, E.C. 3.1.4.1, bovine intestinal mucosa, 0.5–1 U/mg protein), bis(*p*-nitrophenyl) phosphate, α-naphthyl phosphate and 4-phenyl phosphate were all obtained from Sigma (London), Poole, U.K. High-purity alkaline phosphatase (enzyme label



	TERMINAL RING ANALOGUE	R
I	aniline	-H
II	p-aminobenzoic acid	-COOH
III	m/p-aminobenzenesulphonic acid	$-SO_3H$
IV	p-aminobenzyl phosphonic acid	$-CH_2PO_3H_2$
V	m-aminobenzeneboronic acid	$-B(OH)_2$

Fig. 1. Structures of the terminal ring analogues of C.I. Reactive Blue 2.

for enzyme immunoassay-grade I) was obtained from Boehringer Mannheim, Lewes, U.K. and from Biozyme Labs., Gwent, U.K. (ALP I–10 G). All other chemicals were of Analar grade.

Assay of enzyme activities and protein

Alkaline phosphatase and phophodiesterase activities were assayed by following the production of *p*-nitrophenolate anion at 405 nm. For alkaline phosphatase the total assay volume of 1 ml contained: glycine–NaOH buffer, pH 10.4 (0.1 mmol), *p*-nitrophenyl phosphate (6 μmol), $MgCl_2$ (1 μmol) and $ZnCl_2$ (1 μmol). The assay mixture for the phosphodiesterase contained, in a total volume of 1 ml: Tricine–NaOH buffer, pH 8.8 (60 μmol) and bis(*p*-nitrophenyl) phosphate (6 μmol). The molar extinction coefficient of the *p*-nitrophenolate anion was taken as 18 500 l mol^{-1} cm^{-1} at 405 nm. One unit of enzyme activity is defined as that amount of enzyme which catalyses the formation of 1 μmol *p*-nitrophenolate anion per min at 37°C.

Protein concentrations were determined by measurement of the absorbance at 280 nm using a mass extinction coefficient of 1.0 absorbance unit per mg ml^{-1} of protein for alkaline phosphatase[14].

Immobilisation of triazine dyes

Sepharose CL-6B-200 was exhaustively washed with distilled water, sucked moist on a sintered funnel and divided into 5-g portions. The commercial dye powders were washed with diethyl ether and samples (100 mg) dissolved in water (22.5 ml) and added to the moist gel (5 g). The suspension was incubated for 5 min at 20°C prior to the addition of 20% (w/v) NaCl solution (2.5 ml). The suspension was gently agitated for 30 min at 55°C whence solid Na_2CO_3 (250 mg) was added to a final concentration of 1% (w/v). Dichlorotriazinyl dyes (Procion MX series) and monochlorotriazinyl dyes (Procion H series and C.I. Reactive Blue 2 analogues) were incubated for 3 h and 10–50 h respectively in a rotary incubator at 55°C. The dyed gels were thoroughly washed with distilled water with the dichlorotriazinyl dyed gels further tumbled for 2–3 h with 1 *M* NH_4Cl (pH 8.7) in order to aminate unreacted chlorines. All dyed gels were finally washed with 1 *M* NaCl–25% (v/v) ethanol (100 ml), distilled water (200 ml) and equilibration buffer (200 ml). The dyed Sepharose adsorbents were stored as moist gels in distilled water containing 0.01% (w/v) thimerosal at 4°C.

Determination of immobilised ligand concentration

The immobilised dye concentrations were determined by hydrolysis of moist gel (0.2 g) in a known volume of 50% (v/v) glacial acetic acid at 95–100°C for 30 min. The absorbance of the resulting solution was measured at the λ_{max} of each dye tested and the concentration calculated as μmol dye/g moist weight gel. All dyes were immobilised at a ligand concentration of 1–3 μmol dye/g moist weight Sepharose.

Preparation of crude enzyme solutions

All crude phosphatase-containing solutions (*ca.* 6000 U, 5 g solid, 25 ml) were dialysed overnight at 4°C against equilibration buffer (2.5 l) before use.

Screening procedure for immobilised dye adsorbents for alkaline phosphatase and protein binding

Enzyme and protein binding was assessed using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing crude enzyme preparation (25 U alkaline phosphatase) in 20 m*M* HEPES–NaOH buffer pH 7.5 (2 ml). After thorough mixing, the tubes were incubated for 30 min at 25°C whence after gravity sedimentation of the gels, the supernatants were assayed for protein and enzyme activity.

The gel phase was washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatant fraction assayed again for protein and alkaline phosphatase activity. Unmodified Sepharose CL-6B-200 was used as a control.

Screening of the C.I. Reactive Blue 2 analogues for their ability to bind alkaline phosphatase and bovine serum albumin was performed as the above using "Good" buffers (10 m*M*) at their respective pK_a values in the pH range 6.1–10.4.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli[15] using a Bio-Rad Mini-Protean II dual slab cell. Gels were stained for 30 min with 0.1% (w/v) Coomassie Blue R-250 in methanol–glacial acetic acid–water (40:10:50, v/v/v) then destained for 1 h in the same solvent.

RESULTS

Preliminary screen for alkaline phosphatase binding to immobilised triazine dyes

The results of screening a number of commercially available Procion H and MX dyes immobilised to agarose for their ability to bind and purify calf intestinal alkaline phosphatase are summarised in Table I. The screen was designed to encompass the principal categories of dye chromophore (anthraquinone, azo, phthalocyanine and metal complex) exploited in reactive dyes[5] to determine if any one dye category favoured binding of the enzyme. In agreement with the general finding of our other workers[6], all of the immobilised dye adsorbents bound the enzyme from a crude calf intestinal mucosa preparation at pH 7.5. However, in this study the dyes were immobilised at comparable ligand concentrations (1–3 μmol dye/g moist weight gel) thus allowing more precise comparisons of enzyme binding capacities on a U/μmol dye basis. Immobilisation of dyes at similar substitution levels is often overlooked in such studies but is important since high ligand concentrations may increase non-specific interactions[5]. Highest capacities for enzyme binding were displayed by those dyes bearing a significant number of anionic groups, such as immobilised Procion Green H-4G, Procion Green HE-4BD, Procion Turquoise H-A and Procion Blue MX-4GD. However, the most effective for enzyme purification was the azo dye, Procion Red P-3BN as it consistently achieved the highest purification factor, was easily coupled to the matrix and, unlike the large phthalocyanine dyes, was not prone to ligand leakage from the matrix. Interestingly, this dye was unique amongst those tested in possessing both amino phenyl sulphonic and amino benzoic acid groups. Subsequent studies using the Procion Red P-3BN adsorbent in conjunction with 20 m*M* orthophosphate as specific eluent resulted in an alkaline phosphatase preparation of specific activity

TABLE I

PRELIMINARY SCREEN FOR CALF INTESTINE ALKALINE PHOSPHATASE BINDING TO COMMERCIAL TRIAZINE DYES

Dialysed crude alkaline phosphatase preparation (25 U in 2 ml of 20 m*M* HEPES–NaOH, pH 7.5) was added to a small test tube containing immobilised dye adsorbent (0.5 g moist weight gel). After thorough mixing the tubes were incubated for 30 min at 25°C whence after gravity sedimentation of the gels, the supernatants were assayed for protein and enzyme activity. After removal of the supernatant, the gel phase was washed with equilibration buffer (2 × 4 ml), bound protein desorbed by the addition of 0.75 *M* KCl (2 ml) and the supernatant fraction reassayed for protein and enzyme activity. Unmodified Sepharose was used as a control, protein and enzyme recoveries were > 90% and the experiment was performed in duplicate. The specific activity of the crude preparation was 3 U/mg protein.

Immobilised dye (Procion)	*Immobilised ligand concentration (μmol/g moist gel)*	*Binding capacity (U/μmole dye)*	*Specific activity (U/mg protein)*
Control	0.0	–	–
Reactive Blue 2	2.2	6.2	22.6
Blue MX-4GD	2.6	9.8	39.4
Blue HE-RD	2.6	9.6	25.9
Red H8-BN	2.3	5.2	25.6
Red H-3B	2.0	0.7	15.2
Red HE-3B	2.7	6.8	29.4
Red P-3BN	3.0	8.3	51.3
Red HE-7B	3.0	9.7	33.2
Yellow MX-R	3.0	0.6	8.5
Yellow H-A	1.8	1.0	8.2
Green HE-4BD	1.2	23.3	33.2
Green H-4G	1.0	42.3	33.8
Brown H-2G	3.0	3.4	29.7
Brown MX-5BR	2.5	2.0	27.9
Turquoise H-A	1.0	10.8	24.0
Orange MX-G	1.2	0.9	6.2
Scarlet MX-G	2.7	1.4	14.6

180 U/mg and representing a 60-fold purification. Since commercial "high-purity" alkaline phosphatase preparations have specific activities in the range 850–1250 U/mg under these conditions, the preparation reported here was only partially purified and comparable to that obtained by other workers using other commercial dyes for alkaline phosphatase purification[6].

Design, immobilisation and application of specific dyes targeted at alkaline phosphatase

The relative lack of specificity of commercial dyes in their interaction with alkaline phosphatase and the apparent relationship with the number of anionic groups substituted on the dye chromophore suggest that these groups may act as phosphate analogues. Thus, since carboxylate[16], phosphonate[3] and borate[16] are known to be potent competitive inhibitors of alkaline phosphatase and the enzyme generally favours aromatic rather than aliphatic phosphate esters, substitution of these groups onto aromatic dye chromophores would be expected to yield more effective adsorbents. Fig. 1 shows the structure of a series of terminal ring analogues of the anthraquinone dye, C.I. Reactive Blue 2, which were synthesised, immobilised and

TABLE II

PURIFICATION OF CALF INTESTINAL ALKALINE PHOSPHATASE ON IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

Enzyme and protein binding was assessed over the pH range 6.1–10.4 using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing crude enzyme (25 U alkaline phosphatase) in 20 m*M* "Goods" buffer (2 ml). After thorough mixing, the tubes were incubated for 30 min at 25°C, the gels allowed to sediment under gravity and the supernatants assayed for protein and enzyme activity. The gels were washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatants assayed for protein and enzyme activity. Total protein and enzyme recoveries were >90% and the experiment was performed in duplicate.

pH	*Terminal ring analogue*[a]				
	I	*II*	*III*	*IV*	*V*
6.1	5.6[b] (14)[c]	6.1 (19)	6.2 (17)	6.2 (17)	2.1 (11)
7.2	2.9 (30)	5.8 (72)	4.5 (52)	5.9 (83)	1.6 (36)
8.1	1.0 (20)	4.2 (105)	2.4 (62)	4.2 (124)	1.1 (44)
9.3	0.09 (4)	0.13 (5)	0.14 (19)	3.4 (200)	0.3 (33)
10.4	0	0	0	0	0

[a] See Fig. 1 for structures.
[b] Alkaline phosphate bound (U/μmol dye).
[c] Specific activity of eluted enzyme (U/mg protein).

tested for their ability to bind alkaline phosphatase from a crude calf intestinal mucosa extract over the pH range 6.1–10.4 (Table II). These analogues bear terminal rings comprising aniline (I), *p*-aminobenzoic acid (II), *m*-, *p*-aminobenzenesulphonic acid (C.I. Reactive Blue 2) (III), *p*-aminobenzyl phosphonic acid (IV) and *m*-aminobenzeneboronic acid (V). In each case, the capacity of the adsorbents (U enzyme bound/μmol immobilised dye) decreased with increasing pH, whilst the specific activity of the protein eluted with 0.75 *M* KCl increased with increasing pH up to a critical value after which the binding ability sharply declined. This critical value was about a pH unit higher for the phosphonate ligand. As anticipated, the phosphonate ligand was the most effective in yielding enzyme of high specific activity, with the carboxylate, sulphonate, borate and aniline derivatives decreasingly effective. Identification of the phosphonate analogue as an effective affinity ligand was probably attributable to the fact that the methyl phosphonate moiety has approximately the same overall dimensions and charge as the phosphate ester[17]. The excellent performance of the carboxylic acid analogue as an alkaline phosphatase ligand may account for dye Procion Red P-3BN being the most effective of the commercial dyes.

The terminal ring analogues of C.I. Reactive Blue 2 were also screened for their ability to bind bovine serum albumin, a major contaminant of the crude calf intestinal preparation (Table III). Bovine serum albumin bound most effectively to the aniline terminal ring analogue (I) and the monovalent sulphonate (III) and carboxylate (II) species. In sharp contrast to alkaline phosphatase binding, the phosphonate analogue (IV) displayed the lowest affinity for bovine serum albumin, thus, making it an ideal candidate ligand for the purification of calf intestinal alkaline phosphatase.

For these enzyme and protein binding studies, the Good buffers were used at their respective pK_a values in order to maintain a constant ionic environment over the pH range investigated as the buffers would be equally dissociated.

TABLE III

THE BINDING OF BOVINE SERUM ALBUMIN TO IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

Bovine serum albumin binding was assessed over the pH range 6.1–10.4 using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing protein solution (2 mg bovine serum albumin in 2 ml of 20 m*M* Goods buffer). After thorough mixing the tubes were incubated for 30 min at 25°C, the gels allowed to sediment under gravity and the supernatants assayed for protein. The gels were washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatants reassayed for protein. The experiment was performed in duplicate and protein recoveries were in the range 95–110%.

pH	*Terminal ring analogue*[a]				
	I	*II*	*III*	*IV*	*V*
6.1	1.9[b]	1.2	1.2	0.8	1.1
7.2	1.4	1.0	1.1	0.7	0.9
7.5	1.2	0.8	1.0	0.6	0.7
8.1	0.5	0.3	0.4	0.2	0.3
9.3	0	0	0	0	0
10.4	0	0	0	0	0

[a] See Fig. 1 for structures.
[b] Bovine serum albumin bound (mg BSA/μmol dye).

Determination of the capacity of the immobilised phosphonate analogue (IV) of C.I. Reactive Blue 2 for alkaline phosphatase

The interaction between alkaline phosphatase and the agarose-immobilised phosphonate analogue (IV) was strongly dependent on buffer concentration. For example, at a fixed pH value of 8.5 using Tricine–NaOH buffer, 85% of the total activity (140 U; 4 ml) bound when applied to a small column (1 g moist weight gel) of immobilised phosphonate ligand in 10 m*M* buffer, 70% bound in 40 m*M* buffer and only 35% bound when 100 m*M* buffer was used. A pH of 8.5 was chosen as optimum since at this value the phosphonate ligand is very selective for alkaline phosphatase whilst retaining a high binding capacity for the enzyme (Table I). Frontal analysis (Fig. 2) showed binding to be a complex process[18]; in the early phase, the majority of the total protein, but only 50% of the total enzyme activity is adsorbed. In the latter phase, a small amount of additional protein binds, representing the remaining enzyme activity. Alkaline phosphatase represents only 0.3% of the total protein in the crude preparation and the first phase is thought to reflect rapid saturation of potential binding sites on the adsorbent with alkaline phosphatase and contaminating proteins. The second phase may represent a slower time-dependent displacement by alkaline phosphatase of proteins possessing a lower affinity for the immobilised dye.

The volume of crude enzyme preparation necessary to achieve adsorbent saturation was highly dependent on column flow-rate. For example, at a flow-rate of 10 ml/h the volume required for saturation with alkaline phosphatase activity was 18 ml while at 20 ml/h the volume was almost doubled to 35 ml. This finding suggests that a competitive displacement effect may account for the complex binding process discussed above.

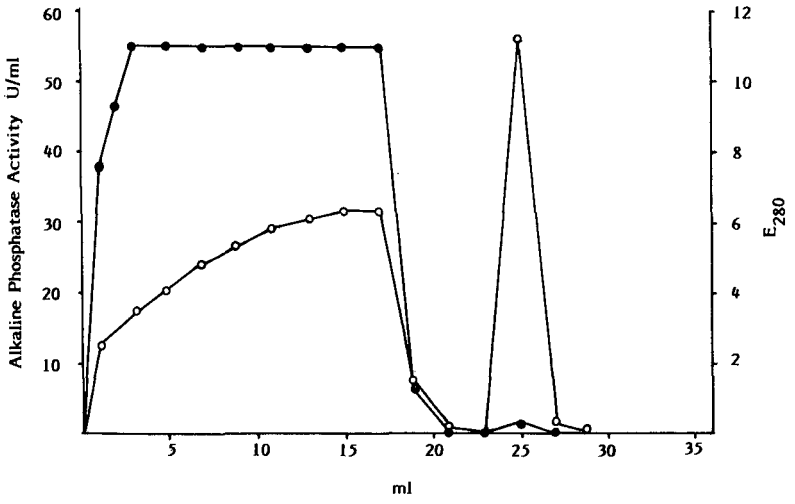


Fig. 2. Determination of maximum binding capacity of the terminal ring phosphonate analogue of C.I. Reactive Blue 2 (IV) when bound to Sepharose CL-6B-200 for alkaline phosphatase by frontal analysis. A crude calf intestinal alkaline phosphatase preparation (30 U/ml in 10 m*M* Tricine–NaOH buffer, pH 8.5) was applied to a glass column (10 cm × 0.5 cm I.D.) packed with phosphonate gel (0.1 g moist weight; 2.6 μmol dye/g moist weight gel) equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 at 4°C at a flow-rate of 10 ml/h. Application was continued until outlet and inlet streams contained identical concentrations of enzyme and protein. The column was washed with the irrigating buffer and bound protein eluted with 0.75 *M* KCl. Fractions (1 ml) were assayed for alkaline phosphatase activity (○) and protein (E_{280}, ●).

A similar frontal chromatogram was observed for adsorption of alkaline phosphatase to immobilised Procion Red P-3BN, although at comparable dye substitution levels (2.6 μmol dye/g moist weight gel) the binding capacity for the immobilised Procion Red P-3BN (751 U/g moist weight gel; 289 U/μmol dye) was markedly lower than that for the immobilised phosphonate analogue (1147 U/g moist weight gel; 441 U/μmol dye). Only 5% of the bound protein appeared to be alkaline phosphatase in the case of immobilised Procion Red P-3BN, compared to 22% in the case of the phosphonate analogue; consequently, the specific activity of the enzyme eluted with 0.75 *M* KCl was 55 U/mg and 195 U/mg respectively. This demonstrates the increased selectivity of the phosphonate ligand over the commercial dyes. SDS-PAGE showed that the contaminating proteins included bovine serum albumin and phosphodiesterase.

Specific elution techniques

Desorption of alkaline phosphatase from the immobilised phosphonate analogue with steps (0.75 *M*) or linear (0–1 *M*) gradients of salt (KCl) resulted in co-elution of contaminating proteins with the enzyme activity. Table IV shows that the use of specific eluents such as phenyl phosphate, α-naphthyl phosphate and inorganic phosphate greatly improved the specific activity of the eluted protein. Specific elution with organic phosphates resulted in sharper elution of alkaline phosphatase although the degree of purification was consistently lower than that obtained with inorganic phosphate. Furthermore, SDS-PAGE showed that preparations eluted with organic

TABLE IV

SPECIFIC ELUTION OF ALKALINE PHOSPHATASE FROM IMMOBILISED PHOSPHONATE ANALOGUE OF C.I. REACTIVE BLUE 2

Total protein and enzyme recoveries were >90%.

Eluent	*Specific activity of eluted enzyme (U/mg protein)*	*Purification (fold)*[a]
0.75 *M* KCl	195	65
5 m*M* α-naphthyl phosphate	487	162
5 m*M* phenylphosphate	520	173
5 m*M* KH_2PO_4	716	238

[a] Crude alkaline phosphatase has a specific activity of 3.0 U/mg protein.

phosphates were highly contaminated with phosphodiesterase, whilst preparations eluted with inorganic phosphate were essentially free of this contaminant. Triazine dyes are competitive inhibitors of bovine phosphodiesterase and have been exploited for its purification[19,20]. Thus, binding of this protein to the phosphonate ligand is to be expected.

Purification of alkaline phosphatases from other sources

To determine the effectiveness of the C.I. Reactive Blue 2 phosphonate derivative as a general alkaline phosphatase affinity ligand, purification from a wide variety of sources was attempted. Table V shows that the ligand is effective for both calf and bovine intestinal alkaline phosphatase even though SDS-PAGE revealed the

TABLE V

PURIFICATION OF ALKALINE PHOSPHATASES FROM A WIDE VARIETY OF SOURCES USING THE C.I. REACTIVE BLUE 2 PHOSPHONATE ANALOGUE AS THE AFFINITY LIGAND

Dialysed crude alkaline phosphatase preparation (1200 U in 4 ml 10 m*M* Tricine–NaOH buffer, pH 8.5) was applied to a small column containing 1.2 g of pre-equilibrated immobilised dye gel (2.6 μmol dye/g gel). The column was washed with 45 ml of buffer, then a 3-ml pulse of 5 m*M* KH_2PO_4 in 10 m*M* Tricine–NaOH buffer, pH 8.5 was applied. Finally any remaining protein was eluted using a 5-ml wash of 1 *M* NaCl.

Alkaline phosphatase source and type	*Specific activity of crude preparation (U/mg protein)*	*Applied alkaline phosphatase bound (%)*	*Total enzyme recovery (%)*	*Specific activity of final preparation (U/mg protein)*
Calf intestine Type I	3.0	76	103	940[a]
Bovine intestine Type I-S	8.8	44	98	850[a]
Chicken intestine Type V	8.7	6	93	350[a]
Pig intestine Type IV	1.0	18	90	107[b]
Sheep intestine Type XII	1.6	17	96	183[b]
Human placental Type XVII	8.6	1	82	40[b]
E. coli Type III-S	4.0	2	71	35[b]

[a] Bound alkaline phosphatase eluted using a 5 m*M* inorganic phosphate pulse.

[b] Bound alkaline phosphatase not eluted using inorganic phosphate pulse but would elute using 1 *M* NaCl.

contaminating proteins present in each crude preparation to be quite different. However, in all other cases both the binding ability of the alkaline phosphatase and the purification factor achieved were disappointingly low. In the case of the pig, sheep, human and *Escherichia coli* isoenzymes the alkaline phosphatase which bound was not eluted using a 5 m*M* inorganic phosphate pulse but was eluted using 1 *M* NaCl suggesting non-specific binding. The lower recoveries of the *E. coli* and human placental enzymes may be attributable to hydrophobic binding.

Affinity chromatography of alkaline phosphatase

A crude alkaline phosphatase preparation (2 g lyophilised powder dissolved in 10 ml of 10 m*M* Tricine–NaOH buffer, pH 8.5; approx. 2000 U) was applied at a flow-rate of 10 ml/h to a column (5 g moist weight gel) of agarose-immobilised C.I. Reactive Blue 2 phosphonate analogue (IV, 2.6 μmol dye/g moist weight gel). Approximately 80% of the applied activity bound to the adsorbent, with loosely bound protein being desorbed by consecutive washes with 60 m*M* Tricine–NaOH buffer pH 8.5 and 10 m*M* Tricine–NaOH buffer pH 8.5 respectively until the background absorbance at 280 nm was negligible. Elution with a linear gradient (0–5 m*M*) of inorganic phosphate (KH_2PO_4) resulted in a broad peak of alkaline phosphatase activity comprising 72% of the bound activity with an overall specific activity of 760 U/mg and representing a 250-fold purification. Subsequent application

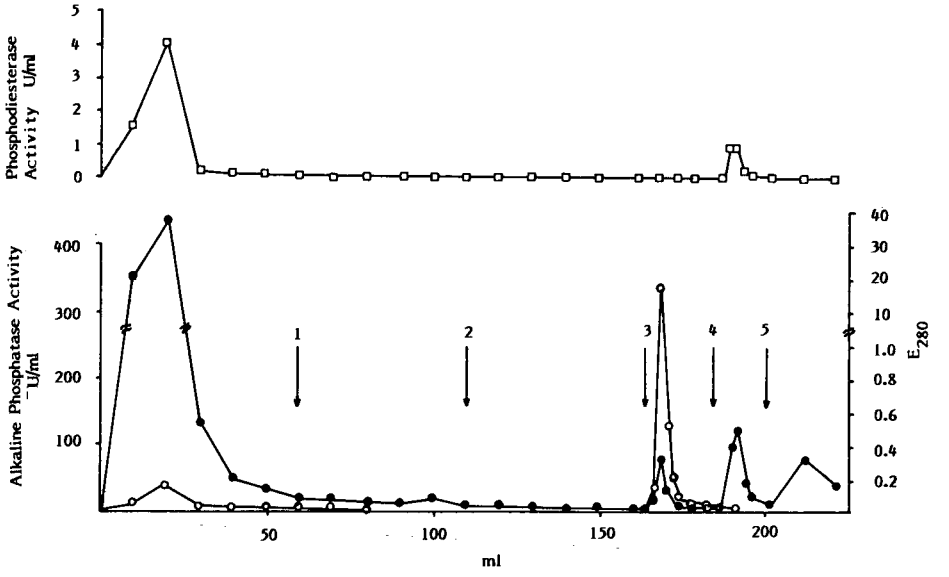


Fig. 3. Chromatography of a crude calf intestinal extract on Sepharose CL-6B-200 immobilised C.I. Reactive Blue 2 phosphonate analogue. A crude alkaline phosphatase preparation [2 g lyophilised powder/10 m*M* Tricine–NaOH buffer pH 8.5 (10 ml); approx. 2000 U] was applied at a flow-rate of 10 ml/h to a column (30 cm × 1 cm I.D.; 5 g moist weight gel) of Sepharose CL-6B-200 immobilised phosphonate analogue (2.6 μmol/g moist weight gel) equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 at 4°C. The column was washed at the points indicated as follows: (1) 60 m*M* Tricine–NaOH buffer pH 8.5; (2) 10 m*M* Tricine–NaOH buffer pH 8.5; (3) 5 m*M* KH_2PO_4 (20 ml); (4) 20 m*M* KH_2PO_4 (15 ml); (5) 0.75 *M* KCl. Fractions (2 ml) were analysed for alkaline phosphatase activity (○), phosphodiesterase activity (□) and protein (E_{280}, ●).

of a pulse (15 ml) of inorganic phosphate (20 m*M*) released the adsorbed phosphodiesterase. Finally, the column was flushed with 0.75 *M* KCl to desorb all remaining protein and then re-equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 prior to reuse.

