ISSN 0021-9673

VOL. 508 NO. 2 JUNE 1, 1990

THIS ISSUE COMPLETES VOL. 508

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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0021-9673/90/$03.50

Printed in The Netherlands

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EJMC-P1

Journal of Chromatography, 508 (1990) 279–287
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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High-performance affinity chromatography of DNA

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SUMMARY

The octadecamer of thymidylic acid, $(dT)_{18}$, was synthesized with a primary amino group on the 5′-terminal phosphate and this was covalently coupled to 300 Å pore macroporous silica. Coupling was performed inside a prepacked column to an activated N-hydroxysuccinimidyl ester silica. The $(dT)_{18}$-silica column succesfully separates mixtures of adenine oligomers differing in length by one nucleotide. The dependence upon salt concentration, temperature and length for elution of oligonucleotides was determined. Methods were also developed to selectively elute such columns using either salt or temperature gradients.

INTRODUCTION

Affinity chromatography has proven to be a powerful technique for the separation of many types of biologically important molecules. While the technique has most often been used for the separation of proteins, the specific base pairing of nucleic acids has also been exploited for affinity-based isolation of DNA and RNA.

Conceptually, polynucleotide affinity chromatography is simple. Polynucleotides naturally form double-stranded duplexes between two strands of complementary nucleotide sequence. In DNA duplexes, adenine (A) specifically base pairs with thymidine (T) while cytosine specifically pairs with guanidine. By attaching one strand of a potential duplex to a chromatographic support, a column can be made which is highly selective for the complementary strand. In mixtures of polynucleotides, this complementary strand alone, of all other possible sequences, is capable of forming the greatest number of base pairs with the attached strand and the duplex formed is the most stable one possible. Such a column could be eluted in several ways: column temperature can be raised until thermal motion dissociates the duplexes. Since DNA is a polyanion, columns can also be eluted by lowering the salt concentration in the mobile phase to the point at which charge repulsion between strands exceeds the

0021-9673/90/$03.50

stability of the base pairs formed. Formamide and other organic solvents can also be used for elution.

Polynucleotide affinity chromatography at low pressure has been in use for over 25 years[1]. The specificity of base pairing allows highly selective chromatography even with the poor resolution which accompanies many low-pressure chromatographic techniques. The superior mass transfer characteristics and higher efficiency of the macroporous silica supports should improve resolution and make possible truly high-performance affinity chromatography (HPAC) of nucleic acids. This type of HPAC has remained virtually unexplored and this is unfortunate since the high resolution of HPAC has potentially significant advantages in both the preparation and the analysis of polynucleotides.

One impediment to the wide-spread use of HPAC arises from the fact that most users of high-performance liquid chromatography (HPLC) do not pack their own columns. Therefore, methods which allow the coupling of nucleic acids inside prepacked columns could speed the investigation of polynucleotide HPAC. Here, we report on such a coupling methodology, using the octadecamer of thymidylic acid [$(dT)_{18}$], applicable to synthetic oligonucleotides and characterize some of the parameters governing polynucleotide HPAC separations.

EXPERIMENTAL

Materials

N-Hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC) and oligonucleotides A_3, A_6 and A_9 were from Sigma (St. Louis, MO, U.S.A.). Aminolink I and other reagents for oligonucleotide synthesis were from Applied Biosystems (San Jose, CA, U.S.A.). 5′-Aminoethyl-$(dT)_{18}$ was synthesized by Dr. Steven Larsen, Department of Microbiology and Immunology, Indiana University School of Medicine (Indianapolis, IN, U.S.A.) and was deblocked using the protocol provided with the Aminolink I reagent and then purified to apparent homogeneity using a modification of the method of Ashman *et al.*[2]. Briefly, a 250 mm × 4.6 mm I.D. Macrosphere C_4 reversed-phase column (Alltech, Deerfield, IL, U.S.A.) was used with 0.1 *M* triethylamine–acetic acid, pH 6.5 (solvent A) and acetonitrile (solvent B) with a linear gradient of 5 to 25% solvent B in 10 min. Oligonucleotides A_{12}, A_{14}, A_{16} and A_{18} as well as mixtures containing A_{12-18} and A_{19-24} were from Pharmacia (Piscataway, NJ, U.S.A.).