Fig. 3 shows that repetition of the chromatographic run with a pulse (20 ml) of inorganic phosphate (5 m*M*) in place of the gradient elution, resulted in the appearance of alkaline phosphatase activity in a sharp peak containing 89% of the bound enzyme with an overall specific activity of 870 U/mg (290-fold purification). Over 60% of the eluted activity was contained in a single fraction (2 ml) with a specific activity in excess of 1000 U/mg and representing a 330-fold purification of the enzyme from the crude preparation in a single step. SDS-PAGE (Fig. 4) and determination of the specific activity revealed that alkaline phosphatase purified using affinity chromatography on the immobilised phosphonate analogue of C.I. Reactive Blue 2 was of equivalent purity to commercially available "high-purity" preparations which had specific activities of 850–1250 U/mg under the assay conditions used in this work. In addition, Fig. 3 shows that specific elution using inorganic phosphate (5 m*M*) separated the alkaline phosphatase activity from the phosphodiesterase activity, which was eluted using higher inorganic phosphate concentrations (20 m*M*). The final alkaline phosphatase preparation contained <0.05 U phosphodiesterase/mg protein. The

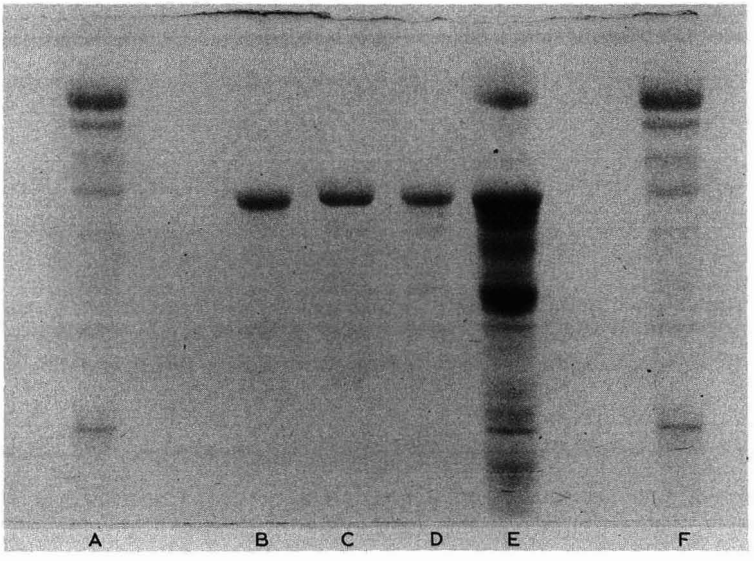


Fig. 4. Comparison of the C.I. Reactive Blue 2 phosphonate purified enzyme with commercial "high purity" preparations by SDS-PAGE. For the comparison equivalent amounts of dye-purified and commercial enzyme were applied to the gel. Tracks A and F, bovine phosphodiesterase I standard; tracks B and C, commercial "high-purity" alkaline phosphatase preparations; track D alkaline phosphatase purified by affinity chromatography on the immobilised phosphonate adsorbent with a specific eluent of inorganic phosphate (5 m*M*; Fig. 3); track E, profile of the total protein binding to the immobilised phosphonate adsorbent from the crude calf intestinal alkaline phosphatase preparation obtained by elution with 0.75 *M* KCl. Electrophoresis of crude calf intestine alkaline phosphatase preparation is not shown since it results in a single, heavily stained band corresponding to bovine serum albumin which accounts for *ca.* 30% of the total protein in the crude extract.

adsorbent could be re-used many times with no apparent reduction in capacity or purification performance being observed over a 12-month period of regular usage. It should also be noted that the alkaline phosphatase loadings used in this process (1000 U/5 ml) are far higher than those reported in other studies. This factor, together with the stability and ease of synthesis and immobilisation makes this process particularly suitable for large scale commercial application.

DISCUSSION

Immobilised triazine dyes have been successfully exploited for the purification of a host of complementary proteins[5], although only in a few cases is the dye–protein interaction well understood[21,22]. The present study indicates that the affinity of calf intestinal alkaline phosphatase for terminal ring analogues of C.I. Reactive Blue 2 (Fig. 1) is governed largely by the anionic substituent on the terminal ring. Substitution of anionic functions such as carboxylate and phosphonate for the ubiquitous sulphonate in commercially available dyes, dramatically enhances the specificity of the dye–protein interaction, presumably because both are competitive inhibitors and bind to the active site of the enzyme[3,16]. It is probable that under the binding conditions used in this study the ligand interacts directly at the active site since the enzyme is specifically eluted with low concentrations of substrates and inhibitors. However, the lack of detailed knowledge of the calf intestinal alkaline phosphatase active site, such as X-ray crystallography data, makes absolute conclusions regarding the dye–protein interaction very difficult. In addition, further rational design of the ligand in order to maximise presentation of the phosphonate or to determine the contribution of the other components of the dye moieties to the dye-protein interaction will be dependent on the availability of such data.

Binding of alkaline phosphatase to the immobilised C.I. Reactive Blue 2 phosphonate ligand appears to be highly ionic in nature since the interaction is greatly affected by pH and ionic strength, the enzyme is easily desorbed using low salt concentrations and is not eluted using 50% (v/v) glycerol. In addition, the uncharged aniline derivative shows very low affinity for the enzyme. Kirchberger *et al.*[23] also found that the binding of calf intestinal alkaline phosphatase to Procion Red HE-3B was mainly stabilised by electrostatic and not hydrophobic interactions.

Phosphonate[3] and arsonate[1] derivatives have been used previously for the purification of alkaline phosphatase. In both cases, presentation of the anionic competitive inhibitors attached to a bulky aromatic/hydrophobic moiety proved the most effective ligands. Similar conclusions have been drawn for the purification of acid phosphatase[24]. Phosphonate derivatives proved superior to arsonate presumably because benzyl phosphonate is iso-spatial to the very high affinity substrate, phenyl phosphate[17].

The use of the Sepharose-immobilised phosphonate analogue of C.I. Reactive Blue 2 in conjunction with specific elution with inorganic phosphate resulted in a calf intestinal alkaline phosphatase preparation purified 330-fold in a single step from a crude extract. Absolute comparisons with results reported by other workers are difficult since quoted specific activities depend on the enzyme assay used and the method of protein determination. For example, use of a phenyl arsonate adsorbent and specific elution with inorganic phosphate probably achieved a homogeneous

preparation of calf intestinal alkaline phosphatase[1]. However, the starting material was a partially purified extract and phenyl arsonates are more labile than phosphonates[2]. Other workers have exploited an immobilised phosphonate derivative to effect an 11-fold purification of calf intestinal alkaline phosphatase[3] and a 400-fold purification of human liver alkaline phosphatase[4] respectively. Commercial dye adsorbents have been exploited by a number of workers for alkaline phosphatase purification. Bouriotis and Dean[6] obtained a 295-fold purification of calf intestinal alkaline phosphatase using α-naphthyl phosphate as specific eluent. In this case, hydrolysis of the organic phosphate eluent led to contamination of the resulting enzyme preparation with α-naphthol which was removed by dialysis. Also, the use of sequential affinity chromatography on immobilised Cibacron Blue F3G-A and Procion Red HE-3B as components in a multi-step purification procedure resulted in apparent homogeneity of calf intestinal alkaline phosphatase[7] and a single step 380-fold purification of human intestinal alkaline phosphatase was achieved using affinity chromatography on immobilised Remazol Yellow GGL[8].

Although these affinity purification techniques are extremely effective in some cases, they cannot be considered generally applicable to alkaline phosphatase purification since this and other studies has shown that alkaline phosphatase isoenzymes differ markedly in their affinity for such ligands[25]. For example, the human placental enzyme shows very low affinity for the C.I. Reactive Blue 2 phosphonate ligand and other immobilised triazine dyes. However, Yasmin and Qadri[10] did obtain a 46-fold purification by precipitating the enzyme onto a column of Cibacron Blue 3G-A-Sepharose 4B using a 50% (w/v) saturated ammonium sulphate solution. Elution was then achieved by lowering the salt concentration. Similarly, Smith and Peters[9] achieved a 244-fold purification of the human polymorphonuclear leukocyte enzyme using solubilisation with Triton X-100 and chromatography on immobilised Cibacron Red F. The bound enzyme was not eluted using high salt concentrations or specific eluents. In these two examples the exact nature of the dye–protein interaction is unknown but is likely to involve mainly hydrophobic rather than ionic interactions. Hydrophobic binding by the human placental and the *E. coli* alkaline phosphatases may account for their low recoveries when screened for their ability to bind the Reactive Blue 2 phosphonate ligand. The inability of isoenzymes, such as the human placental, to bind to triazine dyes under low salt conditions may be due to their high sialic acid content which would increase their overall negative charge and possibly prevent binding to anionic ligands[26].

CONCLUSIONS

The phosphonate terminal ring analogue of C.I. Reactive Blue 2 represents only the second example of the use of specifically designed biomimetic dyes for protein purification[11]. Frontal analysis experiments showed the phosphonate dye adsorbent to be more selective for alkaline phosphatase binding and have a higher binding capacity than the most effective of the commercial dyes tested. Its use in conjunction with specific elution with inorganic phosphate resulted in a single step 330-fold purification of the calf intestinal enzyme from a crude preparation. The dye purified enzyme was of equivalent purity to commercial high purity preparations by SDS-PAGE and specific activity comparisons and contained <0.05 U phospho-

diesterase/mg protein. It is believed that this is the first study in which affinity purified calf intestinal enzyme has been compared directly with commercial "high-purity" preparations.

The phosphonate ligand retains the advantages of commercial dyes in terms of cost, stability, reusability, sterilizability and ease of immobilisation whilst displaying greatly improved specificity for the target protein. It is anticipated that as more information concerning the detailed architecture of enzyme active sites and the nature of enzyme ligand interactions becomes available, the flexibility of dye design and synthesis, particularly using computer aided molecular graphics will accelerate the development of this new approach to affinity chromatography.

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REFERENCES

1 O. Brenna, M. Perrella, M. Pace and P. G. Pietta, *Biochem. J.*, 151 (1975) 291–296.
2 J. M. Trepanier, L. E. Seargeant and R. A. Stinson, *Biochem. J.*, 155 (1976) 653–660.
3 M. Landt, S. C. Boltz and L. G. Butler, *Biochemistry*, 17 (1978) 915–919.
4 L. E. Seargeant and R. A. Stinson, *J. Chromatogr.*, 173 (1979) 101–108.
5 C. R. Lowe, in A. Wiseman (Editor), *Topics in Fermentation Biotechnology, Applications of Reactive Dyes in Biotechnology*, Vol 9, Ellis Horwood, Chichester, 1983, pp. 78–161.
6 V. Bouriotis and P. D. G. Dean, *J. Chromatogr.*, 206 (1981) 521–530.
7 J. Kirchberger and G. Kopperschlager, *Prep. Biochem.*, 12 (1982) 29–47.
8 D. G. Williams, P. G. Byefield and D. W. Moss, *Enzyme*, 28 (1987) 28–32.
9 G. P. Smith and T. J. Peters, *Int. J. Biochem.*, 17 (1985) 209–215.
10 T. Yasmin and F. Qadri, *J. Chromatogr.*, 315 (1984) 425–429.
11 C. R. Lowe, S. J. Burton, J. C. Pearson, Y. D. Clonis and C. V. Stead, *J. Chromatogr.*, 376 (1986) 121–130.
12 C. V. Stead, *J. Chem. Technol. Biotechnol.*, 37 (1987) 55–71.
13 S. J. Burton, S. B. McLoughlin, C. V. Stead and C. R. Lowe, *J. Chromatogr.*, 435 (1988) 127–137.
14 S. J. Kelly, F. Feldman, J. W. Speriow and L. G. Butler, *Biochemistry*, 12 (1973) 3338–3341.
15 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
16 C. A. Zittle and E. S. Della Monica, *Arch. Biochem.*, 26 (1950) 112–122.
17 R. Engel, *Chem. Rev.*, 77 (1977) 349–367.
18 H. A. Chase, *J. Chromatogr.*, 197 (1984) 179–202.
19 M. E. Morrill, S. T. Thompson and E. Stellwagen, *J. Biol. Chem.*, 254 (1979) 4371–4374.
20 A. R. Ashton and G. M. Polya, *Biochem. J.*, 175 (1978) 501–506.
21 D. A. P. Small, C. R. Lowe, A. Atkinson and C. J. Bruton, *Eur. J. Biochem.*, 128 (1982) 119–123.
22 P. Hughes, R. F. Sherwood and C. R. Lowe, *Eur. J. Biochem.*, 144 (1984) 135–142.
23 J. Kirchberger, H. Seidel and G. Kopperschlager, *Biomed. Biochem. Acta*, 46 (1987) 653–663.
24 J. Dissing, O. Dahl and O. Svensmark, *Biochim. Biophys. Acta*, 569 (1979) 159–176.
25 H. Yamamato, M. Tanaka, T. Okochi and S. Kishimoto, *Biochem. Biophys. Res. Commun.*, 111 (1983) 36–40.
26 R. B. McComb, G. N. Bowers and S. Posen, *Alkaline Phosphatase*, Plenum Press, New York, London, 1979.

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OPTICAL RESOLUTION OF AMINO ACIDS, PEPTIDES AND HYDROXYCARBOXYLIC ACIDS USING A NEW CHIRAL COLUMN FOR LIGAND-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The enantiometric resolution of amino acids, amino acid derivatives, peptides and hydroxycarboxylic acids was investigated using a chiral column of the ligand-exchange type. It was found that this column separated all of these compounds satisfactorily. Columns containing either a D- or L-ligand gave almost identical chromatograms, except that the retention order for a pair of chiral isomers was reversed. These columns could be applied to resolve simultaneously ten different types of DL-amino acids. Optically active impurities present in D and L-amino acids could be determined at concentrations down to about 100 ppm.

INTRODUCTION

In recent years, increasing attention has been paid to the development of easier ways of resolving enantiomers, especially using high-performance liquid chromatographic (HPLC) methods. Some of the more important techniques are the use of a stationary phase carrying an optically active compound[1–5], the preparation of a derivative of a diastereomer before resolution[6–8] and the use of a mobile phase containing an optically active compound[9–12].

Each of these approaches has problems, but recently Shinbo *et al.*[5] developed a high-performance resolution column for DL-amino acids. In this column, a crown compound is adsorbed on an octadecylsilyl (ODS)-silica. Although it is silica-based, this column must depend on a strongly acidic eluent, such as perchloric acid. Also, it is ineffective for many DL-amino acids unless it is operated at low temperatures, such as 0°C. In addition, it is unsuitable for simultaneous resolution, because of the similar retention times of these amino acids. Nimura and co-workers[6–8] attempted simultaneous resolution using an ODS column. This approach follows a pre-column method, in which a compound such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) is used. It is suitable for simultaneous resolution, but has several disadvantages: some amino acids do not react to form derivatives, and it requires a time-consuming pretreatment process. Methods in which an optically active compound is

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used are unsuitable for routine analyses and preparations, because the compound is expensive for these purposes.

Using an MCI GEL CRS10W column (N,N-dioctyl-L-alanine-coated, ODS-silica), effective enantiometric resolution of DL-amino acids was obtained under mild operating conditions, and this column does not suffer from the above problems. One of its noteworthy features is that it eliminates pretreatment steps. In this study, we prepared a CRS10WD column, which was the same as the CRS10W column except that the ligand was N,N-dioctyl-D-alanine. We assessed its performance in detail. Optical impurities present in L- and D-amino acids were determined using both the CRS10W and CRS10WD columns. The enantiometric resolution of compounds other than amino acids, such as peptides, amino acid derivatives and hydroxycarboxylic acids, was also investigated. It was shown that the new column can resolve enantiomeric compounds other than amino acids.

EXPERIMENTAL

DL-Amino acids, D-amino acids, L-amino acids, amino acid derivatives, peptides and hydroxycarboxylic acids were supplied by Sigma (St. Louis, MO, U.S.A.) and Tokyo Kasei (Tokyo, Japan). All the other reagents were of special grade, supplied by Wako (Osaka, Japan).

The packed columns used were MCI GEL CRS10W and CRS10WD (a provisional name), both 50 mm × 4.6 mm I.D., supplied by Mitsubishi Kasei (Tokyo, Japan). CRS10WD is a newly developed column, differing from the former in its optical characteristics. The active ligand, N,N-dioctyl-D-alanine, is coated on ODS-silica (pore diameter 100 Å, particle size 3 μm).

The apparatus consists of a pump (Model LC-6A; Shimadzu, Kyoto, Japan), a sample injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a detector (Model SPD-6A; Shimadzu). Fluorimetric trace analysis was based on the post-column method using *o*-phthalaldehyde (OPA). A Model RF530 instrument (by Shimadzu) was used to detect fluorescence.

RESULTS AND DISCUSSION

Basic properties

The enantiometric resolution of DL-amino acids on MCI GEL CRS10W is based on the ligand-exchange reactions of a copper complex of an optically active ligand adsorbed on the column matrix with the amino acid to be separated. The resolution, therefore, is affected by various parameters, including eluent pH, copper (II) ion concentration and column temperature. The effects of these parameters have been described elsewhere[13]. Different copper (II) salts were screened as eluents with respect to the effects of the various anions on the resolution of amino acids. Fig. 1 shows the relationship among the anion species, retention time and resolution. Different amino acids were affected differently according to the anion species used. The retention time changed markedly with acidic amino acids, such as glutamic acid, whereas it changed little with neutral amino acids, such as valine. The resolution was also greatly affected by the anion species, which indicates the possibility of controlling the resolution by varying the anion.

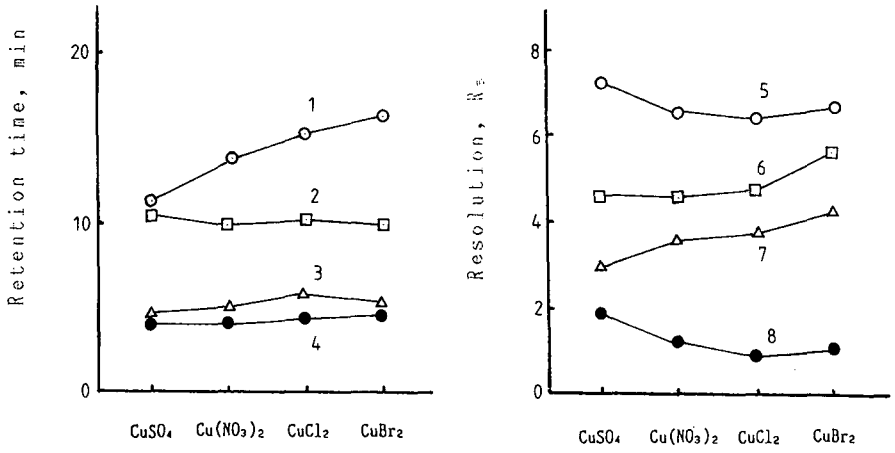

Fig. 1. Effect of salts on retention time and resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* aqueous Cu salt solution; flow-rate, 1 ml/min; pressure, 110 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. (1) L-Glu; (2) L-Leu; (3) L-Asp; (4) L-Val; (5) DL-Leu; (6) DL-Val; (7) DL-Glu; (8) DL-Asp.

Of the copper (II) salts screened, copper sulphate had the shortest retention time and was not deliquescent. Therefore, an aqueous solution of copper sulphate was used as the eluent in the measurements after the screening study. Fig. 2 shows the relationship between load and resolution for DL-valine, having a resolution, R_s, of about 6. Resolution was achieved, but without a considerable decrease in resolution, R_s, as long as the amount separated was 10 μg or less. For an amount of 0.1 mg separated, R_s was still fairly high (2.7). It is therefore possible to use the 50 mm × 4.6 mm I.D. column for preparative purposes.

MCI GEL CRS10W works effectively with an aqueous solution contained an organic solvent as the eluent. Acetonitrile and ethanol gave better chromatograms than methanol (Fig. 3). Fig. 4 shows the relationships among acetonitrile concentration, k' and R_s values for the resolution of DL-phenylalanine. An increase in acetonitrile concentration accelerated elution, sharpened the signals and reduced gradually

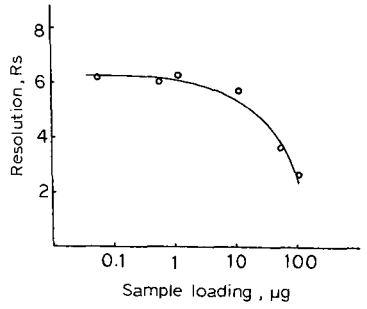

Fig. 2. Effect of sample throughput on resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 100 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, DL-valine.

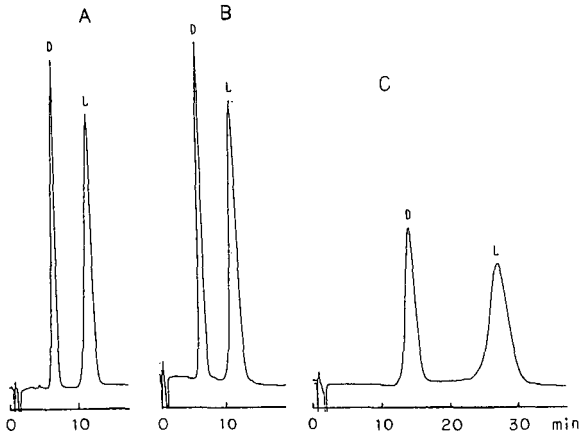

Fig. 3. Effect of addition of organic solvent to the eluent on glycyl-DL-leucine resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$ in (A) 10% acetonitrile solution, (B) 10% ethanol solution and (C) 10% methanol solution; flow-rate, 1 ml/min; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, glycyl-DL-leucine (1 mg/ml).

R_s. Similar results were observed with other compounds. Adding an organic solvent, such as acetonitrile, greatly shortened the retention time of hydrophobic compounds, as discussed above, but its concentration should be limited to 15 vol.-%, because the optically active ligand may be eluted out in the presence of excessive amounts of organic solvent.

Determination of impurities in enantiomers

The analysis of a trace component that elutes after the main component is difficult[14], and a column of reversed activity that reverses the elution order is used to solve this problem. For DL-amino acids with low R_s, it is possible to determine a

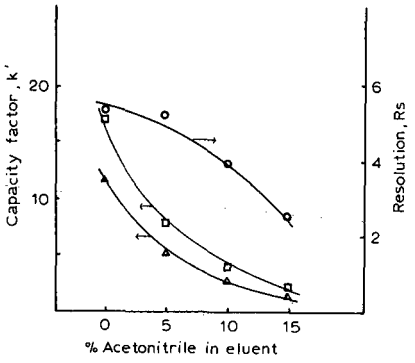

Fig. 4. Effect of acetonitrile content on capacity factor and resolution of DL-phenylalanine. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$–acetonitrile solution; flow-rate, 1 ml/min; pressure, 95 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, DL-phenylalanine (1 mg/ml).

TABLE I

OPERATING CONDITIONS FOR MCI GEL CRS10WD AND RESOLUTION OF AMINO ACIDS

Column temperature: room temperature.

Amino acid	*Eluent $CuSO_4$ concentration (mM)*	*Flow-rate (ml/min)*	*Retention time (min)*		*Separation factor, α*	*Resolution, R_s*
			L-isomer	*D-isomer*		
Lys	0.1	0.5	3.0	3.5	1.17	1.0
His	0.5	0.5	4.1	5.4	1.31	1.4
Ser	0.5	0.5	5.7	7.0	1.22	0.9
Thr	0.5	0.5	5.9	7.6	1.29	1.2
Ala	1.0	0.5	4.3	5.5	1.29	1.2
Cit	1.0	0.5	7.0	10.4	1.49	2.9
Pro	2.0	1.0	3.5	6.7	1.91	5.0
Val	2.0	1.0	4.3	8.0	1.85	5.8
Nval	2.0	1.0	5.3	10.2	1.92	5.7
Asp	2.0	1.0	7.0	8.4	1.20	1.7
Glu	2.0	1.0	15.0	22.7	1.55	4.6
Ileu	2.0	1.0	10.8	22.9	2.13	7.7
Ileu (*allo*)	2.0	1.0	8.4	16.4	1.95	6.6
Leu	2.0	1.0	10.0	19.1	1.91	7.1
Nleu	2.0	1.0	15.2	22.0	1.45	3.0
Met	2.0	1.0	8.7	13.9	1.59	5.0
Tyr	2.0	1.0	16.5	29.8	1.80	6.5
Eth	2.0	1.0	20.8	34.5	1.65	5.4
Phe	2.0	1.0	28.8	52.0	1.81	5.0

D-amino acid impurity present in L-amino acids using CRS10W, but the reverse is difficult using same column. Recently, the determination of D-isomer/L-isomer ratios and trace enantiomers has attracted attention because of their potential for dating[15] and analysis of food production processes. The authors have developed and assessed a packed column that uses an optically active ligand with the reverse optical activity for these purposes.

Table I shows the analytical conditions under which the CRS10WD column

TABLE II

DETERMINATION OF IMPURITIES IN OPTICAL ENANTIOMERS

Amino acid	*Column*	*Manufacturer*	*Concentration of optical impurity (ppm)*
L-Alanine	CRS10W	A	160
L-Alanine	CRS10W	B	80
L-Alanine	CRS10W	C	350
D-Alanine	CRS10WD	C	910
L-Aspartic acid	CRS10W	C	5700
D-Aspartic acid	CRS10WD	C	4750
L-Phenylalanine	CRS10W	C	650
D-Phenylalanine	CRS10WD	C	800

was operated and the resolution results for each amino acid. These columns were used to determine enantiomer impurities present in some L- and D-amino acids. The measurements were based on OPA fluorimetry (post-column method).

The impurities were determined by the standard addition method. The results are given in Table II. The L-alanine samples from different suppliers contained different amounts of impurities. Further, different amino acids had widely varying impurities. Because of this, great care must be taken when these amino acid products are used for analysis. The detection limit will correspond to that of OPA fluorimetry for a compound having a high resolution, and will reach about 100 ppm for a DL-amino acid having a resolution of 1.5–2.

Resolution of DL-*amino acid mixtures*

CRS10WD gives widely varying retention times, depending on the type of amino acid being separated, as shown in Table I. Hence it is possible to effect the resolution of a number of amino acids simultaneously using a single CRS10WD column. Figs. 5 and 6 illustrate examples of simultaneous resolution, in each instance ten different DL-amino acids being separated with a single 50-mm column by isocratic

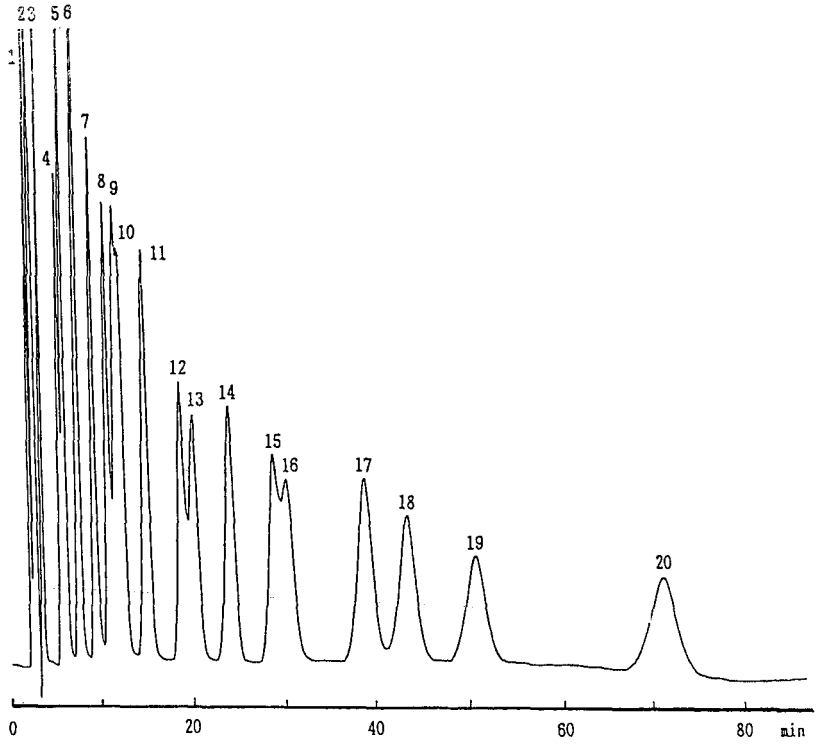

Fig. 5. Resolution of ten amino acid racemates using an MCI GEL CRS10WD column. Column, MCI GEL CRS10WD (50 mm × 4.6 mm I.D.); eluent, 1 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 110 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. Sample: 1, L-Ser; 2, D-Ser; 3, L-Cit; 4, D-Cit; 5, L-Val; 6, L-Nval; 7, L-Asp; 8, D-Asp; 9, D-Val; 10, L-Met; 11, D-Nval; 12, L-Glu; 13, D-Met; 14, L-Tyr; 15, D-Glu; 16, L-Eth; 17, L-Phe; 18, D-Tyr; 19, D-Eth; 20, D-Phe.

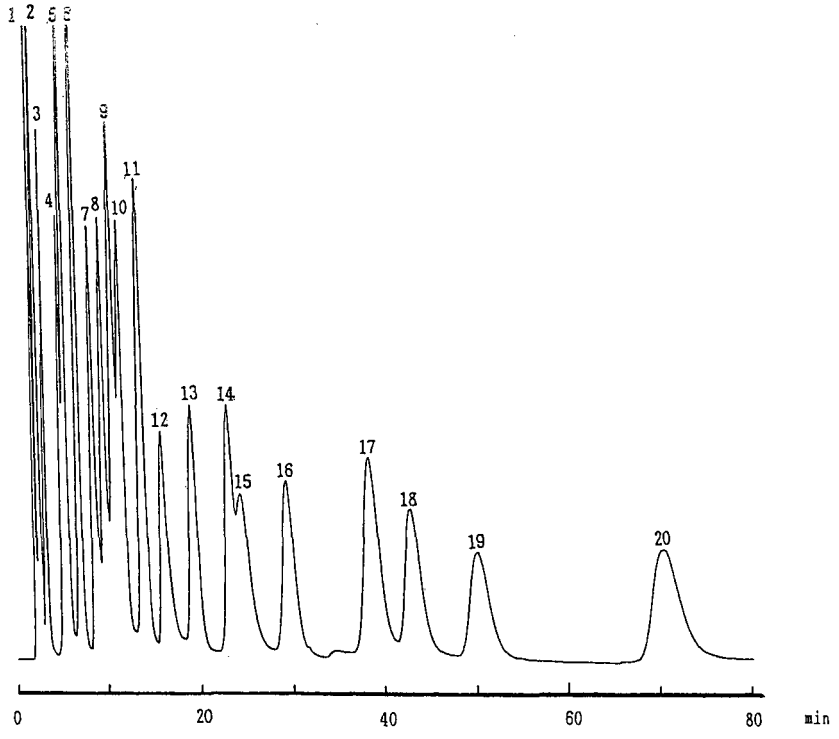

Fig. 6. Resolution of ten amino acid racemates using an MCI GEL CRS10W column. Column: MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 1 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 113 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. Sample: 1, D-Ser; 2, L-Ser, 3, D-Cit; 4, L-Cit; 5, D-Val; 6, D-Nval; 7, D-Asp; 8, L-Asp; 9, L-Val; 10, D-Met; 11, L-Nval; 12, D-Glu; 13, L-Met; 14, D-Tyr; 15, L-Glu; 16, D-Eth; 17, D-Phe; 18, L-Tyr; 19, L-Eth; 20, L-Phe.

elution. CRS10W gave similar chromatograms except that the elution order of the D- and L-isomers was reversed.

Applicability of CRS10W to enantiomers other than amino acids

The applicability of CRS10W to enantiomers other than amino acids was also investigated. The results are shown in Table III. Of amino acid derivatives, N-acetyl derivatives, such as N-acetyl-DL-alanine, DL-leucine,-DL-tryptophan and DL-valine were well separated (Fig. 7). N-carbobenzoxy (CBZ) derivatives, on the other hand, were difficult to elute, even with the aid of an eluent to which an organic solvent had been added. This may have resulted from strong interactions between the CBZ group and hydrophobic groups of the matrix.

Dipeptides, such as glycyl-DL-leucine and DL-leucyl-DL-phenylalanine, were separated well when an organic solvent was added to the eluent, as shown in Fig. 8. Similar results were obtained for tri-peptides, such as DL-alanylglycylglycine and DL-leucylglycyl-DL-phenylalanine. Hence it can be concluded that CRS10W provides a good method for resolving peptide enantiomers.

TABLE III

RESOLUTION OF AMINO ACIDS DERIVATIVES, PEPTIDES AND HYDROXY CARBOXYLIC ACIDS ON MCI GEL CRS10W

Column temperature: room temperature.

Compound	*Optimum conditions*		*Capacity factor*		*Resolution, R_s*
	Eluent $CuSO_4$ concentration (mM)	*Flow-rate (ml/min)*	k'_D	k'_L	
N-Acetyl-DL-alanine	2.0	1.0	7.0	8.4	1.71
N-Acetyl-DL-leucine	2.0[a]	1.0	38.0	54.1	2.90
DL-Ala–Gly–Gly	0.5	0.5	2.3	3.0	0.58
DL-Leu–Gly–Gly	2.0	1.0	10.3	16.4	2.99
Gly–DL-Leu	2.0[a]	1.0	20.9	11.7	2.93
DL-Leu–L-Tyr	2.0[a]	1.0	12.5	15.6	0.96
DL-Leu–DL-Phe	2.0[a]	1.0	16.7	20.6	1.21
Glyceric acid	0.5	1.0	11.0	18.9	2.68
Malic acid	0.5[a]	1.3	44.2	36.4	1.13
Lactic acid	2.0	1.0	12.5	15.9	2.44
Pantothenic acid	2.0	1.0	40.0	45.2	0.41
Mandelic acid	2.0[a]	1.0	52.5	72.3	3.98
2-Hydroxy-*n*-butyric acid	2.0[a]	1.0	9.8	14.2	3.40
Tartaric acid	2.0[a]	1.0	49.5	32.5	0.90
DL-α-Amino-ε-caprolactam	1.0	0.5	1.7	2.4	1.36

[a] In 10% aqueous acetonitrile solution.

Of hydroxycarboxylic acids, DL-mandelic acid, DL-lactic acid, DL-malic acid and DL-tartaric acid were separated well. However, the resolution for DL-pantothenic acid was insufficient, as shown in Fig. 9. For DL-malic acid and DL-tartaric acid, the elution order was reversed.

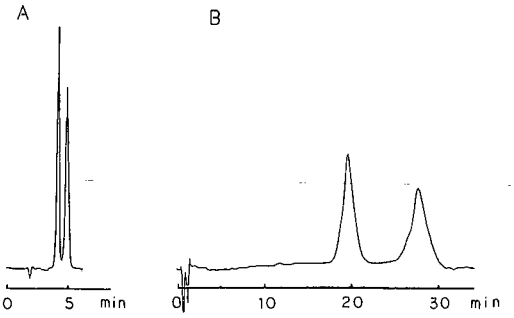

Fig. 7. Resolution of amino acid derivatives. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); flow-rate, 1 ml/min; temperature, room temperature; detector, UV (254 nm). (A) Eluent, 2 m*M* $CuSO_4$ aqueous solution; sample, N-acetyl-DL-alanine (2 mg/ml); injection volume, 15 μl. (B) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; sample, N-acetyl-DL-leucine (0.2 mg/ml); injection volume, 20 μl.

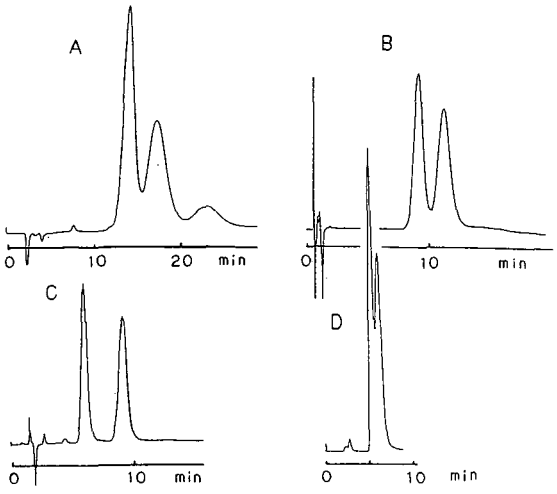

Fig. 8. Resolution of peptides. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); temperature, room temperature; detection, UV (254 nm). (A) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 0.3 ml/min; sample, DL-leucyl-L-tyrosine (2 mg/ml); injection volume, 5 μl. (B) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1 ml/min; sample, DL-leucyl-DL-phenylalanine (1 mg/ml); injection volume, 20 μl. (C) Eluent, 2 *M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-leucylglycylglycine (1 mg/ml); injection volume, 10 μl, (D) Eluent, 0.1 m*M* $CuSO_4$ aqueous solution; flow-rate, 0.3 ml/min; sample, DL-alanylglycylglycine (2 mg/ml); injection volume, 5 μl.

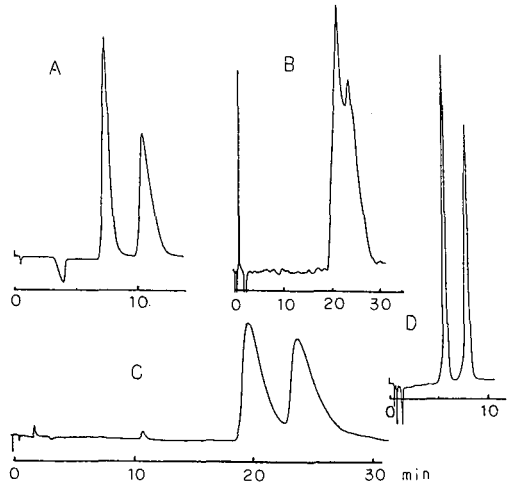

Fig. 9. Resolution of hydroxycarboxylic acids. Column: MCI GEL CRS10W (50 mm × 4.6 mm I.D.); temperature, room temperature; detection, UV (254 nm). (A) Eluent, 0.5 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-glyceric acid, Ca salt (4 mg/ml); injection volume, 20 μl. (B) Eluent, 2 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-pantothenic acid, Ca salt (4 mg/ml); injection volume, 20 μl. (C) Eluent, 0.5 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1.3 ml/min; sample, DL-malic acid (2 mg/ml); injection volume, 2 μl. (D) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1 ml/min; sample, DL-2-hydroxy-*n*-butyric acid, Li salt (2 mg/ml); injection volume, 5 μl.

Of amines, DL-α-amino-ε-caprolactam was separated well, but 1-phenylethylamine was eluted without interacting with the column.

CONCLUSION

When the elution conditions are carefully controlled, MCI GEL CRS10W and CRS10WD can resolve most DL-amino acids. They retain other compounds, including amino acid derivatives, peptides and hydroxycarboxylic acids.

REFERENCES

1 V. A. Davankov, S. V. Rogozhin, A. V. Semechkin, V. A. Baranov and G. S. Sannikova, *J. Chromatogr.*, 93 (1974) 363.
2 V. A. Davankov, A. S. Bochkov, A. A. Kurganov, P. Roumeliotis and K. K. Unger, *Chromatographia*, 13 (1980) 677.
3 C. Corradini, F. Federici, M. Sinibaldi and A. Messina, *Chromatographia*, 23 (1987) 118.
4 Y. Yuki, K. Saigo, H. Kimoto, K. Tachibana and M. Hasegawa, *J. Chromatogr.*, 400 (1987) 65.
5 T. Shinbo, T. Yamaguchi, K. Nishimura and M. Sugiura, *J. Chromatogr.*, 405 (1987) 145.
6 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
7 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
8 N. Nimura, A. Toyama and T. Kinoshita, *J. Chromatogr.*, 316 (1984) 547.
9 P. E. Hare and E. Gil-Av, *Science*, 204 (1979) 1226.
10 P. Masia, I. Nicoletti, M. Sinibaldi, D. Attanasio and A. Messiana, *Anal. Chim. Acta*, 204 (1988) 145.
11 E. Armani, A. Dossena, R. Marchelli and R. Virgili, *J. Chromatogr.*, 441 (1988) 275.
12 E. Armani, L. Barazzoni, A. Dossena and R. Marchelli, *J. Chromatogr.*, 441 (1988) 287.
13 H. Kiniwa, Y. Doi, T. Ishida and H. Katoh, *J. Chromatogr.*, 461 (1989) 397.
14 J. A. Perry, J. D. Rateike and T. J. Szczerba, *J. Chromatogr.*, 389 (1987) 57.
15 G. H. Eduljee, *Chem. Br.*, March (1988) 235.

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DETERMINATION OF SODIUM MONOFLUOROACETATE IN SOIL AND BIOLOGICAL SAMPLES AS THE DICHLOROANILIDE DERIVATIVE

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SUMMARY

A method is described for the determination of trace amounts of sodium monofluoroacetate (MFA-Na) in soil and biological samples. Soil samples were sonicated with distilled water in the presence of basic magnesium carbonate. Biological samples were extracted with distilled water by sonication and the extracts were coagulated by addition of equal volumes of alcohol and centrifuged. MFA-Na in each sample solution was adsorbed on Dowex 1-X8 anion-exchange resin and eluted with 2% (w/v) sodium chloride. The eluate was acidified with hydrochloric acid and treated with 2,4-dichloroaniline and N,N′-dicyclohexylcarbodiimide. The dichloroanilide derivative of MFA-Na was extracted with ethyl acetate and quantified by gas chromatography with electron-capture detection and gas chromatography–mass spectrometry. The detection limits were 0.0015 and 0.003 μg/g in 20 g of soil and 10 g of biological sample, respectively.

INTRODUCTION

Sodium monofluoroacetate (MFA-Na), a highly toxic compound inhibiting a certain step in the tricarboxylic acid cycle, is used for the control of field mice. The potassium salt of monofluoroacetic acid (MFA) is known as a toxic constituent of the South African poisonous plant *Dichapetalum cymosum* ("Gifblaar")[1]. The LD_{50} of MFA-Na is variable in different species and, for example, is 0.06–0.1 mg/kg in canines[2]. The potential toxicity of MFA-Na to non-target animals and the possibility of secondary poisoning require a sensitive method for determining MFA-Na in biological samples.

For chromatographic analysis, MFA-Na is often converted into various ester derivatives with appropriate chromatographic properties[3–12]. However, the concomitant water generally interferes with esterification of MFA-Na. Recently, we have developed a method for determining trace amounts of MFA-Na in water samples by gas chromatography, where MFA-Na in an aqueous solution acidified with hydrochloric acid is converted to the dichloroanilide derivative (MFA-DCA) by reaction with 2,4-dichloroaniline (DCA) and N,N′-dicyclohexylcarbodiimide (DCC)[13]. We have applied this method to the determination of trace amounts of MFA-Na in various

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environmental water samples. We expected this method to be applicable to the determination of MFA-Na in environmental samples such as soil and biological samples. However, the aqueous extracts of these samples contain large amounts of various impurities, which made the direct application of this method difficult.

In this paper, we report a practical method for the determination of MFA-Na in soil and biological samples. The new method involves extraction of the sample with water, ion-exchange pretreatment of the extract, the anilide derivatization of MFA-Na in aqueous solution and quantification of the anilide derivative by gas chromatography with electron-capture detection (GC–ECD). Gas chromatographic–mass spectrometric (GC–MS) analysis of the anilide derivative by using a capillary column for a better separation from impurities and more specific detection is also described.

EXPERIMENTAL

Apparatus

A Model UT-20 ultrasonic cleaner (300 W, 26 kHz; Kokusai Electric, Tokyo, Japan) was used for sample extraction. A Shimadzu Model GC-3BE gas chromatograph equipped with a ^{63}Ni electron-capture detector and a JMS-D-300 mass spectrometer (JEOL, Tokyo, Japan) were used for analyses.

Reagents and materials

MFA-Na was obtained from Wako (Osaka, Japan), DCA from Tokyo Kasei Kogyo (Tokyo, Japan), DCC from Kanto Chemical (Tokyo, Japan) and Wako-gel S-1 silica gel from Wako. Organic solvents and other reagents used were the same as used previously[13]. Basic magnesium carbonate, ammonium sulphate and sodium hydroxide were of analytical-reagent grade. GS25 glass-fibre filter-paper (Toyo Roshi, Tokyo, Japan) was used. Dowex-1-X8 strongly basic anion-exchange resin (50–100 mesh) was treated prior to use as follows. The resin was immersed in distilled water and packed into a glass column. The packed resin was successively washed with 10-fold volumes of 2 *M* sodium hydroxide, 1 *M* ammonium sulphate, 1 *M* hydrochloric acid and distilled water until neutral. A glass column (10 mm I.D.) packed with 10 ml of the resin was used for each experiment.

Ion-exchange pretreatment

MFA-Na was separated from aqueous samples with a 13 cm × 1 cm I.D. ion-exchange column packed with 10 ml of the activated resin, Dowex 1-X8. A 20-μg amount of MFA-Na were loaded on the ion-exchange column and elution of MFA from the resin was investigated in order to choose the eluent.

The influence of the flow-rate of MFA-Na solution through the column on adsorption was examined. A solution of 20 μg of MFA-Na in 100 ml of water was passed through the column at flow-rates in the range 2–18 ml/min. The influence of the pH of MFA-Na solution on adsorption was also examined. The pH of an aqueous solution containing 20 μg of MFA-Na in 100 ml of water was adjusted in the range 2.6–11, and the solution was passed through the column.

Procedure for soil

A mixture of a 20 g of sample, 1 g of basic magnesium carbonate and 50 ml of

distilled water in a 200-ml beaker was sonicated for 20 min. The mixture was allowed to stand for about 15 min, the supernatant was decanted and the residue was extracted with 50 ml of distilled water in the same way. The combined extracts were centrifuged for 15 min at 3000 rpm and filtered by suction through a glass-fibre filter-paper. The filtrate and the washings were combined and passed through the ion-exchange column. The resin was washed with 50 ml of distilled water. MFA was eluted from the column with 50 ml of 2% (w/v) sodium chloride solution. The eluate was subjected to derivatization and instrumental analysis.

Procedure for biological samples

A mixture of 10 g of homogenized animal tissue or plant material or a 2-ml blood sample and 30 ml of distilled water in a centrifuge tube was sonicated for 20 min. After being allowed to stand for a while, the mixture except for the blood sample was centrifuged for 15 min at 2000 *g* and the upper suspension was decanted. The residue was again extracted with 30 ml of distilled water in the same way.

To the combined extract or diluted blood after sonication, an equal volume of ethanol was added. The mixture was stirred slowly, allowed to stand for about 30 min and centrifuged for 15 min at 3000 rpm. The supernatant was filtered by suction through a glass-fibre filter-paper and the filtrate was passed through the ion-exchange column. The resin was washed with 50 ml of distilled water. MFA was eluted from the column with 50 ml of 2% (w/v) sodium chloride solution.

Derivatization[13]

The eluate from the ion-exchange column as mentioned above was transferred into a 100-ml separating funnel. To the eluate 0.25 ml of 10 *M* hydrochloric acid, 2 ml of 0.5 *M* DCA in ethanol, 0.8 ml of 1 *M* DCC in ethanol and 15 ml of ethyl acetate were added. The mixture was shaken vigorously for 1 h on a reciprocating shaker. The aqueous layer was separated after the addition of 5 g of sodium chloride and extracted again with 5 ml of ethyl acetate. The combined organic layer was washed with 5 ml of 3 *M* hydrochloric acid, saturated sodium hydrogencarbonate solution and saturated sodium chloride solution, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in 2 ml of benzene, loaded on to a silica gel (3 g) column slurry-packed with benzene and washed with 50 ml of benzene. The MFA-DCA derivative was eluted with 100 ml of *n*-hexane–diethyl ether (95:5). The eluate was concentrated and subjected to instrumental analysis.

Gas chromatographic and gas chromatographic–mass spectrometric analysis

GC–ECD analysis of the dichloroanilide derivative was performed on a 2.1 m × 3 mm I.D. glass column packed with equal lengths of 5% DEGS–1% H_3PO_4 on Chromosorb W (60–80 mesh) and 5% Apiezone L grease–2% H_3PO_4 on Chromosorb W (60–80 mesh)[13]. The column and detector temperatures were 175°C and the injector temperature was 195°C. The flow-rate of the carrier gas (nitrogen) was 20 ml/min.

For calibration, amounts of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 μg of MFA-Na were dissolved in 50 ml of distilled water and 1 g of sodium chloride was added to each solution. The solution containing sodium chloride was subjected to the derivatization reaction described above.

GC–MS analysis was performed on a 15 m × 0.53 mm I.D. OV-1701 wide-bore

capillary column (Gasukuro Kogyo, Tokyo, Japan). The injector temperature was 250°C, the column oven temperature was 200°C, the ion source temperature was 250°C and the separator temperature was 250°C. Helium was used as the carrier gas at a flow-rate of 15 ml/min. The ionization voltage was 27.5 eV, the ionization current was 300 μA and the ion multiplier voltage was 2.0 kV. The fragment ion peak at *m/z* 186 was monitored for analysis. Aliquots of 2 μl were injected directly. Amounts of 0.1, 0.2, 0.4, 0.6 and 0.8 μg of MFA-Na were used for calibration.

RESULTS AND DISCUSSION

Pretreatment of sample extract

When MFA-Na was extracted from soil or biological samples with water, soil components such as humic substances or tissue constituents such as protein were also extracted in large amounts into the aqueous solution. If the extract is directly subjected to derivatization with DCA and DCC, no successful result is obtained. Purification of MFA-Na in the water extract is essential prior to the derivatization.

Richard and co-workers have successfully used an anion-exchange resin prepared from XAD to separate and concentrate organic acids from aqueous solution[14], and also reported good recoveries of C_2–C_{10} aliphatic carboxylic acids at the ppm level from oil shale process water[15]. Peterson[5] has separated MFA-Na from aqueous solution with Amberlite IR-45 in a batch process. McGary and Meloan[16] used a Bio-Rad AG1-X8 strongly basic resin for the same purpose.

We investigated the separation of MFA-Na from water samples with the strongly basic anion-exchange resin Dowex 1-X8. MFA was adsorbed quantitatively on the resin at flow-rates through the column in the range 2–18 ml/min. Even MFA-Na solution containing 500 mg/l of sodium chloride gave the same result and no influence on the adsorption of MFA-Na was observed. The pH of MFA-Na solution in the range 2.6–11 was also observed to have no influence on adsorption.

Elution of MFA from the resin with various eluents is shown in Table I. Dilute sodium hydroxide solution eluted MFA from the resin, but a large volume of the eluent was required, which is unsuitable for concentration of MFA-Na; for the subsequent derivatization step, an elution volume of less than 50 ml is desirable.

TABLE I

ELUTION OF MFA-Na FROM ANION-EXCHANGE COLUMN

MFA-Na (20 μg) was adsorbed on a Dowex 1-X8 (Cl^-) (50–100 mesh) column (13 cm × 1 cm I.D.) and eluted.

Eluent	*Concentration (M)*	*Recovery (%)*	
		~ 50 ml[a] (1st fraction)	*~ 100 ml[a] (2nd fraction)*
NaOH	0.1	2	10
	0.2	13	45
NaCl	0.1	46	54
	0.25	97	3
	0.5	98	2

[a] The eluate was fractionated into 50-ml volumes.

Sodium chloride solution at concentrations of more than 0.25 *M* is satisfactory for the quantitative elution of MFA from the resin within an elution volume of 50 ml. In derivatizing with DCA and DCC, sodium chloride concentrations in the range *ca.* 1–4% (w/v) gave constant recoveries[13] and higher concentrations of sodium chloride reduced the recovery. Therefore, the eluent for stripping MFA-Na from the ion-exchange resin was selected as 2% (w/v) sodium chloride solution. A 50-ml volume of the eluent could recover MFA-Na quantitatively from the column and the eluate could be subjected to the derivatization procedure without initial addition of sodium chloride.

Extraction from soil sample

The aqueous extract of soil is muddy and contains various inorganic and organic substances such as humic substances. Direct application of this extract to the ion-exchange column, even after filtration, often choked the column owing to precipitation of brown materials.

Calcium hydroxide and magnesium carbonate have been used as reagents to coagulate such materials and precipitate water-soluble organic substances and ions[17,18]. Aqueous extraction of soil by sonication in the presence of the reagents afforded an extract which contained little coloured and supended substances, and this was suitable for subsequent loading on to the ion-exchange column. Basic magnesium carbonate posseses a low water solubility and the extract obtained was weakly basic. The extract obtained by using calcium hydroxide absorbed ambient carbon dioxide, forming a film of calcium carbonate on the surface. This was disadvantageous for the subsequent loading on the ion-exchange column.

Soil with a relatively low ignition loss has a low content of organic substances. Addition of an organic solvent such as an alcohol to the aqueous extract obtained by sonication of such a soil sample caused brown material to precipitate to a certain extent. The extract obtained in this way could be loaded on the column. However, this method was not applicable to soil with a high ignition loss.

Extraction from biological samples

Extraction of MFA-Na from biological samples such as animal tissues, stomach contents, plants and baits has been performed with water or hydrophilic solvents under wet conditions[3–12,18]. Subsequently efforts have often been made to separate MFA from water. In this work, MFA-Na was subjected to derivatization in aqueous solution and no separation from water was required. However, aqueous extracts of animal tissue, blood, and plant samples contained large amounts of protein, etc., and was incapable of being loaded on to the ion-exchange column. Addition of an organic solvent is generally suitable for deproteinization. In this work, ethanol was chosen as a non-ionic protein coagulating agent.

To the aqueous extract of biological samples an equal volume of ethanol was added and the mixture was allowed to stand for about 30 min. Subsequently, precipitates were removed by centrifugation and filtration. The filtrate was applied to the ion-exchange column, and MFA was adsorbed on the resin. Inadequate deproteinization in this step caused the ion-exchange column to choke and gave a low final recovery of MFA-Na.

TABLE II

RECOVERY DATA FOR MFA-Na ADDED TO SOIL SAMPLES

Sample No.	*Ignition loss*[a] *(%)*	*Amount added (μg)*	*Recovery (μg)*	*Average recovery (%)*	*Relative standard deviation (%)*
1	3.5	0.40	0.40, 0.40, 0.41, 0.37, 0.40	99	3.8
2	8.4	0.50	0.38, 0.39, 0.39, 0.37	77	2.5

[a] 600°C, 2 h.

Recovery test

A recovery test on MFA-Na was carried out on several samples according to the analytical procedure. Portions of 20 g of soil were spiked with MFA-Na and analysed. The results are given in Table II. For soil that contained relatively large amounts of organic substances, more than 70% of MFA-Na was recovered. Fig. 1 shows a chromatogram obtained from soil spiked with MFA-Na. The peak of MFA-DCA derivative was well separated from others and successfully determined. However, additional peaks appeared at retention times longer than 10 min, some of which were considered to correspond to short-chain aliphatic carboxylic acids. The relative retention times for the acetic, propionic, formic, and butyric acid derivatives were about 1.4, 1.8, 1.8 and 2.3, respectively, on the column used[13]. The appearance of later peaks required 60–90 min for one GC–ECD analysis.

Portions of 10 g of homogenized biological samples were spiked with MFA-Na and analysed by GC–ECD. The results are given in Table III. Typical chromatograms obtained from these samples are shown in Figs. 2 and 3, which indicate a good

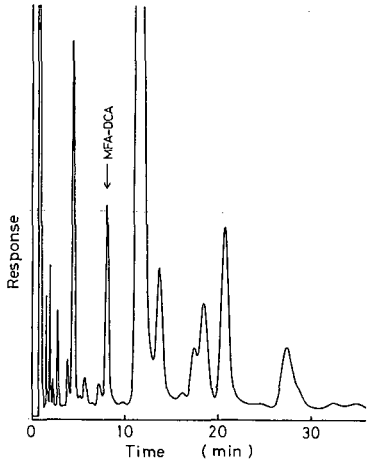

Fig. 1. Gas chromatogram for soil spiked with 0.02 μg/g of MFA-Na.