Chromatography

The chromatograph was a Varian 5000 ternary gradient instrument outfitted with a Varichrome UV–VIS detector. Column temperature was controlled by immersing the column in a Lauda refrigerated circulating water bath (Brinkman Instruments, Westbury, NY, U.S.A.). Between the injector and the column was inserted a 0.5-ml rheodyne sample loop (also immersed in the water bath) to act as a buffer preheater. To detect temperature, a Simpson Model 383 dual-channel digital thermometer (Simpson Electric, Elgin, IL, U.S.A.) was used with two J-type thermocouples (0.5 mm diameter, Cole-Parmer Instrument, Chicago, IL, U.S.A.). The thermocouples were inserted into three-way unions and one union was coupled to the inlet of the column and the other to the column outlet. The temperatures reported are those recorded at the outlet. At heating rates below 0.5°C/min and flow-rates below 0.2 ml/min, the inlet, outlet and bath temperatures were all the same.

Column preparation

The coupling scheme is depicted in Fig. 1. Macrosphere-WCX, 300 Å pore size, 7-μm beads packed in 30 mm × 4.6 mm I.D. cartridge columns were supplied by Alltech. The columns were activated to the N-hydroxysuccinimidyl ester using the procedure and apparatus previously described[3]. After activation, the columns were washed sequentially with 10 ml of 2-propanol, 1 ml of methanol, 1 ml of water and finally 1 ml of 0.5 *M* sodium phosphate, pH 7.5. Immediately, 1 ml of this last buffer containing 5'-aminoethyl-$(dT)_{18}$ was recirculated through the column for 30 min at room temperature. The flow-rate was 1 ml/min throughout. The column was then thoroughly washed with buffer and left in buffer overnight to allow the N-hydroxysuccinimidyl esters to spontaneously hydrolyze back to the parent, unreactive support[3]. The columns were then washed extensively with buffer to remove the hydrolyzed N-hydroxysuccinimide prior to use.

Alternative forms of the procedure in which various other concentrations of sodium phosphate and other column sizes were used are also reported. The coupling time was lengthened to 60 min in some experiments and gave similar results. For one experiment, a 23 mm × 2 mm I.D. column was prepared. The column used was packed from an aqueous slurry of 60 Å pore Adsorbosphere-WCX using a reservoir specially prepared to allow slurry packing of the Alltech direct-connect refillable guard column. The column was packed from a slurry containing 0.1 g silica in 1 ml under a constant flow-rate of 1 ml/min. These columns were found to contain 0.033 ± 0.001 g ($n = 3$) of silica in a total column volume of 0.072 ml. In other experiments, columns were packed

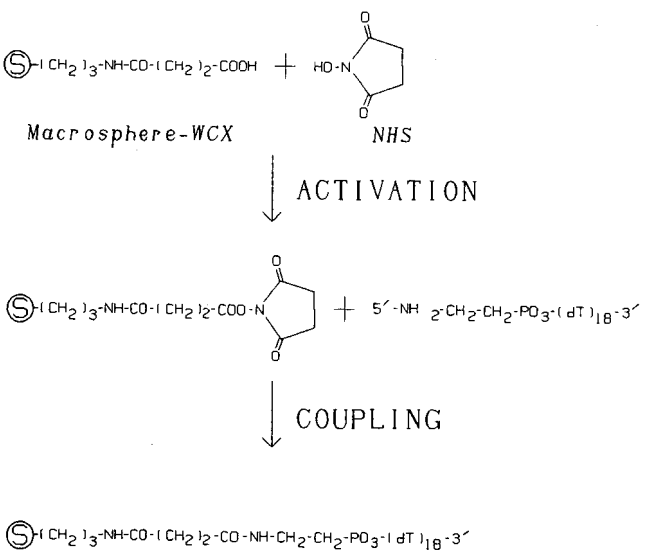

Fig. 1. Synthetic DNA coupled to derivatized silica by an amide bond. Macrosphere-WCX cation-exchange silica in a prepacked cartridge column was activated using N-hydroxysuccinimide (NHS) and a carbodiimide (EDAC) as described previously[3]. 5'-Aminoethyl-$(dT)_{18}$ was then recirculated through the column and coupled via an amide linkage.

at flow-rates as high as 5 ml/min and these columns also gave good chromatographic performance.

Determination of the amount of DNA coupled

During coupling, N-hydroxysuccinimide is released from the column by two processes: as a by-product of the reaction which couples DNA and as a result of spontaneous hydrolysis in aqueous buffers. N-Hydroxysuccinimide and DNA both absorb strongly at 260 nm making it difficult to quantitate the amount of DNA coupled from absorption data at pH 7–8. However, it was found that by titrating solutions to pH 2, the absorption of N-hydroxysuccinimide in the 260-nm range can be greatly reduced while DNA's absorption is barely affected. Thus, after coupling, the recycled DNA solutions were titrated to pH 2 with hydrochloric acid and the absorption at 266 nm was measured and compared to the absorption of starting sample also at pH 2. The amount coupled is expressed in terms of absorption units coupled. In some experiments, the amount coupled was also determined by repurifying and quantitating the 5′-aminoethyl-$(dT)_{18}$ remaining uncoupled by reversed-phase chromatography[2] and these results were similar to the simpler method described above.

RESULTS

Initially, other column coupling protocols were investigated. In model experiments, 5′-TMP was coupled to 3-aminopropyl-silica (Adsorbosphere-Amino, Alltech) using EDAC in 0.25 *M* imidazole, pH 6.1. While TMP was found to couple efficiently, it was latter discovered that DNA binds tightly to unmodified 3-aminopropyl-silica and is only eluted at ionic strengths above *ca.* 0.3 *M*. Since DNA is a highly anionic molecule, this was probably due to anion exchange with the cationic aminopropyl-silica. Thus, DNA-silica prepared by this approach would of necessity be mixed-mode chromatography, and this approach was abandoned for the approach depicted in Fig. 1.

As shown in Fig. 1, DNA which had been synthesized to contain a 5′-amino moiety, 5′-aminoethyl-$(dT)_{18}$, was coupled by way of an amide bond to carboxylic acid-based silica. The chemistry used allows a stable, unreactive silica (Adsorbosphere-WCX) to be activated to the reactive N-hydroxysuccinimidyl ester and reacted to couple DNA all inside a prepacked column. Column preparation only requires a few hours. Unreacted N-hydroxysuccinimidyl ester silica spontaneously hydrolyzes in aqueous buffers[3] and the column is available for use the next day. After *ca.* six months of use, no loss of coupled DNA or column capacity has been detected suggesting that the linkage is stable during routine usage.

The salt concentration used during coupling has an effect on coupling efficiency as shown in Table I. In 0.1 *M* sodium phosphate, pH 7.5, only 18–40% of the applied DNA couples. Increasing the salt concentration increases the fraction of DNA that couples, with 80% coupling efficiency observed at 0.5 *M*. The failure to couple efficiently in low salt may be due to charge repulsion between the DNA and unreacted groups on the Macrosphere-WCX.