TABLE III

RECOVERY DATA FOR MFA-Na ADDED TO BIOLOGICAL SAMPLES

Sample	*Amount added (μg)*	*Recovery (μg)*	*Average recovery (%)*	*Relative standard deviation (%)*
Fish meat	0.50	0.40, 0.37, 0.45	81	10
Fish internal organs[a]	0.50	0.50, 0.48, 0.47	97	3.2
Liver (pig)	0.50	0.42, 0.42, 0.41, 0.39	82	3.4
Bovine blood[b]	0.10	0.090, 0.086, 0.091	89	3.0

[a] Without gall.
[b] Lyophilized, 2 ml.

separation of the MFA-DCA derivative. MFA-Na was successfully recovered from these biological samples. However, as observed with soil samples, additional peaks appeared later than the objective peak and about 90 min were required for one analysis.

A recovery test on bovine blood was conducted with a lyophilized sample. Portions of 2 ml of the blood were spiked with MFA-Na and analysed. In this instance, almost the same results were obtained.

The detection limits of this GC–ECD method were about 0.0015 and 0.003 μg/g with 20 g of soil and 10 g of biological sample, respectively.

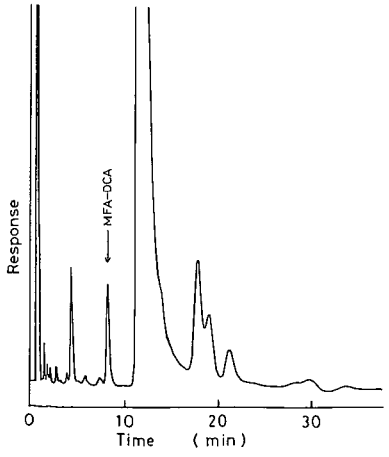

Fig. 2. Gas chromatogram for fish meat spiked with 0.05 μg/g of MFA-Na.

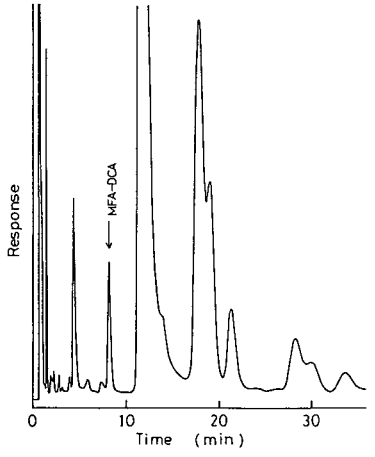

Fig. 3. Gas chromatogram for fish internal organs (without gall) spiked with 0.05 μg/g of MFA-Na.

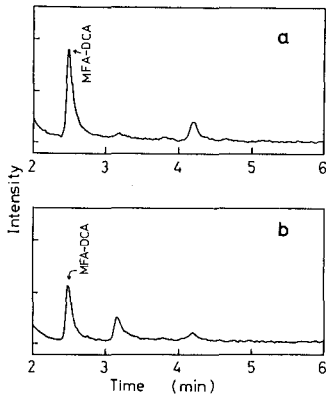

Fig. 4. Selected ion monitoring profiles for (a) soil and (b) pig liver spiked with 0.025 μg/g of MFA-Na. The mass monitored is the fragment ion peak at *m/z* 186.

GC–MS analysis

From the electron-impact mass spectrum of the MFA-DCA derivative[13], the molecular ion peak at *m/z* 221 and the fragment ion peak at *m/z* 186 were selectively monitored. The former was inferior to the latter with respect to quantification and contamination. Quantification was therefore effected by measurement of the area of the fragment ion peak at *m/z* 186. The calibration graph prepared with amounts of 0.1–0.8 μg of MFA-Na was linear.

Soil and pig liver were spiked with MFA-Na, treated according to the analytical procedure and analysed by GC–MS (selected ion monitoring mode). Fig. 4 shows typical chromatograms obtained, and demonstrates the usefulness of monitoring the ion peak at *m/z* 186. The time taken to analyse a sample was substantially reduced.

The recovery test was performed as in the analysis by GC–ECD, and the results are given in Table IV together with data obtained by GC–ECD. MFA-Na quantified by GC–MS was recovered satisfactorily with a lower precision compared with GC–ECD. The detection limit of the GC–MS method was similar to that of the GC–ECD method.

TABLE IV

RECOVERY OF MFA-Na BY GC–MS AND COMPARISON WITH GC–ECD

Sample[a]	*Amount added (μg)*	*GC–MS*		*GC–ECD*	
		Recovery (%)	*Relative standard deviation (%)*	*Recovery (%)*	*Relative standard deviation (%)*
Soil[b]	0.50	88	6.6	86	4.3
Liver (pig)	0.25	91	12	89	5.6

[a] Replicates (n = 4).
[b] Ignition loss (600°C, 2 h) = 4.1%.

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REFERENCES

1 J. S. C. Marais, *Onderstepoort J. Vet. Sci. Anim. Ind.*, 20 (1944) 67.
2 M. B. Chenoveth, *J. Pharmacol. Exp. Ther.*, 97 (1949) 383.
3 H. M. Stahr, *J. Assoc. Off. Anal. Chem.*, 60 (1977) 1434.
4 H. M. Stevens, A. C. Moffat and J. V. Drayton, *Forensic Sci.*, 8 (1976) 131.
5 J. E. Peterson, *Bull. Environ. Contam. Toxicol.*, 13 (1975) 751.
6 I. Okuno and D. L. Meeker, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 49.
7 T. Vartiainen and P. Kauranen, *Anal. Chim. Acta*, 157 (1984) 91.
8 H. H. Casper, T. L. McMahon and G. D. Paulson, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 722.
9 J. J. L. Hoogenboom and C. G. Rammel, *J. Anal. Toxicol.*, 11 (1987) 140.
10 A. C. Ray, L. O. Post and J. C. Reagor, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 19.
11 H. L. Kramer, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 1058.
12 D. M. Collins, J. P. Fawcett and C. G. Rammel, *Bull. Environ. Contam. Toxicol.*, 26 (1981) 669.
13 H. Ozawa and T. Tsukioka, *Anal. Chem.*, 59 (1987) 2914.
14 J. J. Richard and J. S. Fritz, *J. Chromatogr. Sci.*, 18 (1980) 35.
15 J. J. Richard and G. A. Junk, *Anal. Chem.*, 56 (1984) 1625.
16 E. D. McGary and C. E. Meloan, *Anal. Lett.*, 15(A12) (1982) 1051.
17 Dojyo Yobun Sokuteiho Iinkai, *Dojyo Yobun Bunsekiho*, Yokendo, Tokyo, 1975, p. 191.
18 E. G. Cotterill, *Analyst (London)*, 107 (1982) 76.

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CHROM. 21 552

Note

Computation of band shape for strong injection solvent and weak mobile phase combinations in liquid chromatography

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Many workers[1–10] have observed the production of distorted and multiple peaks from the injection of a single eluite dissolved in a solvent stronger than the mobile phase in reversed-phase high-performance liquid chromatography (HPLC). Microcomputer simulation of this production has been attempted[11]. Recently, a systematic study of this phenomenon showed some general patterns of peak development [12]. We have computed, using equilibrium distribution theory, band patterns that develop when a strong injection solvent is used. Although a non-equilibrium process is operative in HPLC, we hoped to find general features in band patterns that were similar to those observed in the above systematic study. Our computer approach was based on that used to examine band development when the injection solvent was the mobile phase[13].

METHOD

The chromatography was viewed as having two contiguous mobile phases. (1) The small volume of strong injection solvent which introduced the eluite into the first few column plates followed by (2) the main weaker mobile phase. Plates had equal volumes, and the total injection volume was an integral multiple of the plate volume. The multiples used varied from 1 to 10. The computation was the same as previously used[13] except that initially two capacity factors (k') were used, one of small value representing the injection solvent plug as mobile phase and one of larger value representing the weaker main mobile phase. Later more than two capacity factors were used to represent a partially diluted injection solvent plug.

An AT&T PC 6300 microcomputer with 640 Kbytes RAM was used. A Lotus 1–2–3 spreadsheet program computed the eluite distribution. Cells were reserved for the fraction of eluite in the stationary phase, $k'/(k' + 1)$, for each k' value used in a given equilibrium distribution. Distributions were extended up to 100 plates. Distributions were displayed with a Hewlett-Packard 7475A plotter.

Table I represents the initial part of a spreadsheet distribution where 100 wt. units were injected into a column whose individual plate volume accepted 10 wt. units. The capacity factors were 0.100 for the injection solvent, giving a stationary phase fraction (rounded to one place beyond the decimal for display purposes) shown

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TABLE I

SPREADSHEET GENERATED FOR INJECTION INTO THE FIRST TEN PLATES[a]

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
1	10.0	0.8	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
2	10.9	9.1										
3	11.0	10.7	8.3									
4	11.0	11.0	10.5	7.5								
5	11.0	11.0	10.9	10.2	6.8							
6	11.0	11.0	11.0	10.9	9.9	6.2						
7	11.0	11.0	11.0	11.0	10.8	9.6	5.6					
8	11.0	11.0	11.0	11.0	11.0	10.7	9.2	5.1				
9	11.0	11.0	11.0	11.0	11.0	10.9	10.5	8.9	4.7			
10	11.0	11.0	11.0	11.0	11.0	11.0	10.9	10.4	8.5	4.2		
11	9.2	2.8	11.0	11.0	11.0	11.0	11.0	10.8	10.2	8.1	3.8	
12	7.6	3.9	1.5	11.0	11.0	11.0	11.0	11.0	10.8	10.0	7.7	3.5

[a] $k' = 5$ and 0.1.

in cells C1 to K1 and 5.00 for the mobile phase, giving a stationary phase fraction shown in B1. All other cells were used for distribution. Increasing numerical values of the rows gives increasing extent of distribution.

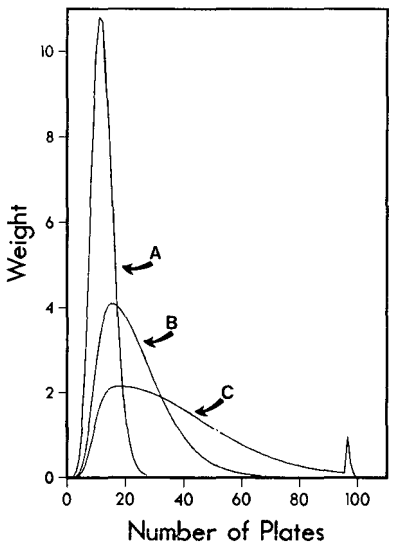
The distributions computed and displayed in this paper are column band distributions. They are not chromatogram simulations because the computation never simulated moving the eluite out of the column. To obtain well developed bands all of the spreadsheet memory was used in computation, and, thus, it was not possible to continue "moving" the plate contents through a "detector".

When simulation of moving a partially developed distribution band through a detector was done the following shape effects occurred and should occur in well developed distributions. The solvent front peak found in Figs. 1–8 retained its height and shape. Other peaks broadened and were somewhat lower. Valleys between peaks were somewhat higher.

RESULTS AND DISCUSSION

Equilibrium distribution predicted that band distortion occurs when the injection solvent strength as measured by the capacity factor was greater than the mobile phase strength. Fig. 1 illustrates the eluite distribution using a weak mobile phase, $k' = 10$. As the strength of the injection solvent increases, k' from 1 to 0.1, the distribution changes from a reasonably symmetric band to a band with a leading edge, and finally to the development of a sharp band near the mobile phase front. Experimentally, chromatographic bands developed a leading edge and a sharp peak at the mobile phase front appeared as the injection solvent strength increased[1,2].

Fig. 2 shows the distribution of an eluite injected in a strong solvent, $k' = 0.1$. The mobile phase strength is decreased from $k' = 1$ to $k' = 10$. This eluite's main band develops a leading edge that produces more asymmetry as the mobile phase strength decreases. In all three cases a sharp band of similar height and identical location is formed near the mobile phase front. The appearance of this sharp band



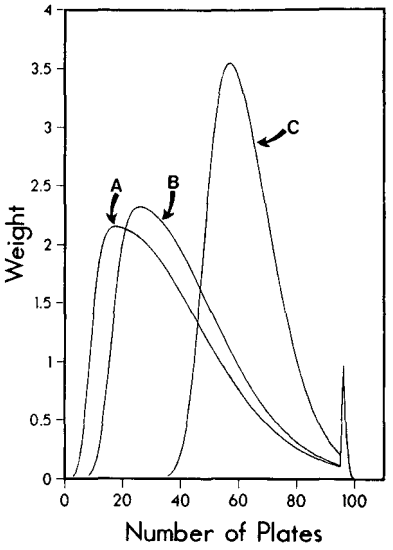


Fig. 1. Distribution of 100 wt. units of eluite after the mobile phase ($k' = 10$) traverses 100 plates. The eluite injection volume was five plate volumes. Injection solvent strength, expressed as capacity factor, was (A) $k' = 1$; (B) $k' = 0.2$; (C) $k' = 0.1$.

Fig. 2. Distribution of 100 wt. units of eluite after the mobile phase [(A) $k' = 10$; (B) $k' = 5$; (C) $k' = 1$] traverses 100 plates. The eluite was introduced in an injection solvent of $k' = 0.1$ and a volume of five plate volumes.

indicates that the strong injection solvent will carry the eluite with it whether the mobile phase is moderately weak or very weak, that is, this peak's appearance is largely dependent on the strength of the injection solvent and not that of the mobile phase.

Fig. 3 attempts to simulate the case of the distribution of three eluites when injected into the same weak mobile phase from the same injection solvent. The eluites have different k' values in the mobile phase, and, as a first approximation, the eluites have proportionally different k' values in the injection solvent giving a constant k' ratio for each eluite of 50. The eluite that is retained the most, has the most symmetric distribution although even here a leading edge appears. Furthermore, the peak near the solvent front is highest for the case of the eluite that has the smallest k' for the injection solvent. This effect is in agreement with the constant appearance of such a peak discussed for Fig. 2. The distributions shown in Fig. 3 are in agreement with the chromatographic peaks observed experimentally[12].

Experimental peak distortion was found to increase with increasing injection volume for HPLC with injection solvents stronger than the mobile phase[12]. Fig. 4 shows the distribution of eluite when 100 wt. units were injected three ways. Distribution A resulted from a small injection volume, one plate volume. The peak had a leading edge. When the injection volume was five plate volumes, the 100 wt. units gave distribution B. In this case the leading edge produced more asymmetry, and the sharp peak at the mobile phase front, illustrated previously in Figs. 1, 2 and 3, appeared. If the injection volume was increased further to nine plate volumes, the

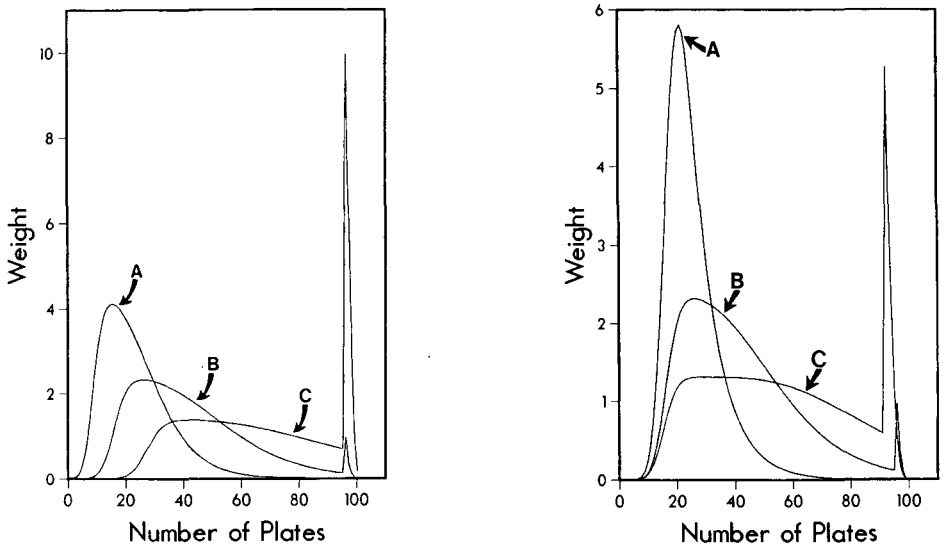


Fig. 3. Distribution of three eluites [(A) $k' = 10$ and 0.2; (B) $k' = 5$ and 0.1; (C) $k' = 2.5$ and 0.05 for mobile phase and injection solvent, respectively] after the mobile phase traverses 100 plates. The eluite injection volume was five plate volumes.

Fig. 4. Distribution of 100 wt. units of eluite ($k' = 5$ and 0.1 for mobile phase and injection solvent respectively) after the mobile phase traverses 100 plates. Injection in a volume of (A) one plate volume, (B) five plate volumes and (C) nine plate volumes.

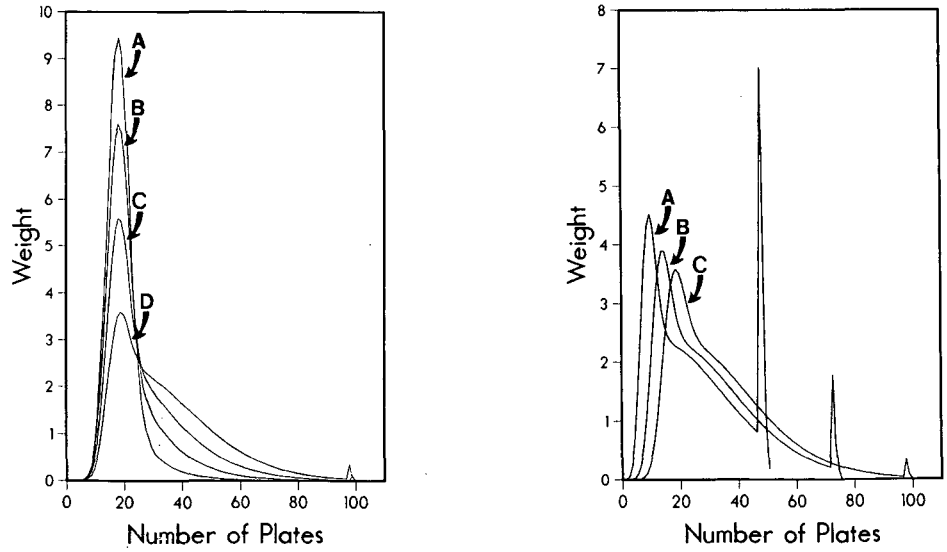


Fig. 5. Distribution of 100 wt. units of eluite ($k' = 5$ and 0.1 for mobile phase and injection solvent respectively) after the mobile phase traverses 100 plates. The volume of the injection solvent plug was five plate volumes. In A the last four fifths of the plug had a $k' = 5$. In B $k' = 5$ was used for the last three fifths, in C the last two fifths and in D the last fifth.

Fig. 6. Distribution of 100 wt. units of eluite ($k' = 10$ and 0.1 for mobile phase and injection solvent respectively) after traversing (A) 50, (B) 75 and (C) 100 plates. The volume of the injection solvent plug was five plate volumes. The last fifth of the plug had a $k' = 10$.

peak was severely distorted and the peak at the mobile phase front was markedly taller.

The general features of these column band patterns were similar to experimentally observed chromatographic peaks. The computed distribution results suggest that, if the volume of strong solvent is large enough, the solvent is capable of carrying part of the eluite with it practically to the end of the column while the remainder of the eluite follows as a peak with a leading edge.

What has been observed experimentally was the development of one or more additional chromatographic peaks between the main peak and the peak near the mobile phase front. It was suggested that one additional peak should develop as the injection solvent volume and strength increased and that more than one additional peak resulted from dilution of the solvent plug[12]. The production of a single additional peak between the two peaks shown in Figs. 1–4 was not supported by distribution theory. However, computation assuming dilution of the plug did give rise to a peak located between the two shown in previous figures.

The consequence of diluting the rear end of the injection solvent plug is illustrated in Fig. 5. When the rear four-fifths of the plug is diluted, the peak develops a slight leading edge. This edge becomes a bulge when only the last fifth of the plug is diluted. A peak near the mobile phase front is present at less dilution.

Fig. 6 shows the effect of traversing an increasing number of plates when the rear fifth of the plug is diluted. The bulge or development of a middle peak is pronounced when only 50 plates have been traversed. The peak near the mobile phase front is very tall early in the distribution development. When 100 plates are traversed, the bulge is smoother and the peak at the mobile phase front is small.

Fig. 7 shows distributions formed when 100 wt. units of eluite are injected into a column whose plate volume is 10% of the injection volume. Both ends of the injection plug were diluted as shown in Table II. These band peaks are very similar to chromatographic peaks observed experimentally by many workers. They illustrate that distri-

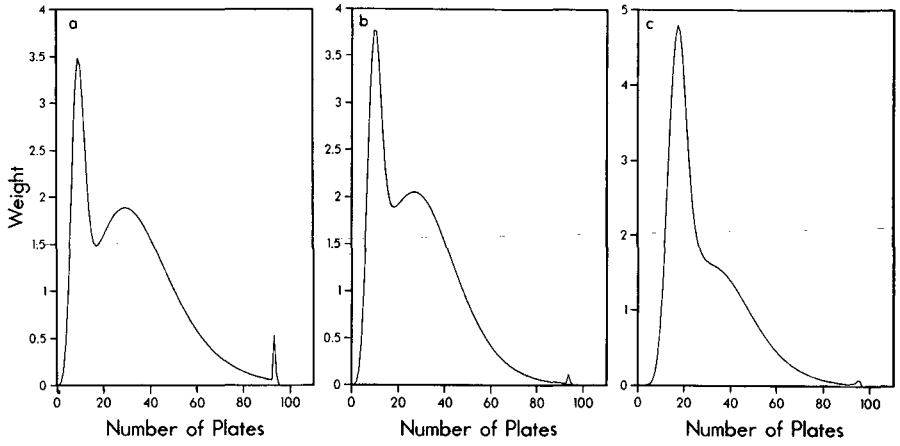


Fig. 7. Distribution of 100 wt. units of eluite after the mobile phase traverses 100 plates. The eluite injection volume was ten plate volumes. Mobile phase $k' = 10$ and injection solvent k' distribution is shown in Table II.

TABLE II

k' DISTRIBUTION IN THE INJECTION PLUG AFTER DILUTION

Plates										*Fig.*
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	
10.0	10.0	0.15	0.15	0.15	0.15	0.15	10.0	10.0	10.0	7a
5.0	2.0	1.2	0.5	0.2	0.1	0.1	0.2	0.4	0.5	7b
10.0	10.0	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5	7c

butions can have three peaks, and their general features can be altered by the dissymmetric dilution of the ends of the strong injection solvent plug.

Finally, Fig. 8 attempts to simulate an experiment in which peak distortion was produced by injecting a strong solvent shortly after the eluite had been injected dissolved in the mobile phase. Experimentally a chromatographic peak with a leading edge was produced[12]. A leading edge in the distribution was produced by computation, and the consequent asymmetry was always present as the volume of strong solvent was varied.

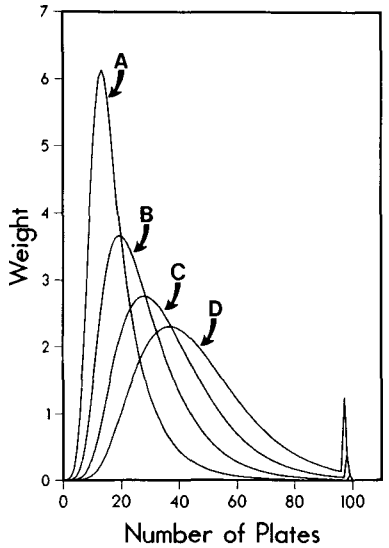


Fig. 8. Distribution of eluite (k' = 10 and 0.1 for mobile phase and injection solvent, respectively). The eluite, 100 wt. units, was introduced into the first plate. For A, B, C and D the injection solvent volumes were one, two, three and four plate volumes, respectively.

CONCLUSION

The general features of the distributions obtained by computation are very similar to the chromatographic peaks obtained experimentally. The similarity suggests the cause of peak distortion and multiplicity is changing partitioning that results

from the short lived movement of a strong mobile phase through the column followed by normal movement of a weak mobile phase. More than two mobile phases are needed, however, to produce more than two peaks. Computation results are consistent with the idea that dilution of the solvent plug gives rise to more than two peaks.

REFERENCES

1 C. Wu and J. J. Wittick, *Anal. Chim. Acta.*, 79 (1975) 308.
2 P. K. Tseng and L. B. Rogers, *J. Chromatogr. Sci.*, 16 (1978) 436.
3 J. Kirschbaum, S. Perlman and R. B. Poet, *J. Chromatogr. Sci.*, 20 (1982) 336.
4 M. Tsimidou and R. Macrae, *J. Chromatogr.*, 285 (1984) 178.
5 C. Yi, J. L. Fasching and P. R. Brown, *J. Chromatogr.*, 352 (1986) 221.
6 G. K. C. Low, A. M. Duffield and P. R. Haddad, *Chromatographia*, 15 (1982) 289.
7 G. K. C. Low, P. R. Haddad and A. M. Duffield, *J. Chromatogr.*, 336 (1984) 15.
8 S. Perlman and J. J. Kirschbaum, *J. Chromatogr.*, 357 (1986) 39.
9 K. C. Chan and E. S. Yeung, *J. Chromatogr.*, 391 (1987) 465.
10 F. Khachik, G. R. Beecher, J. T. Vanderslice and G. Furrow, *Anal. Chem.*, 60 (1988) 807.
11 T. Ng and S. Ng, *J. Chromatogr.*, 329 (1985) 13.
12 N. E. Hofmann, S. Pan and A. M. Rustum, *J. Chromatogr.*, 465 (1989) 189.
13 N. E. Hoffman and A. Rahman, *J. Liq. Chromatogr.*, 11 (1988) 2685.

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CHROM. 21 550

Note

Membership values as indicators of complications in chromatography

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Chromatograms from environmental samples, including those from ambient air, are usually complex. The common presence of coeluting species in such chromatograms and the interpretive problems they pose even when using mass spectral detection with non-interactive computer based data processing are recognized and have been discussed[1–3]. Additional factors which can frustrate the straightforward use of analytical results from adsorbent sampling have also been presented[4].

In such situations data sets in which parameters are distributed can be useful; *e.g.*, chromatograms of one sample from two columns which produce poorly correlated retention times, or multiple adsorbed samples collected simultaneously but with a distribution of air volumes[5]. Practical limits on the number of multiple analyses are understandably severe especially in large-scale applications; but even if such data are available, their usual handling based on statistics can result in the reporting of severely censored databases. An alternative to that, to totally uncensored databases or to those with mixed or unspecified censoring is desirable, especially when those who produce and those who use the data are not the same.

Assuming competent and competently performed procedures, the difficulty becomes one of assigning any datum to a class of essentially uncomplicated results or to others containing data affected by complicating processes. Assignments based on identification of any specific complication with or without a correction for its effect on the observations are generally impractical. Statistically based assignments are not only weak when sets contain few samples, probably only two, but they are binary (membership values in a class can be only zero or one). Severe censoring can result. If multiple grades of membership to the class of uncomplicated results could be obtained from the data themselves, those grades might be part of a report of the uncensored data and make an explicit statement about the conformance of each data pair to expectations for complication free results.

Fuzzy set theory was suggested as an approach for situations such as this where uncertainty about the criteria for class membership exists[6]. Although published chemical applications are not presently numerous, a relevant summary of the fuzzy perspective with some chemical applications can be found elsewhere[7]. Here in this very simple application, a fuzzy relationship is defined to express the "nearness" of pairs of relevant measurements by a membership value in the interval zero to one. A value of one indicates equality and implies a complication free result. Decreasing values imply

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increasing inequality and, therefore, increasing effects of some complications. Two illustrations of this approach follow; one using sets of atmospheric samples adsorbed on Tenax and the other using a synthetic mixture analyzed on serially coupled columns under two different sets of conditions.

EXPERIMENTAL[a]

Data

The data from sets of atmospheric samples adsorbed on Tenax and analyzed by gas chromatography–mass spectrometry have been reported previously (see Table 1 in ref. 5). Only data from the front tubes are used here.

Synthetic gaseous mixtures for the chromatographic experiments were prepared by neat liquid injection and subsequent volatilization into a suitable vessel[8]. Expected retention times and flame ionization detector responses were determined for a calibration mixture of hydrocarbons and halogenated hydrocarbons of some environmental interest. The test mixture of 75 species contained in addition to those, a hydrocarbon mixture not characterized but containing gasoline components.

Separations were done using a Perkin-Elmer Model 8500 gas chromatograph with serially coupled 30 m × 0.32 mm I.D. fused-silica columns; 0.2-μm DB-1 followed by 0.2-μm DB-210. Control of helium pressures at the inlet and column coupling points determined relative residence times of the carrier in each column. Two sets of pressure settings were chosen arbitrarily. Calibration and test mixtures were analyzed at each setting. In all cases temperatures were programmed at 4°C/min starting at 40°C at the time of injection.

Peak areas and locations were determined by the chromatograph's data system and identified with a substance in a conventional way. The ratios of detector outputs from the test mixture to those from calibrations are shown in Tables II and III as normalized areas.

Fuzzy approach

Expectations were modeled as fuzzy relations. Assuming no complications, all Tenax samples in a set should yield nearly equal concentrations for any given substance. Similarly, in these chromatographic experiments, each substance should appear near its expected retention times and the quantities found should be nearly equal as well.

Membership values (MV) for pairs of observations in the Tenax sets were calculated using a one-dimensional Gaussian function:

$$\mathrm{MV}_i = \exp - [(x_{ij} - x_{ik})^2/2s_i^2]$$

where x_{ij} and x_{ik} are the pairs of concentrations for substances i and s_i is a scale factor. Chromatographic data were described by a membership function of the same form but in three or in some cases four dimensions; two or three retention time differences and one difference in areas. Computations are straightforward manipulations of data as shown in the argument of the one-dimensional function or a sum of such terms to

[a] Mention of trade names does not constitute endorsement or recommendation for use.

comprise the argument of the multidimensional function. They were done here on a Compaq Deskpro 286 using PC-Matlab; however, any spreadsheet or other program capable of such manipulations would suffice.

RESULTS AND DISCUSSION

Substantial latitude exists in the choice of the membership function and its parameters' values. Different choices will yield somewhat different membership values. Choice of the Gaussian was for convenience both in computations and selecting the scale factors, s_i. Those used were 0.15 min for retention time terms and

TABLE I

MEMBERSHIP VALUES (MV) AND NET APPARENT ATMOSPHERIC CONCENTRATIONS ($\mu g/m^3$) FROM THREE DISTRIBUTED AIR VOLUME SETS

	MV Mx Mn	*MV 6 & 27*	*Air volume sampled (l)*			
			6	*11.4*	*26.9*	*54*
Benzene	0.00	0.00	7.0	4.9	3.9	2.1
Methylbenzene	0.00	0.92	57.0	50.4	53.6	20.7
1,2-Dimethylbenzene	0.00	0.01	3.8	3.8	2.4	1.9
Ethylbenzene	0.00	0.00	3.4	2.7	2.0	1.6
Trichloroethene	0.00	0.00	0.65	0.44	0.39	0.22
1,1,1-Trichloroethane	0.00	0.00	5.5	2.9	2.1	1.0
Tetrachloroethene	0.11	0.95	2.2	2.0	2.1	1.6
Benzaldehyde	0.00	0.00	32.4	20.0	11.1	5.6
1-Phenylethanone	0.00	0.00	32.4	29.3	10.4	9.1
Benzonitrile	0.00	0.00	2.9	1.7	1.0	0.0
Benzene	0.27	0.83	17.1	15.9	15.6	13.4
Methylbenzene	0.00	0.00	122	126	22.6	10.4
1,2-Dimethylbenzene	0.63	0.72	9.6	9.3	8.5	8.3
Ethylbenzene	0.98	0.99	6.3	6.1	6.2	6.1
Ethenylbenzene	0.28	0.85	1.2	1.2	1.1	1.4
Trichloroethene	0.71	0.98	3.4	3.3	3.3	3.0
1,1,1-Trichloroethane	0.00	0.18	8.6	8.8	6.5	4.2
Tetrachloroethene	0.82	0.82	38.4	35.8	34.9	35.1
1,2-Dichloropropane	0.70	0.77	18.5	19.2	20.6	21.0
Benzaldehyde	0.00	0.00	11.3	10.7	5.6	4.6
1-Phenylethanone	0.00	0.00	0.39	0.14	0.00	3.2
Benzene	0.00	0.01	4.6	3.7	2.8	2.5
Methylbenzene	0.08	0.89	7.9	9.4	8.5	6.7
1,2-Dimethylbenzene	0.00	0.01	2.6	2.4	1.6	1.3
Ethylbenzene	0.41	1.00	1.0	1.1	1.0	0.90
Ethenylbenzene	0.00	0.00	0.75	0.85	0.41	0.23
Trichloroethene	0.00	0.03	0.39	0.37	0.26	0.17
1,1,1-Trichloroethane	0.00	0.92	1.7	1.9	1.6	0.80
Tetrachloroethene	0.77	0.97	2.8	2.9	2.7	2.6
1,2-Dichloropropane	0.27	0.36	2.9	3.2	3.6	3.7
Benzaldehyde	0.00	0.00	27.7	16.3	6.1	4.0
1-Phenylethanone	0.00	0.00	34.4	0.00	6.8	6.0
Benzonitrile	0.00	0.00	2.3	1.4	0.00	0.00

15% of the average concentration (Table I) or average normalized area (Tables II and III) taken as reasonable standard deviations of these differences. Although the considerations which shaped these choices are chemically plausible and statistically based, the membership values are not statistical results.

If distributed air volume samples are taken at all, sets of two are more likely to be collected than the sets of four shown in Table I. Therefore, a treatment using only two of the four samples was done. Two different membership values are shown; one using the extreme values for each substance at whatever sampling volume they appear (perhaps the easiest way to assign the whole set of four to a class) and the other using just the results from the 6- and 26.9-l samples as a more usual application.

Although the values reflect the behavior of the relevant observations, inspection will reveal some striking differences as well as similarities resulting from these choices. This underscores that each membership value describes only the fuzzy relationship of the data pair. Clearly, any indicator of the importance of complications discernable from the data (here, only factors with a strong non-linear dependence on air volume) must depend critically on the data used.

TABLE II

MEMBERSHIP VALUES (MV) AND OBSERVATIONS FOR 24 ANALYTES FROM PAIRS OF CHROMATOGRAMS OF A 75-COMPONENT MIXTURE

	MV	*Chromatogram A*			*Chromatogram B*		
		Expected retention time (min)	*Observed retention time (min)*	*Normalized area*	*Expected retention time (min)*	*Observed retention time (min)*	*Normalized area*
2-Bromo-1-propene	0.00	3.89	3.92	2.04	6.00	6.03	0.90
1-Bromopropane	0.00	4.89	4.88	1.94	7.44	7.48	1.15
Bromodichloromethane	0.00	6.35	6.40	4.83	9.66	9.76	8.26
2,2-Dichlorobutane	0.00	7.52	7.49	1.05	11.00	11.04	2.54
Methylbenzene-d_8	0.80	8.51	8.48	1.03	12.34	12.40	1.11
2,2-Dibromopropane	0.72	8.70	8.67	1.04	12.56	12.64	1.13
1,3-Dichlorobutane	0.78	11.12	11.10	1.00	14.98	15.05	1.08
1,1,2-Trichloropropane	0.82	11.29	11.27	1.00	15.52	15.59	0.94
1,1,1,2-Tetrachloroethane	0.69	11.40	11.37	0.98	15.89	15.96	1.08
1,4-Dichlorobutane	0.00	14.31	14.28	0.99	18.30	18.36	4.74
1,2,3-Trichloropropane	0.00	14.45	14.42	0.97	18.82	18.88	2.71
(1-Methylethyl)benzene	0.86	14.70	14.68	0.99	19.75	19.83	1.00
1,1,1,2-Tetrachloropropane	0.87	15.06	15.04	1.00	20.06	20.13	0.97
1,3-Dibromopropane	0.83	15.58	15.54	1.01	20.20	20.27	0.97
1-Methyl-2-chlorobenzene	0.81	15.98	15.95	1.01	21.05	21.12	1.08
1-Methyl-4-chlorobenzene	0.87	16.31	16.28	1.02	21.31	21.38	0.99
1,3-Dibromobutane	0.00	17.59	17.49	3.38	22.43	22.50	0.96
1,4-Dichlorobenzene	0.69	18.52	18.49	0.98	23.76	23.84	0.89
Benzonitrile	0.73	19.28	19.23	0.98	22.61	22.67	0.90
1,2-Dichlorobenzene	0.79	19.62	19.60	0.96	24.90	24.97	0.89
Butylbenzene	0.16	20.37	20.36	1.01	25.61	25.68	0.76
1,4-Dibromobutane	0.35	21.20	21.18	1.07	26.18	26.25	0.87
1-Ethenyl-4-chlorobenzene	0.72	21.28	21.28	0.92	26.66	26.73	0.83
1-Phenylethanone	0.60	22.30	22.26	0.83	26.40	26.46	0.73

TABLE III

MEMBERSHIP VALUES (MV) AND OBSERVATIONS FOR ANALYTES COELUTING IN ONE OF A PAIR OF CHROMATOGRAMS

		Chromatogram A			*Chromatogram B*		
		Expected retention time (min)	*Observed retention time (min)*	*Normalized area*	*Expected retention time (min)*	*Observed retention time (min)*	*Normalized area*
1-Bromo-2-chloroethane	0.01	7.17	7.12	3.40	10.45	10.55	1.76
1-Chloro-2,3-epoxypropane		7.92	7.88	1.07			
Dibromochloromethane	0.90	8.96	8.94	1.06	13.17	13.23	1.13
3,4-Dichloro-1-butene		9.38	9.35	1.20			
Tribromomethane	0.83	12.29	12.26	1.00	17.18	17.26	1.00
1,4-Dimethylbenzene		12.48	12.45	1.02			
1,1,2,2-Tetrachloroethane	0.00	13.64	13.61	3.39	18.08	18.15	0.98
1,4-Dichloro-2-butene		13.87	13.84	1.05			
1,3,5-Trimethylbenzene	0.87	16.62	16.60	1.05	21.84	21.91	0.99
1,2,2,3-Tetrachloropropane		16.75	16.72	0.92			

Illustrative data for some of the analytes in the two chromatograms of the gaseous test mixture are shown in Tables II and III. Table II contains results for those analytes which, during calibration, eluted singly under both sets of conditions. These are describable by the three-dimensional membership function. Normalized areas differing greatly from one indicate coeluents from the hydrocarbons and are reflected appropriately in the membership values. Even though mixtures were chosen to cause clear effects from coelution, a membership value range is exhibited. Even more subtle gradations are to be expected from environmental samples with a greater range of causes.

Optimization of the two sets of conditions was deliberately avoided to insure that some potential analytes coeluted. Table III displays some that separated under just one set of conditions and can be described by a membership function of four dimensions. (Because of the way the normalization was done here, in a complication free case, the sum of individual areas should equal twice the area when they coeluted.) In these instances, all of the results from the separated species could be reported accompanied by the membership value for the pair. These values reflect conformance to the expectation that all observations are free of further complications. Clearly, the same idea can be applied if larger numbers coelute under one set of conditions.

CONCLUSIONS

Membership values calculated quite simply using a fuzzy approach can reflect the relative importance of some common complications in analyses of complex mixtures. The membership value depends on the choice of membership function, values of its parameters and the data pairs. In this application, the function and its parameters' values must be made explicit. Any inadequacies in the pairs of experiments which fail to test the relationships (*e.g.*, nearly equal air volumes or columns too similar in separation characteristics) would not be apparent; however, in an

application to chromatogram pairs, the adequacy of the retention time observations (sometimes overlooked by data users) are included. Within these limits, each value can be a useful index of complications eliminating the need for the sometimes severe censoring of data.

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REFERENCES

1 D. Rosenthal, *Anal. Chem.*, 54 (1982) 63.
2 J. M. Davis and J. C. Giddings, *Anal. Chem.*, 57 (1985) 2168 and 2178.
3 M. Martin, D. P. Herman and G. Guiochon, *Anal. Chem.*, 58 (1986) 2200.
4 J. F. Walling, *Atmos. Environ.*, 18 (1984) 855.
5 J. F. Walling, J. E. Bumgarner, D. J. Driscoll, C. M. Morris, A. E. Riley and L. H. Wright, *Atmos. Environ.*, 20 (1986) 51.
6 L. A. Zadeh, *Inform. Control*, 8 (1965) 338.
7 H. Bandemer and M. Otto, *Mikrochim. Acta*, 2 (1987) 93.
8 C. Morris, R. Berkley and J. Bumgarner, *Anal. Lett.*, A20 (1983) 1585.

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Note

High-performance liquid chromatography on continuous polymer beds

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Current research in this laboratory is directed toward the investigation of various possible means to increase performance in electrophoresis and chromatography. These methods are studied in parallel because the separation mechanisms are analogous[1–3], which means that solutions of methodological problems in electrophoresis are often applicable to analogous problems in chromatography and *vice versa*. In the chromatographic field we give an high priority to practical solutions (with the aid of theoretical considerations) of the following fundamental questions:

(1) Is it possible to design chromatographic beds such that the resolution is independent of or even increases with an increase in the flow-rate and bead size? Classical chromatographic theory says no. However, the experiments described in refs. 4–6 and forthcoming papers show that compressed beds of non-porous agarose beads have the desired unique relationship between the resolution, flow-rate and bead-size.

(2) Is it possible to design a chromatographic bed by bulk polymerization directly in the chromatographic tube? It has been taken for granted that a chromatographic bed must be built up of granulated particles, preferably in spherical form. Even when the spheres are monodisperse the packing is never perfect. The theoretical maximum resolution can therefore never be attained. Further disadvantages of packed beds are the time-consuming and expensive steps required for preparation of the beads, the sieving of the beads to select the desired size (if not monodisperse in the preparation) and the packing of the column with the beads.

A continuous gel plug with channels sufficiently large to permit an hydrodynamic flow might be the ideal chromatographic column. One could then expect the zones to be almost as sharp as those obtained in agarose or polyacrylamide gel electrophoresis. Unfortunately, the latter continuous gels cannot be used for chromatography, since they collapse when pressure is applied, *i.e.*, water cannot be pressed through them. However, more than 20 years ago we prepared a polyacrylamide gel (cross-linked in a special way) directly in a glass tube and on this gel plug separated monomers and dimers of albumin by molecular-sieve chromatography. The flow-rate was relatively low, which limited its usefulness. We have now resumed these experiments and at the same time have tried to improve the mechanical properties of the gel plug so that it will withstand higher pressures and thereby permit higher flow-rates. The polymerization technique is still under development and will be published else-

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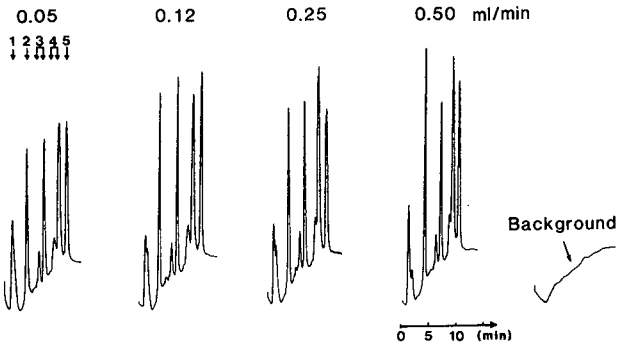

Fig. 1. High-performance cation-exchange chromatography of model proteins on a compressed continuous gel at the flow-rates indicated.

where. However, since the experiments are promising we present some preliminary results in order to focus interest on columns of continuous polymers.

EXPERIMENTAL AND RESULTS

The amphiphilic, macroporous gel plug consisted of a copolymer of acrylic acid and N,N′-methylenebisacrylamide. The gel plug was strongly compressed to a bed height of 3 cm (the importance of compressing a bed to increase its resolution is discussed in ref. 7). The diameter of the gel plug was 0.6 cm. This gel column was utilized for a cation-exchange chromatography experiment performed in the following way.

After equilibration with 0.01 *M* sodium phosphate, pH 6.4, a 40-μl sample [about 10–15 μg of each of the proteins alcohol dehydrogenase (1), horse skeletal muscle myoblobin (2), whale myoglobin (3), ribonuclease A (4) and cytochrome *c* (5)] was applied. Elution was performed with a linear gradient formed from the equilibration buffer and 0.01 *M* sodium phosphate, pH 6.4, containing 0.25 *M* sodium chloride. The flow-rates were 0.50, 0.25, 0.12 and 0.05 ml/min. The gradient volume was constant at 5.0 ml. The chromatograms are shown in Fig. 1. The relationship between the pressure and flow-rate is presented in Fig. 2.

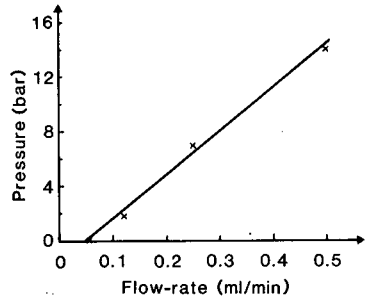

Fig. 2. The relationship between the flow-rate and pressure for the gel column used in the experiment shown in Fig. 1.

DISCUSSION

From Figs. 1 and 2 one can draw several conclusions:

(1) It is possible to prepare by bulk polymerization a continuous gel with channels large enough to permit passage of buffer when a pressure is applied to the bed.

(2) The bed is sufficiently rigid to give high flow-rates at moderate pressures.

(3) It is possible to prepare directly in the chromatographic tube a gel bed useful for ion-exchange chromatography. No subsequent step is required for attachment of ligands.

(4) The resolution on the continuous gel is roughly independent of the flow-rate, which is in sharp contrast to what is observed on columns of macroporous beads[8]. The reasons are probably that a gel plug has a more homogeneous structure than a packed bed of beads and that the gel plug was compressed, which, analogously to a compressed bed of agarose beads[7], has a favourable effect on the resolution. It is also likely that the gel plug is non-porous, *i.e.*, the "walls" of the channels in the gel are impermeable to proteins, which in combination with compression of the bed gives a resolution with the attractive flow-rate dependence mentioned above[4–6].

We are working on the preparation of continuous polymer beds for both hydrophobic-interaction and anion-exchange chromatography as well as for other kinds of chromatography.

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REFERENCES

1 S, Hjertén, *J. Chromatogr.*, 270 (1983) 1–6.
2 S. Hjertén, in H. Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1984, pp. 71–79.
3 S. Hjertén, K. Elenbring, F. Kilár, J.-L. Liao and L. Valtcheva, *Pittsburgh Conference and Exposition, New Orleans, February 22–26, New Orleans, 1988*, Abstract No. 478.
4 S. Hjertén, K. Yao and J.-L. Liao, *Makromol. Chem., Macromol. Symp.*, 17 (1988) 349–357.
5 S. Hjertén and J.-L. Liao, *J. Chromatogr.*, 457 (1988) 165–174.
6 J.-L. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 175–182.
7 S. Hjertén, Z.-q. Liu and D. Yang, *J. Chromatogr.*, 296 (1984) 115–120.
8 K. Yao and S. Hjertén, *J. Chromatogr.*, 385 (1987) 87–98.

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CHROM. 21 487

Note

Gas chromatographic analysis of sulphonic acids as their sulphonamide derivatives

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The analysis of aliphatic and aromatic sulphonic acids by gas chromatography (GC) has been carried out after their conversion into the volatile derivatives by desulphonation with acids[1–3], alkali fusion[4–6], sulphonyl chlorination[7–9] or fluorination[10], sulphonyl esterification[7,11–15], trimethylsilylation[16], and reduction to the thiol[17,18]. However, the usefulness of these methods may be limited by the time-consuming derivatization process. Further, the sulphonyl halides and the esters of sulphonic acids are generally unstable and are apt to undergo thermal decomposition during the GC analysis.

It appeared to us that dibutylamide derivatives of sulphonic acids would be potentially useful for GC, especially as we had already succeeded in the GC analysis of taurine (2-aminoethanesulphonic acid) as its N-acyldibutylamide derivatives[19,20]. The sulphonic acid function of N-acyltaurine was converted into the corresponding dibutylamide derivative in nearly quantitative yield by a convenient procedure involving ion-pair extraction, followed by chlorination and amidation. We have therefore studied the GC analysis of a variety of sulphonic acid compounds as their dibutylamide derivatives and report the results in this paper.

EXPERIMENTAL

Reagents

Methane-(C_1) and 2,4,6-trimethylbenzenesulphonic acids as the free acids and 1-propane-(C_3), 1-pentane-(C_5), 1-heptane-(C_7), 1-nonane-(C_9), 1-tridecane-(C_{13}), 1-octadecane-(C_{18}), benzene-, *p*-toluene-, 2,4-dimethylbenzene- and α-naphthalenesulphonic acids as the sodium salts were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). C_1–C_9 and aromatic sulphonic acids were dissolved in water and C_{13} and C_{18} sulphonic acids were dissolved in 30% ethanol. These standard solutions were prepared so as to contain 0.1 mg/ml of each acid. Anthracene (Nakarai Chemicals, Kyoto, Japan) was dissolved in methylene chloride at a concentration of 0.05 mg/ml and used as an internal standard (I.S.). Tetraalkylammonium salts were purchased from Nakarai Chemicals and used as 10% methanolic solutions. Tetraalkylammonium hydroxide solutions were prepared from their salts with silver oxide as described earlier[19]. Thionyl chloride (Nakarai Chemicals) was used after distillation. Dibutylamine (DBA) (Nakarai Chemicals) was used as a 2 *M* solution in acetonitrile. All other chemicals were of analytical-reagent grade.

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Gas chromatography

GC analysis was performed using a Shimadzu Type 4CM-PF gas chromatograph equipped with a 1.5 m × 3 mm I.D. glass column packed with 1.0% silicon SE-54 on Uniport HP (100–120 mesh) and with flame ionization detection (FID). The packed column was conditioned at 290°C for 24 h with nitrogen at a flow-rate of 30 ml/min. The operating conditions are given in the caption of Fig. 3. Peak heights for sulphonic acids and the I.S. were measured and the peak-height ratios were calculated for the construction of a calibration graph.

Gas chromatography–mass spectrometry

A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer with the same type of column as used for GC–FID was employed under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion-source temperature, 250°C; separation temperature, 240°C; and helium flow-rate, 40 ml/min.

Analytical derivatization procedure

An aliquot of the sample solution (containing 2–50 μg of each sulphonic acid) was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. After the total reaction volume had been made up to 1 ml with distilled water, 0.05 ml of 10% tetrahexylammonium chloride[a] or tetrabutylammonium bromide[a] and 2 ml of methylene chloride were added and the tube was shaken with a shaker set at 300 rpm (up and down) for 3 min at room temperature. After centrifugation for 1 min, the organic layer was transferred to another tube and the solvent was evaporated to dryness under a stream of nitrogen. To the residue was added 0.2 ml of thionylchloride, and the tube was tightly capped and heated at 80°C for 10 min. The excess of thionyl chloride was removed at 50°C under a stream of nitrogen. To the residue was added 0.2 ml of 2 *M* DBA solution, and the mixture was incubated at 80°C for 5 min after tightly capping. The reaction mixture was acidified with 1 ml of 20% orthophosphoric acid and then extracted twice with 3 ml of diethyl ether. To the ether extracts was added 0.4 ml of anthracene (I.S.) solution and the solvent was evaporated to dryness at 50°C. After the residue had been dissolved in 0.2 ml of ethyl acetate, the solvent was

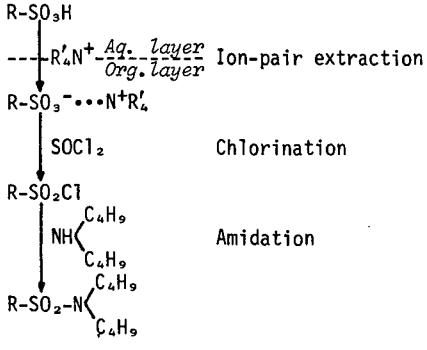

Fig. 1. Derivatization of sulphonic acids. R, R′ = alkyl or aryl.

[a] Tetrahexylammonium chloride and tetrabutylammonium bromide were used for the hydrophilic sulphonic acids (C_1–C_9) and the lipophilic sulphonic acids (C_{13}, C_{18} and aromatic), respectively.

dried over anhydrous sodium sulphate and 2 μl of this solution were injected into the GC–FID system. The derivatization process is summarized in Fig. 1.

RESULTS AND DISCUSSION

In the first step of the derivatization, sulphonic acids are extracted from the aqueous phase into methylene chloride by an ion-pair extraction technique[21] using tetraalkylammonium as counter ion. The influence of the nature of the counter ion on this extraction was investigated. Table I shows that tetrahexylammonium and tetrabutylammonium are the most satisfactory counter ions for the hydrophilic sulphonic acids (C_1–C_9 aliphatic) and the lipophilic sulphonic acids (higher aliphatic and aromatic), respectively, although the optimum counter ion varies with the class and type of sulphonic acid. On the other hand, it was found that the Cl^- and Br^- forms of the tetraalkylammonium salts were as effective here as the OH^- form, whereas the I^- and ClO_4^- forms were not useful. Chlorination of the sulphonic acid function with thionyl chloride was accomplished within 10 min at 80°C. The reaction of sulphonyl chlorides with DBA proceeded rapidly at 80°C and gave the corresponding sulphonamide derivatives. The total derivatization process could be performed within 30 min.

The structures of the derivatives were confirmed by GC–MS. The mass spectrum of the dibutylamide derivative of *p*-toluenesulphonic acid is shown in Fig. 2. The molecular ion peak (M^+) with the postulated *m/z* 283 and the prominent fragment ion peaks, *m/z* 240 ($M^+ - C_3H_7$), 155 [$M^+ - N(C_4H_9)_2$] and 91 [$M^+ - SO_2N(C_4H_9)_2$] were observed and were useful for structure elucidation.

The derivatives were found to be very stable under normal laboratory conditions and no thermal decomposition was observed during the GC analysis. Typical chromatograms are shown in Fig. 3.

In order to test the linearity of the calibration graph, various amounts of sulphonic acids ranging from 2 to 50 μg were derivatized in a mixture and aliquots representing 40–1000 ng of the acids were injected into the GC system. In each in-

TABLE I

INFLUENCE OF THE NATURE OF THE ALKYLAMMONIUM AS COUNTER ION ON THE ION-PAIR EXTRACTION OF ALIPHATIC AND AROMATIC SULPHONIC ACIDS

Alkylammonium hydroxide	*Peak-height ratio[a] for sulphonic acids[b]*											
	C_1	C_3	C_5	C_7	C_9	C_{13}	C_{18}	*B*	*T*	*X*	*M*	*N*
Tetraethylammonium	0	0	0	0.030	0.217	0.990	0.720	0.020	0.019	0.019	0.022	0.039
Tetrapropylammonium	0	0	0.014	0.275	0.841	0.985	0.720	0.057	0.150	0.253	0.388	0.441
Tetrabutylammonium	0.017	0.083	0.587	0.828	0.957	0.954	0.682	0.909	1.068	0.991	0.882	0.700
Tetrapentylammonium	0.387	0.618	0.904	0.946	1.030	0.953	0.682	1.048	1.123	1.021	0.921	0.663
Tetrahexylammonium	0.850	0.935	1.056	0.963	0.981	0.832	0.595	0.530	0.679	0.586	0.697	0.366
Tetraheptylammonium	0.932	0.978	0.973	0.663	0.490	0.624	0.446	0.332	0.314	0.218	0.331	0.129
Tetraoctylammonium	0.780	0.650	0.656	0.417	0.281	0.352	0.321	0.308	0.304	0.221	0.328	0.113

[a] Peak-height ratios are given relative to the internal standard (anthracene).