Table I also shows that columns containing 72 units of DNA per g of silica can be made by this coupling chemistry. This corresponds to about 2.7 mg of $(dT)_{18}$ per g of

TABLE I

EFFICIENCY OF DNA COUPLING

Experiment No.	*Ionic strength in total Na^+ (M)*	*Column dimensions (mm × mm I.D.)*	*DNA reacted (units)*	*DNA coupled (%)*	*Load[a] (units/g)*
1	0.1	30 × 4.6	1.3	18	0.78
2	0.1	30 × 4.6	1.8	40	2.5
3	0.14	30 × 4.6	19.2	59	38
4	0.5	30 × 4.6	2.0	80	5.3
5	0.42	23 × 2.0	3.0	78	72

[a] The units of 5'-aminoethyl-$(dT)_{18}$ coupled per gram of silica assuming 0.3 g silica for 30 mm × 4.6 mm I.D. columns and 0.033 g for the 23 mm × 2 mm I.D. column.

silica or about 87 μg of DNA coupled inside the small 23 mm × 2 mm I.D. column. Similarly, the highest amount coupled inside a 30 mm × 4.6 mm I.D. column (37 units/g) corresponds to *ca.* 400 μg of DNA coupled to the 0.3 g of silica these columns were found to contain. If a significant fraction of this DNA can participate in specific hybridization during chromatography, adequate capacity for many types of genetic engineering experiments is feasible.

In Fig. 2 is shown the separation of a mixture of A_{12}–A_{18} using temperature-dependent elution. At 8°C, the mixture was loaded onto the column and the column was slowly raised to 44°C over the next 2 h. Essentially all of the 0.3 U of oligoadenines applied was retained by the column. At the flow-rate and column size used, the loaded sample volume would traverse the column in about 1 min and this time was evidently sufficient for complete hybridization at 8°C. The high resolution is apparent from the seven separations shown in Fig. 2 which result from a difference in length of a single base pair.

When adenine oligomers longer than 18 were applied to the column, they could

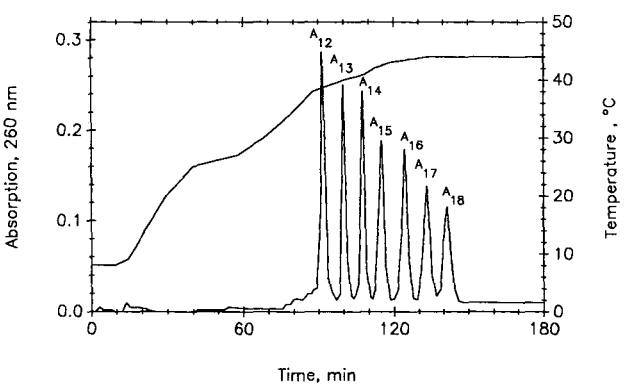

Fig. 2. Mixture containing seven different oligomers of adenine resolved using a temperature gradient. The 30 mm × 4.6 mm I.D. $(dT)_{18}$-silica column used for this experiment contained 11 U of oligo-(dT). The column was immersed in an 8°C water bath and at time 0, 0.3 U of an A_{12}–A_{18} mixture was loaded onto the column. The mobile phase was 0.49 *M* sodium chloride, 0:01 *M* sodium phosphate, pH 6.5, and the flow-rate was 0.2 ml/min throughout. Temperature was controlled by manually adjusting the temperature of the water bath and is shown on the right hand ordinate.

not be separated from one another but rather all elutd near where A_{18} would elute (data not shown). Thus, a column can separate based on length only up to the length of the coupled DNA, in this case, octadecamers. This was not necessarily a predictable result. Longer adenine oligomers should have more alternative ways of forming hybrids with the column-coupled octadecamer of thymidine. The larger degrees of freedom should contribute to make such hybrids more thermally stable but the effect is apparently too small to affect the chromatography.

In a separation analogous to that shown in Fig. 2, the A_{12}–A_{18} mixture was loaded onto the column at 8°C and the temperature rapidly raised to 30°C followed by rapid gradient to 47°C in 30 min. Using this protocol, all seven of the oligomers were also separated within 35 min, albeit with some decrease in resolution (data not shown). Thus, the long gradient shown in Fig. 2 is not necessary to obtain separation but does demonstrate the resolution obtainable.