[b] C_1–C_{18} = aliphatic; B = benzene-; T = toluene-; X = 2,4-dimethylbenzene-; M = 2,4,6-trimethylbenzene-; N = α-naphthalenesulphonic acids.

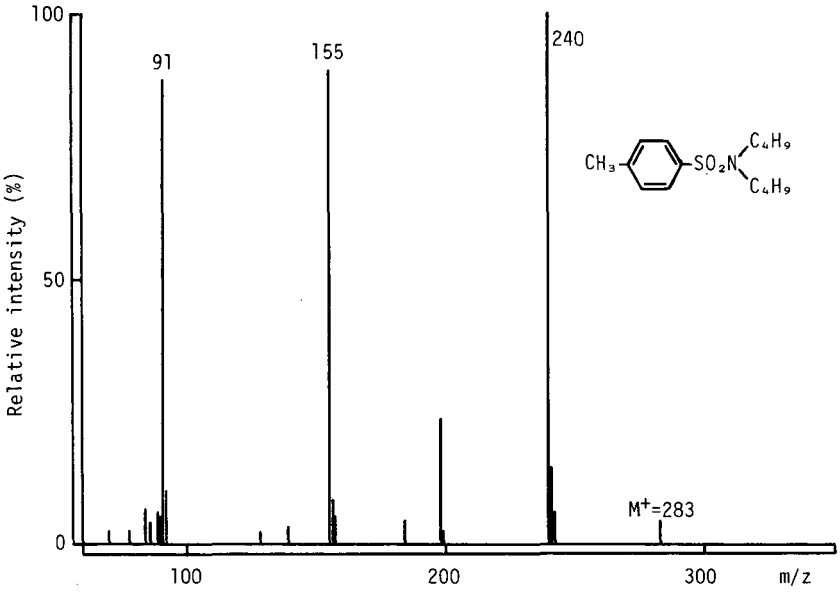

Fig. 2. Mass spectrum of dibutylamide derivative of *p*-toluenesulphonic acid.

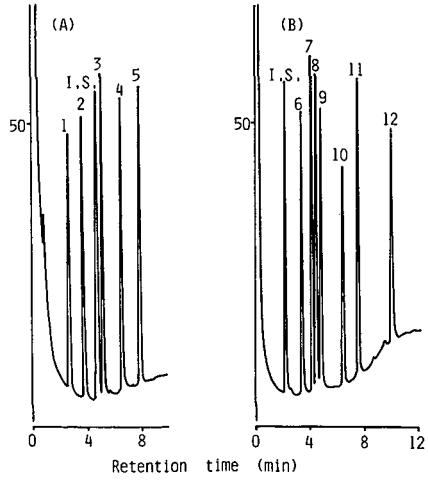

Fig. 3. Gas chromatography of mixtures of sulphonic acids as the corresponding dibutylamide derivatives. GC conditions: column, 1.0% silicone SE-54 on Uniport HP (100–120 mesh), 1.5 m × 3 mm I.D., glass; column temperature, (A) programmed at 15°C/min from 100 to 250°C and (B) programmed at 15°C/min from 140 to 285°C; injection and detector temperatures, 290°C; nitrogen flow-rate, 45 ml/min. Peaks: 1 = methane-; 2 = 1-propane-; 3 = 1-pentane-; 4 = 1-heptane-; 5 = 1-nonane-; 6 = benzene-; 7 = *p*-toluene-; 8 = 2,4-dimethylbenzene-; 9 = 2,4,6-trimethylbenzene-; 10 = α-naphthalene-; 11 = 1-tridecane; 12 = 1-octadecanesulphonic acid; I.S. = anthracene. Each peak represents 400 ng of the acid.

TABLE II

LINEAR REGRESSION DATA FOR SOME SULPHONIC ACIDS

Sulphonic acid	*No. of data*	*Regression line*[a]	*Correlation coefficient (r)*
C_1	15	$y=43.09x-6.95$	0.9963
C_7	15	$y=48.30x-3.90$	0.9977
C_{13}	15	$y=46.93x+5.47$	0.9995
p-Toluene-	15	$y=53.80x-5.85$	0.9996
α-Naphthalene-	15	$y=34.63x-6.17$	0.9973

[a] y = Peak-height ratio; x = amount of each sulphonic acid.

stance, a linear relationship was obtained and the reproducibility was found to be satisfactory (Table II).

These experiments have conclusively demonstrated that aliphatic and aromatic sulphonic acids can be succesfully analysed at the microgram level by GC of their sulphonamide derivatives.

REFERENCES

1 J. D. Knight and R. House, *J. Am. Oil Chem. Soc.*, 36 (1959) 195.
2 E. A. Setzkorm and A. B. Carel, *J. Am. Oil Chem. Soc.*, 40 (1963) 57.
3 S. Nishi, *Bunseki Kagaku*, 14 (1965) 912.
4 S. Nishi, *Bunseki Kagaku*, 14 (1965) 917.
5 T. Nakagawa, K. Miyajima and T. Uno, *J. Gas Chromatogr.*, 6 (1968) 292.
6 S. Siggia, L. R. Whitlock and J. C. Tao, *Anal. Chem.*, 41 (1969) 1387.
7 J. J. Kirkland, *Anal. Chem.*, 32 (1960) 1388.
8 J. B. Himes and I. J. Dowbak, *J. Gas Chromatogr.*, 3 (1965) 194.
9 J. S. Parson, *J. Chromatogr. Sci.*, 11 (1973) 659.
10 J. S. Parson, *J. Gas Chromatogr.*, 5 (1967) 254.
11 W. Funasaka, T. Kojima and T. Toyota, *Bunseki Kagaku*, 14 (1965) 815.
12 A. Heywood, A. Mathias and E. Williams, *Anal. Chem.*, 42 (1970) 1272.
13 K. M. Baker and G. E. Boyce, *J. Chromatogr.*, 117 (1976) 471.
14 A. El Homsi, B. Gilot and J. P. Canselier, *J. Chromatogr.*, 151 (1978) 413.
15 A. M. Nardillo, R. C. Castells, E. L. Arancibia and M. L. Caslla, *Chromatographia*, 25 (1988) 618.
16 J. Eagles, *Anal. Chem.*, 43 (1971) 1697.
17 T. Ishii, K. Yamane, H. Kato and K. Kunishiro, *Yukagaku*, 24 (1975) 23.
18 H. Oka and T. Kojima, *Bunseki Kagaku*, 28 (1979) 410.
19 H. Kataoka, S. Yamamoto and M. Makita, *J. Chromatogr.*, 306 (1984) 61.
20 H. Kataoka, N. Ohnishi and M. Makita, *J. Chromatogr.*, 339 (1985) 370.
21 E. V. Dehmlow and S. S. Dehmlow, *Phase Transfer Catalysis*, Verlag Chemie, Weinheim, 1982.

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CHROM. 21 485

Note

Coupling of proteolytic quenching and high-performance liquid chromatography to enzyme reactions

Application to bovine pancreatic ribonuclease

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The elucidation of an enzyme mechanism implies the identification and temporal resolution of the reaction intermediates. Many systems can be studied by convenient optical methods (*e.g.*, stopped-flow), but not all give a signal and even if there is one it may be difficult to assign it to a given chemical species.

High-performance liquid chromatography (HPLC) is a powerful analytical technique which has been much used to identify chemically the components of enzyme reaction mixtures. In such studies the investigator is faced with three problems. First, the conditions must be found under which the reaction mixture can be stopped ("quenched", *e.g.*, by acid) without introducing artifacts. Second, the enzyme must not be reactivated during the subsequent treatment (*e.g.*, pH adjustment) often necessary for HPLC. These problems have been discussed[1]. Finally, the chemical species of interest must be cleanly separated by the HPLC method used.

It was our aim to extend previous studies[2,3] on bovine pancreatic ribonuclease (RNase) with cytidine 2′:3′-cyclic phosphate (C>p) as the substrate to a detailed chemical analysis of the two reactions of this system: synthesis to cytidylyl-3′:5′-cytidine 2′:3′-cyclic phosphate (CpC>p) and hydrolysis to 3′-CMP. In particular, we wished to study the transient kinetics of the RNase–C>p systems with the aim of detecting any relationship between the synthetic and hydrolytic pathways. Such a study necessitates the sampling of reaction mixtures milliseconds old, which can be carried out by the rapid flow quench method[4,5].

Here we report an HPLC procedure for the rapid separation and determination of the two products of RNase in the presence of very high concentrations of the substrate C>p. The success of this analysis depends on an effective way of immedi-

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ately stopping the reaction and destroying the enzyme without decomposing the labile substrate and product CpC>p. With RNase this was a problem. Thus, by decreasing the pH to 2 the reaction is stopped but on increasing it for the subsequent HPLC analysis the enzyme is reactivated. It is not possible to use a stronger acid as this decomposes C>p and CpC>p. Finally, it was found that the RNase is destroyed by adding pepsin to the reaction mixture at pH 2. The pH could now be increased and the mixture then analysed by HPLC. By this procedure, which we term proteolytic quenching, we were able to obtain progress curves for the formation of CpC>p and 3'-CMP at high substrate concentrations. We propose that proteolytic quenching could be of use with other systems involving robust enzymes.

EXPERIMENTAL

Materials

Bovine pancreatic ribonuclease (twice crystallized) was purchased from Biozyme (Blaenavon, U.K.). The RNase A component was obtained as described previously[6]. The enzyme concentration was calculated by using $\varepsilon_{278} = 9800$ l mol^{-1} cm^{-1} (ref. 7).

C>p was synthesized from the isomeric mixture of cytidine 2'- and 3'-phosphates (Sigma) according to the method of Szer and Shugar[8] and its purity checked as described[9]. Concentrations of C>p were calculated by using $\varepsilon_{268} = 6850$ l mol^{-1} cm^{-1} (ref. 10).

Pepsin from porcine stomach mucosa was purchased from Sigma. Ammonia and acetic acid (HPLC grade) were obtained from Scharlau (FEROSA, Barcelona, Spain). All other reagents were of analytical-reagent grade.

A Nucleosil 10 SB anion-exchange column (300 mm × 8 mm O.D. × 4 mm I.D.) and Vydac-310SB precolumn stationary phase were purchased from Macherey, Nagel & Co. (Düren, F.R.G.).

Apparatus

All the HPLC experiments were carried out with a modular HPLC apparatus (LKB, Bromma, Sweden) consisting of a pump (Model 2150) controlled by an automated gradient controller (Model 2152) and a sample injector with a 20-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.). Nucleotides were detected by monitoring the effluent at 254 nm with an absorbance detector (Uvicord SD, 2158) and an integrator–recorder unit (Chromatopac C-R3A; Shimadzu, Kyoto, Japan).

^{31}P NMR measurements were carried out on a Bruker AM 500 NMR spectrometer with a resonance frequency of 202.46 MHz for the phosphorus nucleus.

Reaction conditions

All reactions were carried out at constant temperature (25°C), pH (5.5) and ionic strength (0.2). Both the enzyme and substrate were dissolved in 0.2 *M* acetic acid–sodium acetate buffer (pH 5.5). Typically, the reaction mixture contained 25 μl of substrate dissolved in the buffer to which 5 μl of RNase solution was added.

Identification of nucleotides by means of ^{31}P NMR

The samples to be assayed were obtained by HPLC separation. Both samples

and standards were dissolved in 0.1 *M* Tris–acetic acid (pH 7.0). Phosphoric acid was used as an external standard and was assigned a chemical shift value of 0 ppm.

RESULTS AND DISCUSSION

Proteolytic quenching

The RNase reactions are readily stopped by the addition of acid but even with acids of relatively low strenght, such as 4% trichloroacetic acid or 0.2 *M* HCl, the 2′:3′-cyclic phosphodiester bond is cleaved to the 2′- and 3′-phosphomonoesters. Finally, it was found that 0.2 *M* H_3PO_4 in 0.1 *M* HCl (pH 1.8) added to an equal volume of the reaction mixture (0.2 *M* acetic acid–sodium acetate, pH 5.5) gave a solution with a final pH of 2. At this pH the acid hydrolysis of the substrate and the product CpC>p is low and the reactions are stopped.

Separate studies showed that the substrate and reaction products of the RNase–C>p system could be separated by ion-exchange HPLC. This requires the adjustment of the pH of the mixture to that of the original reaction mixture (5.5), which almost completely reactivated the enzyme, making the analysis of reaction mixtures a few minutes old impossible.

Furthermore, active enzyme is adsorbed on the HPLC precolumn, where its accumulation causes problems with succeeding samples. Means of irreversibly destroying the acid-quenched reaction mixture were therefore sought.

The irreversible inactivation of RNase at pH 2 was finally achieved by incubating it with pepsin. This protease cleaves the Phe 120–Asp 121 bond in RNase, giving a derivative which is known as "pepsin-inactivated RNase". This material is inactive[11].

The quenching procedure finally adopted was as follows. RNase reaction mixture (30 μl; see Figs 1 and 2 for details) were incubated for the desired times and quenched by the addition of 25 μl of 0.2 *M* H_3PO_4 in 0.1 *M* HCl. The final pH was 2. This was followed by the immediate addition of 5 μl of a pepsin solution (3.6 mg/ml) and the mixture was left for 15 min at 25°C. Under these conditions, complete and irreversible inactivation of the RNase was obtained. The mixture was either analysed immediately or kept at −20°C. Immediately before injecting the sample on to the HPLC column, the pH was increased to 5.5 by the addition of 4 μl of 2 *M* NaOH. Storage of the samples for several months at −20°C did not affect the reproducibility of the results provided that adequate blanks were carried out.

HPLC analysis

The assay method is based on the fast chromatographic separation of nucleotides by an anion-exchange HPLC column at pH 5.5. The conditions were as follows: 20 μl of the sample at pH 5.5 were injected on to the column, previously equilibrated with 0.1 *M* ammonium acetate solution (pH 5.5). Elution was carried out immediately at a flow-rate of 1 ml/min with a linear salt gradient from 0.1 to 0.6 *M* ammonium acetate solution (pH 5.5). To prevent clogging of the HPLC column with pepsin and digested RNase, a precolumn was used which was reconditioned after approximately 20 runs. Fig. 1 shows a typical chromatogram. Although the hydrolytic action of RNase A on C>p produces only 3′-CMP[3], a small amount of 2′-CMP appeared in the chromatograms. Both monophosphates are produced by the hydrolysis of C>p in the acid quench.

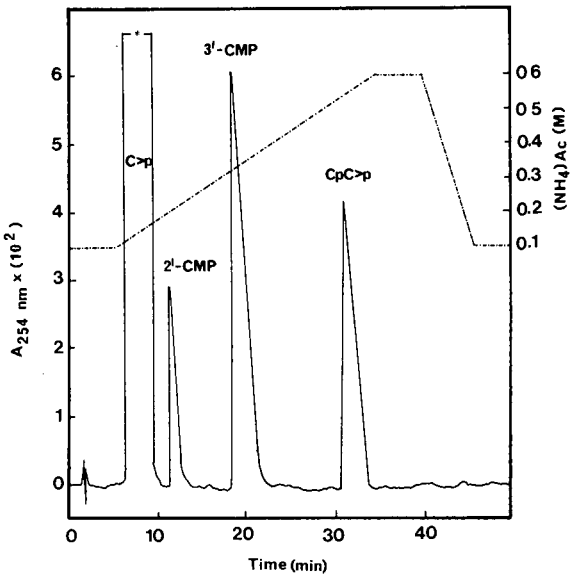

Fig. 1. Chromatography of the components of a reaction mixture (C>p = 40 m*M*, RNase A = 0.6 μM) incubated for 5 min. The reaction was stopped by addition of H_3PO_4–HCl and then pepsin and the products were separated on an anion-exchange HPLC column. Ac = Acetate.

The peaks C>p, 2'-CMP and 3'-CMP in the chromatogram were identified with the use of respective standards. The identity of CpC>p (retention time 34 min), the product of the synthetic activity of RNase, was ascertained in two ways: by digestion with RNase and by ^{31}P NMR. The material with a retention time of 34 min was incubated with RNase and subjected to HPLC analysis. After incubation for 2 min only a peak corresponding to C>p was observed. Longer incubation times

TABLE I

REPRODUCIBILITY OF THE ANALYSIS OF RNase REACTION MIXTURES BY HPLC

Reaction mixtures with initial C>p concentrations of (A) 40 m*M* or (B) 10 m*M* and 0.6 μM RNase were incubated for the times shown, quenched by acid proteolysis and analysed by HPLC. Each point is the average of three determinations. For further details, see the text and Fig. 1.

	Nucleotide	*Reaction time (min)*				
		0	*1*	*5*	*10*	*15*
A	C>p (m*M*)	36.7±1	35.1±1	34.6±1	33.1±1	32.8±1
	2'-CMP (μM)	111±15	114±18	115±20	102±14	106±17
	3'-CMP (μM)	161±16	243±19	374±19	462±21	582±20
	CpC>p (μM)	0	121±12	301±14	312±12	296±15
B	C>p (m*M*)	10.5±0.4	9.80±0.4	9.65±0.40	9.55±0.45	9.03±0.35
	2'-CMP (μM)	29±1	27±4	24±2	27±2	23±4
	3'-CMP (μM)	27±2	69±5	138±5	192±2	244±7
	CpC>p (μM)	0	14±3	14±2	13±5	15±3

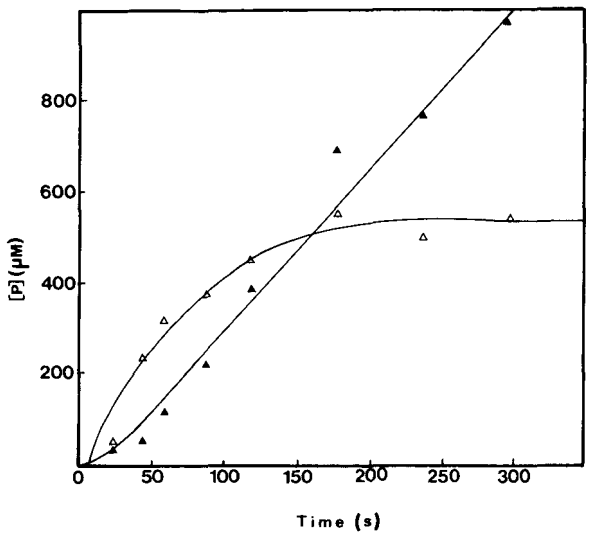

Fig. 2. Progress curves of the products of the RNase reaction obtained with acid proteolytic quenching. The reaction mixtures (50 m*M* C>p plus 0.55 μM RNase A) were incubated for the times shown, quenched with H_3PO_4–HCl and pepsin and separated by HPLC. ▲ = 3′-CMP; △ = CpC>p.

resulted in the progressive appearance of 3′-CMP with the concomitant disappearance of C>p. This is what would be expected from the hydrolysis by RNase of a dinucleotide of cytidine ending in a 2′:3′-cyclic phosphate.

Second, the structure of the dinucleotide was confirmed by ^{31}P NMR spectroscopy, which unambiguously showed the presence of both a 3′–5′-phosphodiester bond and a terminal 2′:3′-cyclic phosphate as judged by a resonance found at 20.576 ppm ascribed to the cyclic phosphate of the dinucleotide and another at −0.560 ppm ascribed to 3′–5′ internucleotide phosphodiester bond with reference to 3′–5′-CpC.

The reproducibility of the HPLC analysis of acid protease-quenched RNase reaction mixtures at two concentrations of C>p is assessed in Table I.

Kinetic analysis of the synthetic and hydrolytic reactions of RNase

By using the procedure described above, we obtained progress curves for CpC>p and 3′-CMP formation by RNase at a concentration of C>p of 50 m*M* (Fig. 2). The curves have the following features.

First, CpC>p synthesis is initially more rapid than 3′-CMP production, but a plateau is reached within 3 min. The size of this plateau increased with increasing C>p concentration, but it was independent of RNase concentration. Bernfield[12,13] also studied the synthetic reactions of RNase and obtained plateaux, but his conditions were different from ours (pH 7 and a time scale of several hours).

Second, there is a lag in the production of 3′-CMP before the steady state (k_{cat} = 3.2 s^{-1}) was attained. It is possible that there is a correlation between this lag and the burst phase observed in the dinucleotide formation. Hence there could be a relationship between the synthetic and hydrolytic pathways; this possibility is under further study.

In conclusion, proteolytic quenching coupled with a convenient HPLC analysis provides a means of studying the mechanism of RNase.

ACKNOWLEDGEMENTS

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REFERENCES

1 T. E. Barman and H. Gutfreund, *Biochem. J.*, 101 (1966) 411–416.
2 F. M. Richards and H. W. Wyckoff, in P. D. Boyer (Editor), *The Enzymes*, Vol. IV, Academic Press, New York, 3rd ed., 1971, pp. 647–806.
3 P. Blackburn and S. Moore, in P. D. Boyer (Editor), *The Enzymes*, Vol. XV, Academic Press, New York, 3rd ed., 1982, pp. 317–433.
4 H. Gutfreund, *Methods Enzymol.*, 16 (1969) 228–249.
5 T. E. Barman and F. Travers, *Methods Biochem. Anal.*, 31 (1985) 1–59.
6 G. Taborsky, *J. Biol. Chem.*, 234 (1959) 2652–2656.
7 M. Sela and C. B. Anfinsen, *Biochim. Biophys. Acta*, 24 (1957) 229–235.
8 W. Szer and D. Shugar, *Biochem. Prep.*, 10 (1962) 139–144.
9 J. A. Biosca, F. Travers and C. M. Cuchillo, *Eur. J. Biochem.*, 124 (1982) 151–156.
10 D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952) 2040–2049.
11 C. B. Anfinsen, *J. Biol. Chem.*, 221 (1956) 405–412.
12 M. R. Bernfield, *J. Biol. Chem.*, 240 (1965) 4753–4762.
13 M. R. Bernfield, *J. Biol. Chem.*, 241 (1966) 2014–2023.

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Note

Trifluoroacetic anhydride–sodium iodide as a reagent for the selective detection of nitrones and nitroxide radicals by thin-layer chromatography

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The importance of N-oxo compounds is connected with their technological applicability and use as reagents in organic and bioorganic chemistry[1]. In this class of nitrogen derivatives, special interest is focused on the properties and applications of nitroxide radicals and nitrones. Nitroxide radicals play a special role in spin labelling[2,3], whereas nitrones are important reagents in organic synthesis[4] and also serve as excellent spin traps in electron paramagnetic resonance techniques[5,6]. Hence the selective detection and determination of nitrones and nitroxide radicals are still important problems in nitrogen analytical chemistry[7–9].

Recently, we reported the application of trifluoroacetic anhydride–sodium iodide reagent (TFAA–I) for the microdeterminations of sulphoxides[10], nitrones[11] and nitroxide radicals[12]. We have also used this reagent for the selective detection of sulphoxides and sulphimides by thin-layer chromatography (TLC)[13].

Here we report a selective procedure for the detection of nitrones and nitroxide radicals in the presence of amine N-oxides, based on the application of TFAA–I reagent. This reagent is able to convert amine N-oxides (**1**) into the corresponding amines (**1b**), nitrones (**2**) into the corresponding trifluoroacetamidoalkyl trifluoroacetate (**2c**) and nitroxide radicals (**3**) into the corresponding hydroxylamine trifluoroacetates (**3c**), with the simultanous formation of elemental iodine, according to the equations[12] on the next page.

Because the rate of reaction of TFAA–I reagent and related systems with N-oxo compounds is influenced by their structures, there is the possibility of establishing conditions which allow the selective detection of nitrones and nitroxide radicals in the presence of aliphatic amine N-oxides.

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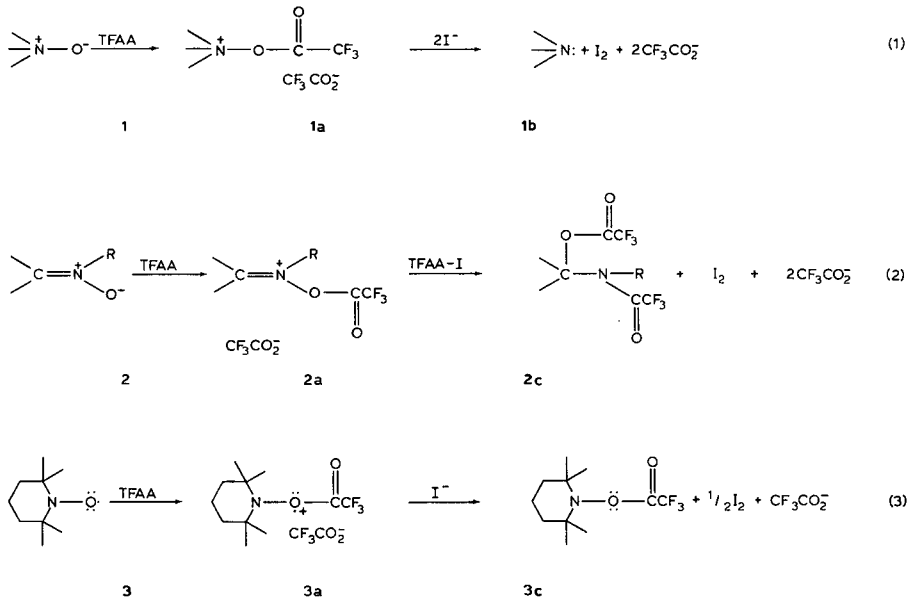


EXPERIMENTAL

Materials

Trifluoroacetic anhydride (TFAA), trimethylamine N-oxide dihydrate, pyridine N-oxide and quinoline N-oxide hydrate were purchased from Aldrich (Milwaukee, WI, U.S.A.). Trimethylamine N-oxide was dehydrated according to ref. 14 and pyridine N-oxide and quinoline N-oxide were dehydrated by double vacuum distillation.

Nitroxides **3a–i** were purchased from Aldrich. Nitrones **2a–h** were prepared according to ref. 15 and were all of the same purity as reported previously.

Solutions

A 0.8 *M* solution of sodium iodide (NaI) in anhydrous acetone and a 0.8 *M* solution of TFAA in anhydrous acetone (prepared immediately before use) were used.

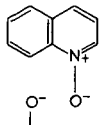
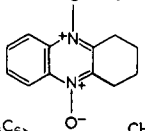
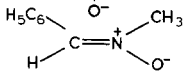
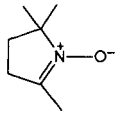
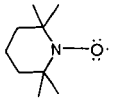
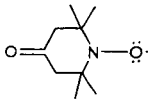
The concentrations of the compounds **1**, **2** and **3** chromatographed were *ca.* $5 \cdot 10^{-2}$–10^{-3} *M* in anhydrous acetone.

Dragendorff reagent (Bi) was a 2% solution of potassium bismuth tetraiodide in 0.01 *M* hydrochloric acid. DDQ reagent was a 2% solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in benzene. Other solutions were iron(III) chloride (Fe), 2% aqueous; potassium permanganate (Mn), 0.1% aqueous; 4-dimethylaminobenzaldehyde (DAB), 5% methanolic; ninhydrin reagent (NH), 2% in butanol saturated with water; bromophenol blue (Ind), *ca.* 0.05% in ethanol; and reduced sodium nitroprusside (SN), for which a 1.2% aqueous solution of sodium nitroprusside (10 ml) was reduced with sodium borohydride to give a clear, deep-red solution, 0.8 ml of acetic acid (1 *M*) was added and, after 2 min, 5 ml of water were added.

TABLE I

COMPARISON OF VARIOUS ACYLATING AGENT–HALIDE SALT SYSTEMS FOR THE DETECTION OF AMINE OXIDES **1**, NITRONES **2** AND NITROXIDE RADICALS **3** BY TLC

Silica gel plates. Solvents: acetone and methanol (**2g**). +/−, Spot is detectable; +, distinct detection; ++, strong detection.

Compound applied			*Ac_2O–NaI*	*AcCl–NaI*	*Ac_2O–LiBr*	*AcCl–LiBr*[a]	*TFAA–I*	*TFAA–LiBr*[a]
No.	*Structure*	*μg/spot*						
1c		1	−	−	−	−	−	−
		10	+	+	−	−	+	−
1d		1	−	+	−	−	+	−
		10	+/−	++	−	−	++	−
2a		1	−(+[b])	−(+[c])	−	−	+	−
		10	−(+[b])	−(+[c])	−	−	++	−
2g		1	−	−(+[b])	−	−	−	−
		10	+	−(+[b])	−	−	+	−
3a		1	−	+/−	−	−	+/−	−
		10	+/−	+	−	+	+	+
3b		1	−	+	−	−	+	−
		10	+/−	+	−	+/−	+	+

[a] Red-brown spots.
[b] After 30 min exposure.
[c] A spot of iodine appeared after 10 min exposure.

Thin-layer chromatography

Pre-coated silica gel 60 F_{254} aluminium sheets and pre-coated aluminium oxide 60 F_{254} aluminium sheets (10 cm × 5 cm), with a 0.2-mm thick layer (Merck, Darmstadt, F.R.G.), were used for all TLC experiments. The plates were spotted with an appropriate amount of compound (see tables), developed for a distance of 8 cm with acetone, air-dried and sprayed with sodium iodide solution and subsequently with TFAA solution. Nitrones **2** and nitroxides **3** appeared almost immediately as brown spots on a white background, and were stable for more than 20 min.

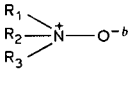
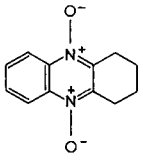
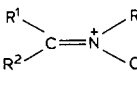
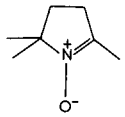
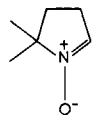
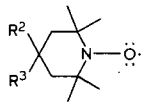
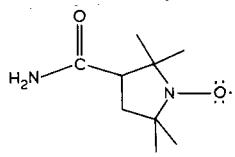
RESULTS AND DISCUSSION

The results of the application of various reagents, related to TFAA–I detection systems, for the detection N-oxo compounds by TLC are presented in Table I. These

results reveal that the most sensitive system for the detection of nitrones and nitroxide radicals is TFAA–I. Comparable properties were observed for acetyl chloride–sodium iodide reagent, which is slightly less sensitive for the detection of nitrones, however.

TABLE II

DETECTION LIMITS FOR AMINE OXIDES **1**, NITRONES **2** AND NITROXIDE RADICALS **3** USING TFAA-I AS THE DETECTION REAGENT

No.	*Structure*	*Substituents*			SiO_2			Al_2O_3		
		R^1	R^2	R^3	*Detection limit*		R_F[a]	*Detection limit*		R_F[a]
					μg	*nmol*		*μg*	*nmol*	
1d					5.0	23.3	0.52	10.0	46.6	0.68
2a		C_6H_5	H	CH_3	1.04	7.7	0.26	22.4	17.8	0.62
2b		C_6H_5	H	C_6H_5	1.0	5.0	0.70	2.3	11.7	0.72
2c		*m*-ClC_6H_4	H	C_6H_5	1.04	4.5	0.65	2.1	9.0	0.73
2d		*p*-$CH_3OC_6H_4$	H	C_6H_5	1.0	4.4	0.67	2.0	8.8	0.72
2e		*p*-ClC_6H_4	H	*tert.*-C_4H_9	5.7	22.3	0.61	11.4	44.5	0.79
2f		*p*-$O_2NC_6H_4$	H	*tert.*-C_4H_9	1.2	5.2	0.58	2.3	10.4	0.72
2g					5.0 5.0	31.0 31.0	0.05 0.75[c]	7.5	45.5	0.81[c]
2h					3.0 3.0	26.4 26.4	0.05 0.75[c]	5.0	44.0	0.76[c]
3a			H	H	1.1	7.1	0.66	2.2	14.2	0.68
3b			–O–		1.0	5.9	0.65	2.0	11.8	0.70
3c			H	OH	0.96	5.58	0.65	3.0	17.6	0.71
3d			CH_3	OH	1.08	5.8	0.71	2.2	12.0	0.78
3e			*tert.*-C_4H_9	OH	1.08	4.7	0.72	2.2	9.6	0.77
3f			$C_6H_5CH_2$	OH	1.1	4.27	0.73	2.2	8.5	0.80
3g			H	TosO	0.9	2.75	0.7	2.0	6.2	0.75
3h			H	NH_2	2.8	16.0	0.06	5.2	30.0	0.16
3i					1.04	5.6	0.51	2.08	11.2	0.59

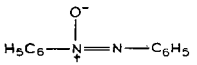
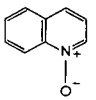
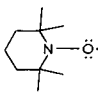
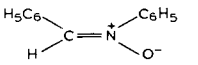
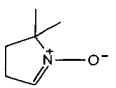
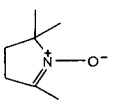
[a] Acetone.

[b] Trimethylamine N-oxide (**1a**) did not give a positive test with TFAA–I even for applied amounts ≤ 50 μg/spot, whereas pyridine N-oxide (**1b**) and quinoline N-oxide (**1c**) did for applied amounts ≥ 10 μg/spot.

[c] Methanol.

TABLE III

COMPARISON OF REAGENTS FOR THE DETECTION OF N–O COMPOUNDS BY TLC

Compound		*Detection reagent*										
Structure	*Amount applied (μg/spot)*	*UV*	I_2	*TFAA-I*	*Bi*[a]	*Mn*[b]	*Fe*	*NH*	*Ind*	*SN*[c]	*DDQ*	*DAB*[d]
	1	+/−[e]	−	−	−	−	−	−	−	−	−	−
	10	++[e]	−	−	−	−	−	−	+/−[f]	−	−	+
	50	++[e]	+/−	−	−	+/−	−	−	+/−[f]	−	−	+
	1	+	−	−	−	−	−	−	−	−	−	−
	10	+	+	−	+	+	+		+/−[g]	−	−	−
	50	+	+	−	+	+	+	−	+[g]	−	−	−
	1	−	−	+/−	−	+	−	−	−	+	−	−
	10	+/−	+	+	−	+	−	−	−	+	−	−
	50	+	+	++	−	+	−	−	−	+	−	−
	1	+	+	+	−	−	−	−	−	−	−	−
	10	+	+	+	−	+	−	+[h]	+/−[g]	−	−	+/−
	50	+	+	++	+	+	−	+[h]	+/−[g]	−	+[i]	+
	1	+	+	−	−	−	−	+/−[j]	−	−	−	−
	10	+	+	+	−	+	−	+[j]	−	−	−	−
	50	+	+	+	−	+	−−	+[j]	−	−	−	+/−
	1	+	+	−	−	−	−	+/−[k]	−	−	−	−
	10	+	+	+	−	+	−	+[k]	−	−	−	−
	+	+	+	−	+	−	+[k]	−	−	−	−	−

[a] Red spots.
[b] Grey-green.
[c] Blue spots on a grey-green background.
[d] Yellow spots.
[e] Brown spots after UV irradiation.
[f] Grey-blue spots on a blue-violet background.
[g] Grey-blue spots on a pink-blue background.
[h] Grey spots after preheating to *ca.* 100°C.
[i] Red spots, turning to green after saturation with ammonia vapour.
[j] Grey-blue spots after preheating to *ca.* 150–200°C.
[k] Yellow-brown spots after preheating to *ca.* 150–200°C.

The replacement of sodium iodide with lithium bromide leads to the more selective detection of nitroxide radicals, but a decrease in sensitivity also occurs. These results clearly indicate the superiority of TFAA–I as a detection reagent for nitrones and nitroxide radicals by TLC.

Results for the application of TFAA–I for the detection of amine N-oxides **1**, nitrones **2** and nitroxide radicals **3** are summarized in Table II. It is evident that the detection limits of N-oxy compounds are strongly influenced by their structures. Thus, aliphatic amine N-oxides do not react at all with TFAA–I, heterocyclic amines N-oxides react slowly with high detection limits, the acyclic nitrones are detectable at 5–10 nmol and cyclic aliphatic nitrones (both aldo and keto nitrones) are detectable at *ca.* 30–40 nmol. The detection limits of nitroxide radicals vary from 5 to 10 nmol.

Results are given in Table III for the evaluation of TFAA–I reagent in comparison with other spray reagents for the detection of N-oxy compounds.

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REFERENCES

1 E. Breuer, in S. Patai (Editor), *The Chemistry of Amino, Nitroso and Nitro Compounds and their Derivatives*, Wiley, New York, 1982.
2 B. J. Gaffney, in L. H. Berliner (Editor), *Spin Labelling —Theory and Applications*, Academic Press, New York, 1976, p. 184.
3 E. G. Rozentsev and V. D. Stolle, *Synthesis* (1971) 190 and 401.
4 J. J. Tufarinello, in A. Padva (Editor), *1,3-Dipolar Cycloaddition Chemistry*, Vol. 2, Wiley-Interscience, New York, 1984, Ch. 9.
5 C. A. Evans, *Aldrichim. Acta*, 12 (1979) 23.
6 *Can. J. Chem.*, 60 (1982) 1379 (special collection of papers on spin trapping chemistry).
7 A. F. Krivis, in G. A. Streuli and P. R. Averell (Editors), *The Analytical Chemistry of Nitrogen and its Compounds*, Vol. 28 (1), Wiley-Interscience, New York 1970, p. 253.
8 L. Reio, *J. Chromatogr.*, 88 (1974) 119.
9 L. A. Damani, L. H. Patterson and J. W. Gorrod, *J. Chromatogr.*, 155 (1978) 337.
10 W. Ciesielski, J. Drabowicz, W. Jędrzejewski, Z. H. Kudzin and R. Skowroński, *Talanta*, 35 (1988) 969.
11 W. Ciesielski, J. Drabowicz, W. Jędrzejewski, Z. H. Kudzin and R. Skowroński, paper presented at the *31st IUPAC International Congress, Sophia, 1987.*
12 W. Ciesielski, J. Drabowicz, W. Jędrzejewski, Z. H. Kudzin and R. Skowroński, paper presented at the *Polish Chemical Society Congress, Łódź, 1988.*
13 J. Drabowicz, A. Kotyński and Z. H. Kudzin, *J. Chromatogr.*, 447 (1988) 225.
14 J. A. Soderquist and C. L. Anderson, *Tetrahedron Lett.*, 27 (1986) 3961.
15 W. Rundel, in D. Klaman (Editor), *Methoden der Organischen Chemie (Houben-Weyl)*, Vol. X-4, Georg Thieme, Stuttgart, 1968, p. 309.

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

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Note

Efficient clean up of non-aqueous plant extracts using reversed-phase cartridges

Applications to the determination of phytoalexins from *Brassica* spp. by high-performance liquid chromatography

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Phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized *de novo* by plants in response to microorganism challenge[1]. These metabolites are a chemically heterogeneous group of compounds but certain chemical types tend to be associated with particular plant families[2].

A phytoalexin, brassilexin (Fig. 1), has been recently isolated from *Brassica juncea* and *B. napus*[3,4]. Its accumulation was related to resistance to *Leptosphaeria maculans*, a fungus which causes the blackleg disease of crucifers[4,5]. Brassilexin has been related to sulphur-containing indol phytoalexins, which were isolated from Chinese cabbage (*Brassica campestris* spp. *pekinensis*), Japanese radish (*Raphanus sativus*) and oilseed rape (*B. napus*)[4,6–9] (Fig. 2).

Extraction of phytoalexins from leaves and further steps of purification and determination are usually complicated by the presence of pigments, waxes, sterols and other interfering compounds. All these compounds are also highly damaging to high-performance liquid chromatography (HPLC) equipment and columns. Many authors bypass this problem using non-chlorophyllous or etiolated plant tissues, *e.g.* refs. 9–14, or diffusates from elicited plant tissues, *e.g.* refs. 15 and 16. Extraction with light petroleum or hexane and preparative chromatography have also been used to remove pigments, *e.g.* refs. 17–20. These methods were usually inefficient or their yields were too low and insufficiently consistent for quantitative analysis of brassilexin from small plant samples (fresh weight 1–2 g)[4].

In this paper, we present a new and unusual use of reversed-phase cartridges to eliminate these interfering compounds from non-aqueous plant extracts, prior to HPLC quantitative analysis of sulphur-containing indol phytoalexins.

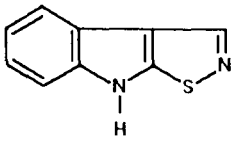

Fig. 1. Chemical structure of brassilexin[3].

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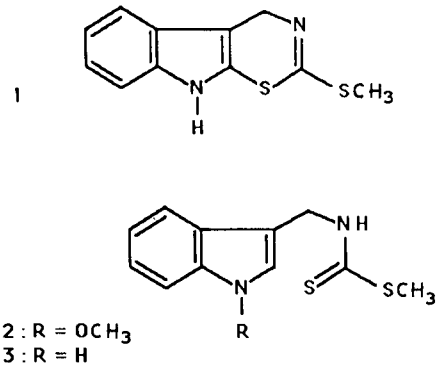

Fig. 2. Chemical structures of cyclobrassinin (**1**), methoxybrassinin (**2**) and brassinin (**3**)[8].

MATERIALS AND METHODS

Plant material and elicitation

B. juncea cv. Aurea and *B. napus* (cvs. Brutor and Primor) plants were grown in the glasshouse, under the conditions previously described[5].

To elicit phytoalexin accumulation, plants in the greenhouse or leaf disks were contaminated with pycniospore suspensions of *L. maculans* or sprayed with a 10 m*M* aqueous solution of $CuCl_2$ containing Tween 80 as described previously[5].

Instrumentation and chemicals

Extra-Sep reversed-phase cartridges (sorbent mass/column volume : 100 mg/1.0 ml) were obtained from Lida Manufacturing Corp. and Bond Elut endcapped reversed-phase cartridges from Analytichem International. The sorbents tested were C_{18}, C_8, C_2, CH, PH and CN.

A Waters 600 multisolvent delivery system equipped with a Waters U6K universal injector was used for analytical HPLC. Chromatographic and spectral data from the eluate were acquired with a Waters 990 photodiode array detector. Data were then stored in the memory and treated by a NEC APC III computer loaded with the Waters 990 software. Reversed-phase HPLC took place through a Brownlee C_{18} column (220 mm × 4.6 mm, particle size 5 μm) which was directly coupled with a 30-mm Brownlee guard column packed with the same stationary phase.

Solvents were obtained from Carlo Erba [ethanol 95% (v/v) RPE-ACS, methanol RS HPLC] or Prolabo (hexane R. P. Normapur, dichloromethane HPLC). Twice distilled water was produced in the laboratory. HPLC solvents were filtered through 0.45 μm Millipore filters and degassed by flushing through with helium.

Extraction of phytoalexins

To extract brassilexin from *B. juncea*, elicited leaves or leaf disks (fresh weight 1–2 g) were macerated in 95% (v/v) ethanol at 80°C for 15 min as previously described[5]. To extract methoxybrassinin and cyclobrassinin from *B. napus*, elicited leaves or leaf disks (1–2 g) were homogenized in 95% (v/v) ethanol at room temperature (Waring blender)[5]. The ethanolic extracts were evaporated to dryness under reduced pressure at 40°C and the residues taken up in hexane (2 × 2 ml).

Sample clean up (Fig. 3)

The cartridges were dried in an oven (40°C, 4 h) before use. Hexane samples were passed through the *unsolvated* cartridges and the sorbent washed with hexane until elution of a colourless solution. Cartridges were then eluted with 3 × 1 ml methanol–water (50:50, v.v). The colourless eluates containing the phytoalexins were dried under reduced pressure (40°C) and recovered in dichloromethane (3 × 3 ml). Dichloromethane was evaporated under a stream of nitrogen (50°C, Reacti-therm and Reacti-vap, Pierce) to prevent the strong oxidation of these compounds in the air. Finally the residue was taken up in 100 μl 95% (v/v) ethanol.

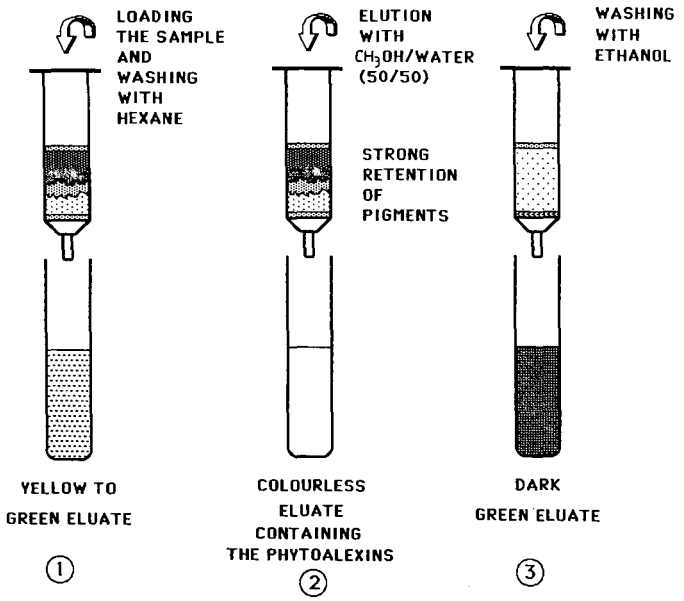

Fig. 3. Sample clean up protocol using reserved-phase cartridges.

Quantitative analysis of phytoalexins

Samples (20 μl) were chromatographed on the analytical C_{18} column eluted with a methanol–water gradient as previously described[5]. Known quantities of brassilexin (purified in the laboratory), brassinin, cyclobrassinin and methoxybrassinin (calculated from their absorption coefficients recorded with a Pye Unicam SP8-200 spectrophotometer) were chromatographed under the same conditions as standards.

Protocol efficiency

The efficiency of the protocol was estimated by adding 5–10 μg of purified phytoalexins to the ethanol during the extraction of unelicited plant samples. The quantities of phytoalexins recovered after sample clean up and HPLC analysis were compared to the quantities added to the ethanol.

RESULTS

Extraction of phytoalexins

Under the analysis conditions, the retention times of brassilexin, brassinin, methoxybrassinin and cyclobrassinin were 7.4 (± 0.5), 8.2 (± 0.3), 9.2 (± 0.2) and 9.7 (± 0.2) min respectively.

The extraction of elicited *B. napus* tissues with hot ethanol allowed the elimination of a compound with a retention time close to that of brassilexin. As this phytoalexin was usually present in small amounts in these plants[4,5], this extraction facilitated the determination of brassilexin. The extraction led to important losses of brassinin, methoxybrassinin and cyclobrassinin and was inadequate for quantitative analysis of these phytoalexins (Table I). Extraction by homogenization of leaves in ethanol at room temperature permitted an excellent recovery of all phytoalexins (Table I) but quantitative analyses of brassilexin was less precise in elicited *B. napus* tissues.

TABLE I

RECOVERY (%) OF PHYTOALEXINS AFTER EXTRACTION, CLEAN UP AND HPLC ANALYSIS

Amounts of 5–10 μg of phytoalexins were added to ethanol at 80°C or ethanol at room temperature during the extraction of unelicited plant samples. Plant extracts were then cleaned up using various reversed-phase cartridges. The recovery is expressed as percent of the quantity of phytoalexin added to the ethanol. Each value is the mean of 12–15 measurements (three experiments) and the standard deviation is noted.

Cartridge	*Brassilexin*	*Brassinin*	*Methoxy-brassinin*	*Cyclo-brassinin*
C_{18} (extraction with ethanol at 80°C)	77.8 ± 5.7	47.0 ± 3.4	27.6 ± 7.8	51.8 ± 9.4
C_{18}	83.9 ± 5.7	82.9 ± 5.9	82.5 ± 5.6	67.2 ± 6.0
C_8	82.0 ± 4.9	82.4 ± 7.2	80.7 ± 9.9	66.5 ± 4.8
C_2	75.6 ± 3.0	82.8 ± 3.4	68.2 ± 12.4	65.8 ± 5.2
Ph	67.3 ± 8.9	87.5 ± 9.3	84.2 ± 7.2	71.3 ± 11.7

Efficiency of various reserved-phase cartridges in sample clean up

As shown in Fig. 3, washing by hexane, whatever the volume introduced, gave a yellow to green eluate containing no traces of brassilexin, brassinin, methoxybrassinin and cyclobrassinin. A nearly colourless eluate containing these phytoalexins was obtained upon elution with methanol–water. Pigments and other interfering compounds were strongly retained on the sorbent from which they were eluted by 95% (v/v) ethanol. In this case, the cartridges could be used again with the same efficiency if carefully dried (with the exception of endcapped reversed-phase, see below). This sample clean up (Fig. 4) permitted the elimination of polar compounds, which were not dissolved with hexane and dichloromethane, and of non-polar compounds (retention time > 11 min) showing a strong absorbance at 420 nm.

All types of bonded silicas tested, whether endcapped or not, cleaned up the *Brassica* extracts equally efficiently (Fig. 5) without interfering with brassilexin. The

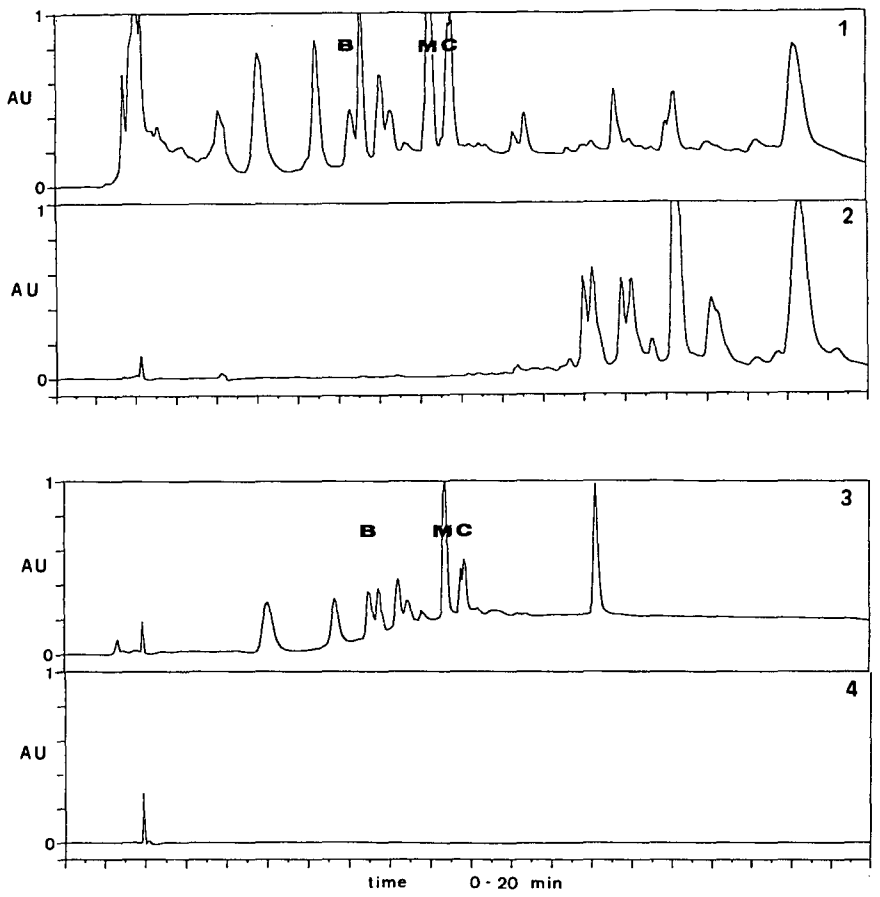

Fig. 4. Chromatograms at 218.2 (1 and 3) and 420 nm (2 and 4) of an ethanolic extract of elicited *B. napus* before (1 and 2) and after (3 and 4) sample clean up. The leaves were macerated in ethanol 80°C and the extract was cleaned up using C_{18} cartridges (see Fig. 3). The cleaned up samples (20 μl) were injected on a C_{18} analytical column (5 μm particle size). The solvent delivery consisted of a linear gradient from 50% (v/v) methanol in water to 100% methanol in 5 min, then maintained for 10 min. The flow-rate was constant at 1.5 ml min^{-1}. B = brassilexin; M = methoxybrassinin; C = cyclobrassinin.

total yield of the method was very high for brassilexin, brassinin and methoxybrassinin, using C_{18} or C_8 cartridges to clean up samples (Table I). It was lower for cyclobrassinin, whatever the sorbent used.

DISCUSSION

Dahiya and Rimmer recently described procedures for determining methoxybrassinin and cyclobrassinin from elicited *B. napus* or *B. juncea* tissues. Since these authors used callus tissues[14] and leaves or stem segments incubated in the dark[9], their analyses were less complicated by interfering compounds, *e.g.*, pigments than ours (see Fig. 4), and the use of a guard column was sufficient to protect the column.

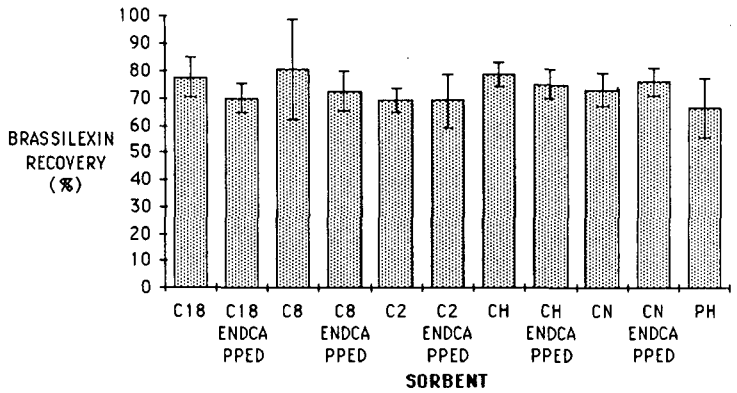

Fig. 5. Brassilexin recovery after sample clean up and HPLC analysis. Amount of 5–10 μg of the phytoalexin were added to ethanol during the extraction of unelicited plant samples. These samples were then cleaned up using various endcapped or non-endcapped reversed-phase cartridges. The recovery is expressed as percent of the phytoalexin added to the ethanol. Each value is the mean of 12–24 measurements (3–6 experiments) and the standard deviation is noted.

Brassilexin appears to be less apolar than methoxybrassinin and cyclobrassinin. We thus need a more polar elution system than that used by Dahiya and Rimmer[14] to get a good separation of indol phytoalexins. With this elution system, chlorophyllous pigments and other interfering compounds precipitate in aqueous solvents, and with repeated injections, are damaging to HPLC apparatus and columns (see Fig. 4). The filters and guard column used for analysis were inappropriate to protect the column and pipes.

We previously proposed a protocol for brassilexin analysis, in which ethanolic extracts were fractionated between water and diethyl ether prior to HPLC analysis[5]. This permitted us to discard polar compounds but most of the pigments were still present in the samples and interfered with the analysis. We therefore tested classic uses of silica or bonded silica cartridges to clean up samples. Neither silica cartridges with non-polar solvents (hexane or dichloromethane) nor bonded silica cartridges with polar solvents (methanol–water) allowed us to eliminate undesirable compounds.

Finally, we considered a less classic separation. First hexane was used to dissolve samples, thus discarding most polar compounds. The separation obtained with the novel strategy, described here using bonded silicas and appropriate solvents, may first be due to polar interactions between some compounds of the sample and non-bonded silanol groups in the non-polar solvent (Fig. 3, No. 1). In this case, the most non-polar metabolites, *e.g*, lipids or carotenoids are not retained on the sorbent and are directly eluted with hexane. Other compounds are retained by polar interaction with silanol groups. Elution with a polar solvent (methanol–water) solvates the sorbent and suppresses polar interactions between the metabolites and silanol groups (Fig. 3 No. 2). The most non-polar compounds still present, *e.g*, chlorophyllous pigments will then interact with the functional groups of bonded silicas (non-polar interaction in a polar solvent) and be retained. Moderately non-polar compounds, like the phytoalexins, are eluted with the solvent. Finally, the retained compounds are eluted with ethanol (Fig. 3, No. 3).

To support this hypothesis it is important to note that all bonded silicas tested were equally efficient for the separation of phytoalexins from unwanted compounds (Table I, Fig. 5). On the other hand, the use of endcapped bonded silicas (mostly C_{18}) showed that chlorophyllous pigments, which were previously retained on the sorbent, are profusely eluted with hexane. As for brassilexin, it was identically retained on endcapped and non-endcapped sorbents (Fig. 5). This suggests, if our hypothesis is sound, that all silanol groups are not methylated during the endcapping process. In this respect we note that non-endcapped cartridges can be used many times with equal efficiency. On the contrary, endcapped cartridges can never be used more than twice to clean up samples. On the third use of the same cartridge, phytoalexins and pigments are no longer retained on the sorbent. This may mean that irreversible bonding to silanol groups occurred during the cleaning up the samples. The fewer free silanol groups present in endcapped bonded silicas than in non-endcapped ones would then explain why the latter can be reused many times, which is not the case with the former. The same phenomenon was observed with insufficiently dried sorbent. In this case, unactivated silanol groups led to important losses of phytoalexins and pigments.

The protocol presented here for quantitative analysis of *Brassica* phytoalexins is easy to use, rapid and accurate. Its yield is excellent. It would then be interesting to see whether it can be used to separate other metabolites from various kind of matrices. In our case, this protocol is particularly efficient for comparison of the accumulation of phytoalexins between single plantlets or even single leaves, and to screen for plants possessing the genes conditioning the hypersensitive resistance to *L. maculans* in the population of interspecific crosses between *B. juncea* and *B. napus*[4,21].

ACKNOWLEDGEMENTS

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REFERENCES

1 J. D. Paxton, *Phytopath. Z.*, 101 (1981) 106.
2 J. W. Mansfield, in J. A. Callow (Editor), *Biochemical Plant Pathology*, Wiley, Chichester, 1983, p. 237.
3 M. Devys, M. Barbier, I. Loiselet, T. Rouxel, A. Sarniguet, A. Kollmann and J. F. Bousquet, *Tetrahedron Lett.*, 29 (1988) 6447.
4 T. Rouxel, *Thèse de l'Université de Paris Sud-Orsay*, Paris, 1988.
5 T. Rouxel, A. Sarniguet, A. Kollmann and J. F. Bousquet, *Physiol. Mol. Plant. Pathol.*, in press.
6 M. Takasugi, N. Katsui and A. Shirata, *J. Chem. Soc., Chem. Commun*, (1986) 1077.
7 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Chem. lett.*, (1987) 1631.
8 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Bull. Chem. Soc. Jpn.*, 61 (1988) 285.
9 J. S. Dahiya and S.R. Rimmer, *Phytochemistry*, 27 (1988) 3105.
10 N. Sato, K. Tomiyama, N. Katsui and T. Masamune, *Ann. Phytopathol. Soc. Jpn.*, 34 (1968) 140.

11 J. A. Bailey and J. L. Ingham, *Physiol. Plant Pathol.*, 1 (1971) 451.
12 N. T. Keen, *Phytopathology*, 65 (1975) 91.
13 A. O. Latunde-Dada, R. A. Dixon and J.A. Lucas, *Physiol. Mol. Plant Pathol.*, 31 (1987) 15.
14 J. S. Dahiya and S. R. Rimmer, *J. Chromatogr.*, 448 (1988) 448.
15 I. A. M. Cruickshank and D. R. Perrin, *Nature (London)*, 187 (1960) 799.
16 N. T. Keen, *Phytopathology*, 68 (1978) 1237.
17 P. M. Dewick, W. Barz and H. Grisebach, *Phytochemistry*, 9 (1970) 775.
18 C. H. Fawcett, R. D. Firn and D. M. Spencer, *Physiol. Plant. Pathol.*, 1 (1971) 163.
19 F. M. Lyon and R. K. S. Wood, *Physiol. Plant Pathol.*, 6 (1975) 117.
20 S. Mayama, T. Tani, Y. Matsuura, T. Ueno and H. Fukami, *Physiol. Plant Pathol.* 19 (1981) 217.
21 T. Rouxel, M. Renard, A. Kollmann and J. F. Bousquet, *Euphytica*, submitted for publication.

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CHROM. 21 515

Note

Determination by high-performance liquid chromatography of clenbuterol in commercial syrup formulations

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Clenbuterol (4-amino-3,5-dichloro-α-*tert.*-butylaminomethylbenzyl alcohol hydrochloride) is a beta-adrenergic drug used as a bronchial dilating agent. It is recommended for the treatment of asthmatic disease and, due to its long-term high activity, it is used especially in the case of chronic illness[1–7]. Beacuse of its antidepressive like activity in animals, it is also suggested for human endogenous depression[8].

Various methods for the assay of clenbuterol in biological samples have been reported. Radiological [9,10], enzymatic[11] and chromatographic methods [thin-layer chromatography[12], gas chromatography[13,14], high-performance liquid chromatography (HPLC)[15–17]] have been used for pharmacokinetic studies and for routine analysis in racehorses. Moreover, an HPLC method was developed for pharmaceutical gel formulations[18]. As part of our research on clenbuterol pharmaceutical preparations [19,20], we now describe a rapid and easy to reproduce procedure which allows an assay of clenbuterol in syrups, directly on the sample itself, without any previous extraction of the drug. In this way, possible recovery problems are avoided. Direct injection of the sample appears particularly opportune for an adequate and reliable quality control of these preparations which contain a very small quantity of an highly active drug in the presence of the large number of excipients usually contained in the syrups (1–2 μg clenbuterol per ml syrup).
This method is applicable to all the commercial formulations available in Italy, even when the excipients are different and numerous. It consists in a modification of the method proposed by Hamann *et al.*[18] for gel formulations.

EXPERIMENTAL

Materials

Clenbuterol was provided by Resfar (Milan, Italy). HPLC grade methanol and isopropanol were from Carlo Erba (Milan, Italy) and heptanesulphonic acid sodium salt monohydrate was from Fluka (Buchs, Switzerland). All solutions and solvents were filtered through a Millipore filter, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.).

HPLC instrumentation

Analytical liquid chromatography was performed using a Waters HPLC appa-

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ratus (Waters Assoc., Milford, MA, U.S.A.) consisting of a Model 510 Pump, equipped with an U6K injector, a variable-wavelength UV detector (Waters 450). Chromatographic data were collected and processed with a LC 100 Perkin-Elmer integrator (Norwalk, CT, U.S.A.). A stainless-steel column (25 cm × 4.6 mm I.D.) was packed with 7-μm CN stationary phase (LiChrosorb CN; Merck, Darmstadt, F.R.G.).

Operating conditions

The following chromatographic conditions were used. Mobile phase: water–methanol–isopropanol (70:29:1, v/v) containing 0.10% (w/v) heptanesulphonic acid sodium salt, degassed before use. Flow-rate: 1 ml/min. Column temperature: ambient. Volume injected: 100 μl. Detector wavelength: 246 mm. Detector sensitivity: 0.01 a.u.f.s. The column was carefully washed every day with 30 ml water and 30 ml methanol.

Linearity of detector response vs. standard concentration

A calibration graph was obtained by injecting clenbuterol solution in a concentration range of 0.5–10 μg/ml.

Sample preparation

The syrup samples (100 μl) were injected as such without any purification or dilution.

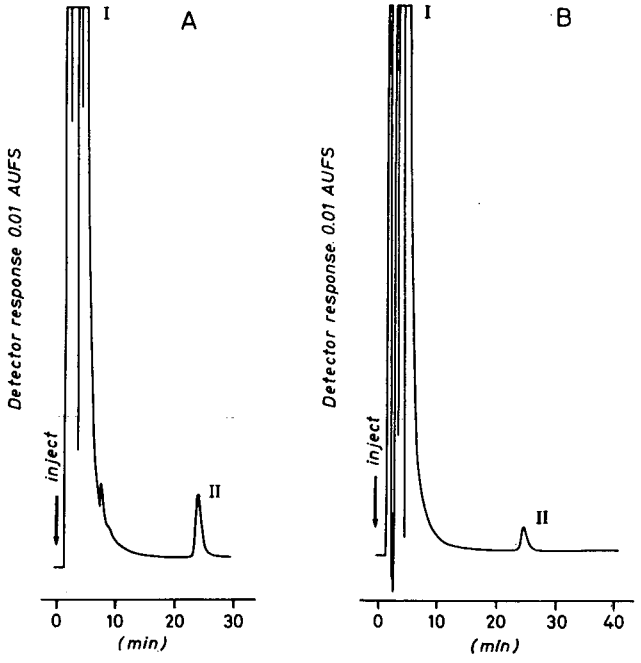

Fig. 1. Chromatograms of two different commercial clenbuterol syrups (A, 2 μg/ml; B, 1 μ/ml). Peaks: I = excipients; II = clenbuterol.

TABLE I

DAY-TO-DAY REPRODUCIBILITY OF THE CALIBRATION GRAPH FOR CLENBUTEROL

Day	*Slope*	*Correlation coefficient*
1	1698.6	0.9953
2	1716.0	0.9996
3	1739.6	0.9997
Mean	1718.1	
S.D.	20.6	
C.V. (%)	1.2	

RESULTS AND DISCUSSION

Our modifications of the Hamann method[18] permitted a good separation between clenbuterol and the other components of all the pharmaceutical syrup formulations commercially available in Italy, without any purification of the sample. Fig. 1 shows the chromatograms of two (A, B) of the seven syrups analyzed. For all the other formulations examined, a comparably good separation of the clenbuterol from the excipients was obtained.

The calibration graph showed good linearity with a correlation coefficient of 0.9997: $y = 1739.6x - 0.277$.

A day-to-day reproducibility test was performed over 3 days. A good reproducibility of the slope of the calibration graph was obtained (coefficient of variation, C.V. = 1.2%). The slopes and the linear correlation coefficients are reported in Table I.

The detection limit was approximately 0.1 ng, calculated on a response of twice the noise level.

The reproducibility of the determination of clenbuterol in syrups was satis-

TABLE II

REPRODUCIBILITY FOR DETERMINATION OF CLENBUTEROL IN A SYRUP

Injection	*Concentration (μg/ml)*
1	1.08
2	1.02
3	1.01
4	1.01
5	1.07
6	1.15
7	1.09
8	1.09
Mean	1.06
S.D.	0.05
C.V. (%)	4.72

TABLE III
RECOVERY STUDY

Placebo	*Clenbuterol added (μg/ml)*	*Recovery (% ± S.D.)*
A	1.08	96.8 ± 2.3
B	1.00	98.2 ± 2.0
C	1.94	99.0 ± 2.1
D	1.85	98.0 ± 2.2

factory, as shown in Table II which reports the response to repeated injections of one of the syrups analyzed (C.V. 4.72%).

Four different placebo syrups were prepared according to some commercial formulations. No interfering peaks were present at the retention time of clenbuterol for all these placebos. Known amounts of clenbuterol (1–2 μg/ml) were added and recovery experiments were carried out (Table III).

The column was carefully washed every day, as mentioned. In this way, no efficiency loss was observed throughout our work.

In conclusion, this HPLC method for the determination of clenbuterol in syrups appears to be reproducible and sensitive. In addition, it allows clenbuterol to be determined in these pharmaceutical formulations without any sample preparation, making this procedure very easy to perform. Finally, it provides a reliable quality control of these preparations.

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REFERENCES

1 G. Engelhardt, *Arnzneim.-Forsch.*, 26 (1976) 1404.
2 P. Thorpe, *Drugs Fut.*, 1 (1976) 221.
3 P. L. Kamburoff and F.J. Prime, *Brit. J. Clin. Pharmacol.*, 4 (1977) 67.
4 S. R. Bäring-Kuhlmey, *Med. Actual./Drug Today*, 14 (1978) 62.
5 G. Bonzi, G. Reguzzoni and L. Negri, *Gazz. Med. Ital.*, 138 (1979) 383.
6 N. Carnimeo, *Clin. Europea*, 18 (1979) 3.
7 A. Baronti, A. Grieco and C. Vibelli, *Int. J. Clin. Pharmacol.*, 18 (1980) 21.
8 R. Jouvent, Y. Lecrubier, A. J. Puech, J. F. Alliaire and A. Des Lauriers, *13th C.I.N.P. Congress, Jerusalem, June 20–25, 1982*, Collegium Internationale Neuro-Psychopharmacologicum, Abstracts, Vol. 1, p. 365.
9 E. Osada, T. Murai, Y. Ishizaka and K. Sanai, *Arzneim.-Forsch.*, 34 (1984) 1614.
10 T. W. Guentert, J. N. Buskin and R. L. Galeazzi, *Arnzneim.-Forsch.*, 34 (1984) 1691.
11 I. Yamamoto, *J. Immunoassay*, 3 (1982) 155.
12 J. Henion, G. A. Maylin and B. A. Thomson, *J. Chromatogr.*, 271 (1983) 107.
13 D. E. Bradway and T. Shafiq, *J. Chromatogr. Sci.*, 15 (1977) 322.
14 G. A. Maylin, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY, personal communication, 1983.
15 B. Diquet, L. Doare and P. Simon, *J. Chromatogr.*, 336 (1984) 415.
16 C. Eddins, J. Hamann and K. Johnson, *J. Chromatogr. Sci.*, 23 (1985) 308.
17 G.A. Qureshi and A. Eriksson, *J. Chromatogr.*, 441 (1988) 197.
18 J. Hamann, K. Johnson and D. T. Jeter, *J. Chromatogr. Sci.*, 23 (1985) 34.
19 E. Ciranni Signoretti, A. Dell'Utri, A. De Salvo and L. Donini, *Drug Dev. Ind. Pharm.*, 12 (1986) 603.
20 E. Ciranni Signoretti, C. De Sena, A. Dell'Utri and S. Alimonti, *Drug Dev. Ind. Pharm.*, 14 (1988) 1167.

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Note

Determination of sugars in polysaccharide hydrolysates by anion-exchange chromatography

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The analysis of sugars and sugar mixtures is of considerable and growing importance in many fields[1]. Various techniques have been investigated for the separation and quantitative determination of sugars, but most have several disadvantages, *e.g.*, difficulty in quantitating results for paper and thin-layer chromatography, the appearance of anomeric peaks and the necessity for some derivatization for gas chromatography. Problems with detection, difficulty in the separation of the sugars present in hemicellulose hydrolysates and the care that must be exercised to protect the chromatographic columns with precolumns or sample pretreatment are disadvantages of high-performance liquid chromatography (HPLC). Refractive index detectors are probably the most efficient for the detection of sugars in HPLC, but no gradient elution system can be used under these conditions. Detection by refractive index measurement can therefore only be used in special cases[2,3].

Many of these disadvantages can be avoided by the use of methods based on ion-exchange chromatography, where the sugars are separated as borate complexes on anion-exchange resins[4–9]. Common objections to this conventional liquid chromatographic technique are the long separation times and the need to regenerate the resin column after each analysis.

In the course of studies on the sugar composition of isolated hemicellulose A and B fractions from grass species and sugarcane bagasse, difficulties were encountered in finding a suitable method for the separation and determination of especially rhamnose, mannose, arabinose, galactose, xylose and glucose. Excellent separations can be achieved with an anion-exchange method using gradient elution with four buffer solutions, but a single determination needed 8 h[10]. A major disadvantage is that the column has to be regenerated after each run.

After the investigation of various buffer systems and other methods, including HPLC, we developed an anion-exchange chromatographic technique which is currently in use in our laboratory. The method is based on the system of Floridi[6]. The separation and determination of different sugar mixtures takes 90–240 min with minimal pretreatment of hydrolysates and no regeneration of the resin column after each run.

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EXPERIMENTAL

Equipment

The analyses were made with a Technicon AutoAnalyzer system. A flow diagram of the chromatographic modules and analytical manifold is shown in Fig. 1. Sugars were detected with a Technicon single-channel colorimeter (420-nm filter for orcinol reagent) and identified according to retention times. Peaks were recorded and integrated with a Varian Model 4270 integrator. An adjustable spindle supplied by Technicon was mounted on top of the column to minimize the dead volume. Sample application was facilitated with a Waters UK6 injection system. Interconnections consisted of glass tubes (1.6 mm I.D.) where possible. When glass could not be used it was replaced with Acidflex transmission tubes. Reagent flow was segmented by air bubbles, but debubbled just before detection in the colorimeter. Stepwise elution, when used, was performed by means of a peristaltic valve (Perivalve) and tape programmer supplied by Technicon.

Chromatographic conditions

Technicon Chromobeads Type S (product No. T15-0357-42), a 10% cross-linked styrene–divinylbenzene ion exchanger, was slurry-packed at 1375 kPa to a height of 69 cm in a glass column (75 × 0.5 cm I.D.) maintained at 55°C. The column was initially treated with 0.5 *M* boric acid (eluate pH *ca.* 4.2) an equilibrated with buffer A.

Two separation systems could be used: with system I the column was eluted only with buffer A whereas with system II elution started with buffer B for 90 min and was then followed by buffer A. The flow-rate was maintained at 0.5 cm^3/min.

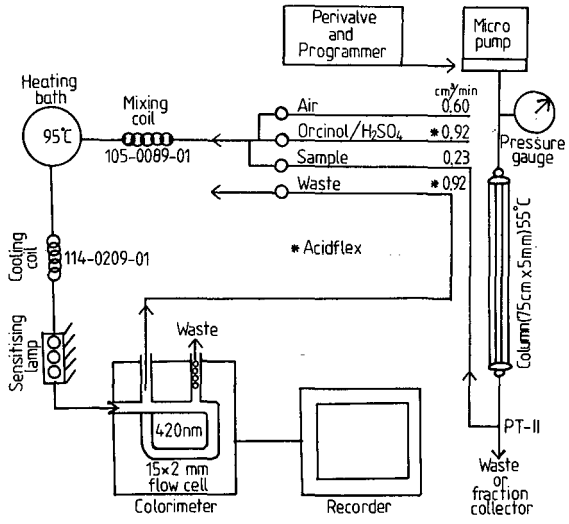

Fig. 1. Flow diagram of chromatographic modules and analytical manifold.

Chemicals and reagents

All reagents were of the best grade available and of various brands. Sugar standards were 2 m*M* solutions of the relevant sugars in deionized water. The colour reagent consisted of 1 g of orcinol (3,5-dihydroxytoluene) per dm^3 of 70% sulphuric acid and was protected from light. Buffer solutions were prepared as follows: buffer A, 24.44 g of dipotassium tetraborate tetrahydrate (0.08 *M*) and 11.00 g of boric acid (0.18 *M*) per dm^3 (pH 8.8); and buffer B, 7.79 g of dipotassium tetraborate tetrahydrate (0.0255 *M*) and 7.73 g of boric acid (0.125 *M*) per dm^3 (pH 8.4).

After dissolving the reagents in deionized water the solution was filtered (0.45 μm) and de-aerated. If necessary, the pH was adjusted with 1 *M* potassium hydroxide solution.

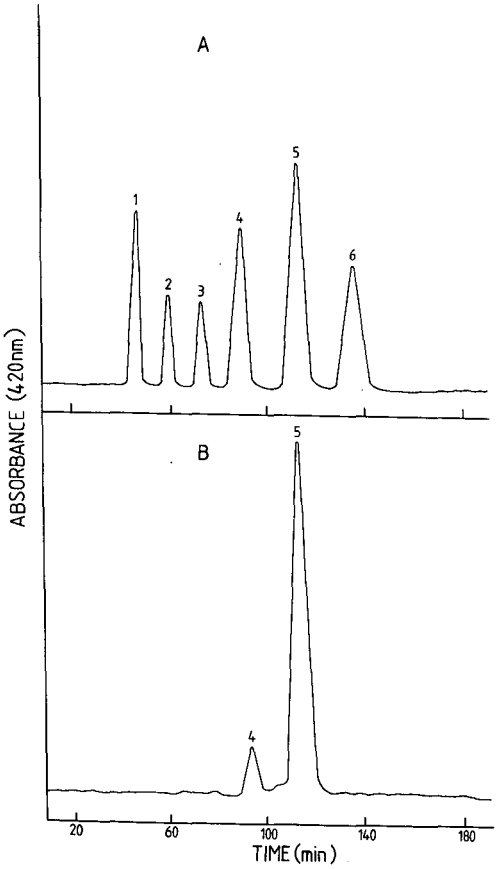

Fig. 2. Chromatogram (system I) of (A) a calibration mixture of sugars and (B) a hemicellulose A hydrolysate from sugarcane bagasse. Peaks: 1 = cellobiose; 2 = L-rhamnose; 3 = D-mannose; 4 = L-arabinose; 5 = D-xylose; 6 = D-glucose.

RESULTS AND DISCUSSION

Separations with the one-buffer system (I) can be used for certain mixtures of sugars such as sucrose, fructose and glucose, the major free sugars in most plants and mixtures or hydrolysates containing rhamnose, mannose, xylose, glucose and either arabinose or galactose. These determinations take about 120 min, including *ca.* 30 min residence time in the analytical system. As no regeneration of the resin is required, a large number of samples can be fractionated successively. Fig. 2 shows chromatograms of (A) a calibration mixture of known sugars and (B) a hydrolysate of a hemicellulose A fraction isolated from sugarcane bagasse.

Determinations with system II, employing stepwise elution with the two buffer solutions, need more time (*ca.* 240 min). Chromatograms of a calibration mixture and a hemicellulose B hydrolysate from bagasse are shown in Fig. 3A and B, respectively. System II can also be used without regeneration of the resin for at least 120 consecutive runs and with no special pretreatment of the hydrolysates or extracts. Sulphuric acid (1 *M*)-hydrolysed polysaccharide fractions were only neutralized with sodium

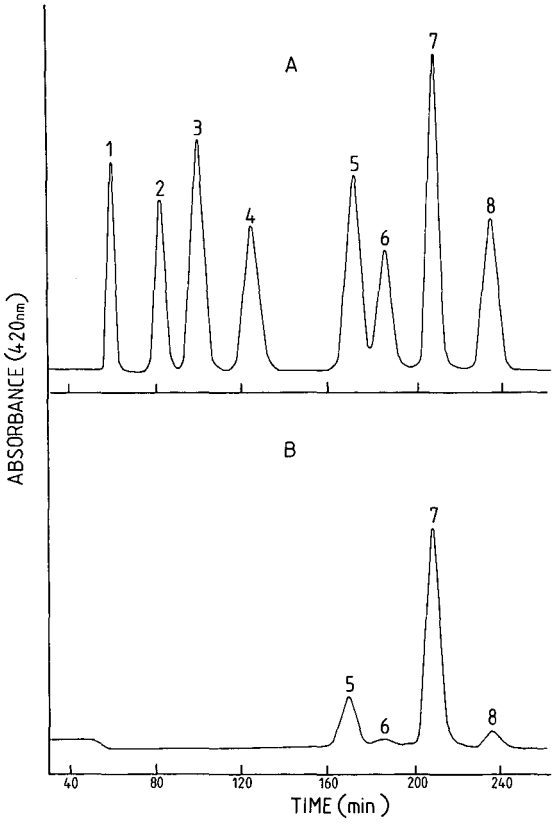

Fig. 3. Chromatogram (system II) of (A) a calibration mixture of sugars and (B) a hemicellulose B hydrolysate from sugarcane bagasse. Peaks: 1 = cellobiose; 2 = L-rhamnose; 3 = D-ribose; 4 = D-mannose; 5 = L-arabinose; 6 = D-galactose; 7 = D-xylose; 8 = D-glucose.

hydroxide and filtered prior to injection. The only complication experienced with such untreated hydrolysates was the blackening of the top 10 mm of resin, with a resulting higher operating pressure. Replacement of this portion with new resin remedied the problem. When necessary the resin could be regenerated in the column with 0.5 *M* boric acid until the pH of the eluate reached 4.2 and then again equilibrated with buffer A.

The variations in retention times that may occur can largely be attributed to degeneration of the pump tubes, which have to be changed after about 240 h. Fresh batches of buffer or sulphuric acid solutions can also lead to slight changes in retention times. This causes no problem if a calibration mixture is run at regular intervals.

Most of the free sugars in plants and especially the component monosaccharides of hemicellulose fractions could be separated and quantified by either of the chromatographic systems described, the main advantages being no regeneration of the resin column after each run and no special pretreatment of samples to be chromatographed.

REFERENCES

1 T. Kawamoto and E. Okada, *J. Chromatogr.*, 258 (1983) 284–288.
2 S. Honda, *Anal. Biochem.*, 140 (1984) 1–47.
3 T. Okada and T. Kuwamoto, *Anal. Chem.*, 58 (1986) 1375–1379.
4 R. B. Kesler, *Anal. Chem.*, 39 (1967) 1416–1422.
5 Y. C. Lee, J. F. McKelvy and D. Lang, *Anal. Biochem.*, 27 (1969) 567–574.
6 A. Floridi, *J. Chromatogr.*, 59 (1971) 61–70.
7 L Hough, J. V. S. Jones and P. Wusteman, *Carbohydr. Res.*, 21 (1972) 9–17.
8 L. A. Th. Verhaar and M. H. Dirkx, *Carbohydr. Res.*, 59 (1977) 1–10.
9 R. S. Ersser and J. D. Mitchell, *J. Chromatogr.*, 307 (1984) 393–398.
10 P. L. van Biljon and P. L. le Roux, *Agroplantae*, 15 (1983) 1–6.

PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications*: Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

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AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY

edited by **P.L. DUBIN,** *Indiana-Purdue University*

(Journal of Chromatography Library, 40)

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.

These developments in principles and applications are assembled for the first time in this book.

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- High-performance packings based on derivatized silica are reviewed in detail.
- Special techniques are thoroughly described, including SEC/LALLS, inverse exclusion chromatography, and frontal zone chromatography.
- Attention is focussed on special applications of size-exclusion methods, such as the characterization of micelles, separations of inorganic ions, and Hummel-Dreyer and related methods for equilibrium systems.
- Protein chromatography is dealt with in both dedicated sections and throughout the book as a whole.

This is a particularly comprehensive and authoritative work - all the contributions review broad topics of general significance and the authors are of high repute.

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