To further characterize the temperature dependence of elution, single adenine oligomers were applied to the column individually and the column was heated at a very low rate (1°C per 10 min or less) and the position of the peak more precisely measured. Control experiments showed that the rate of heating was low enough that thermal equilibrium across the column was obtained and reproducible melting temperatures were measured. This is a more stringent requirement than that needed to simply separate mixtures. The results are in Fig. 3.

Elution temperatures were measured at both 0.1 and 0.5 *M*, as shown in Fig. 3. As expected[4,5], decreasing salt concentration lowers the temperature at which duplex hybrids melt and elution occurs. At either salt concentration, the data over this range of lengths fit a straight line. The slope of the lines show that increasing oligoadenine length by one base increases the melting temperature by 2.5–3.5°C at 0.1–0.5 *M* salt, respectively. Other studies of DNA hybrids in solution or immobilized to nitrocel-

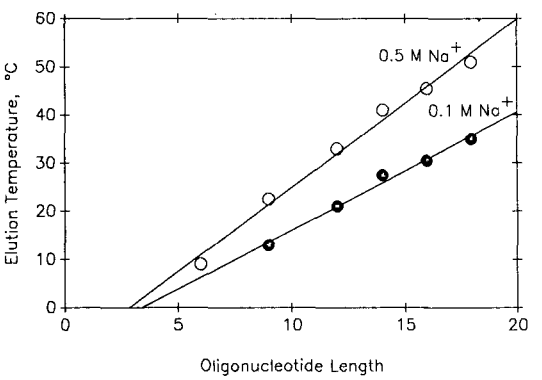

Fig. 3. Dependence of temperature on oligomer length and ionic strength at which an oligomer elutes. For this experiment, the 30 mm × 4.6 mm I.D. column containing 1.6 U of DNA was used and the various oligomers of adenine used had been previously kinased using γ-$^{32}PO_4$-ATP and polynucleotide kinase[8]. The flow-rate was 0.2 ml/min throughout and the mobile phase was 0.01 *M* sodium phosphate, pH 6.5, containing either 0.49 *M* sodium chloride (0.5 *M* Na^+) or 0.09 *M* sodium chloride (0.1 *M* Na^+). The individual oligoadenines were applied to the column at a temperature at least 4°C below the anticipated temperature of hybrid melting, and the temperature was slowly raised while collecting fractions. The absorption at 260 nm of the fractions was then measured, and a portion was mixed with scintillation fluid and counted for ^{32}P. The temperature at which each oligomer eluted in either mobile phase is shown.

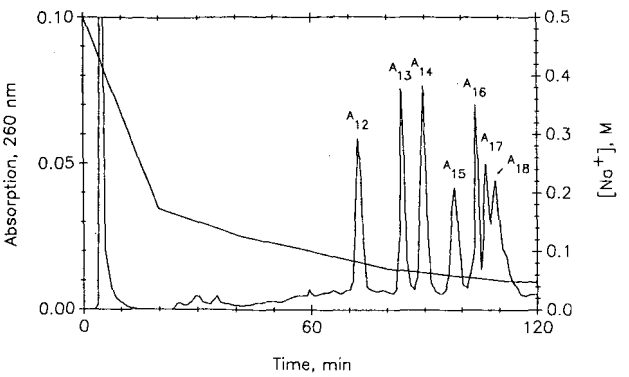

Fig. 4. Oligoadenine mixtures resolved using salt gradients. The 30 mm × 4.6 mm I.D. column containing 11 U of DNA was immersed in the water bath which was maintained at 35°C throughout. At time 0, 0.3 U of the oligoadenine mixture A_{12}–A_{18} was injected and eluted using a ternary salt gradient. Buffer A (0.5 *M* Na^+) and buffer B (0.1 *M* Na^+) were the buffers described in Fig. 3; buffer C was water. The gradient, shown on the right ordinate, was a linear rate of change between the following times and percentages: 0 min, 100% A; 20 min, 18% A, 82% B; 40 min, 7% A, 93% B; 60 min, 100% B; 80 min, 70% B, 30% C; 100 min, 60% B, 40% C; 140 min, 50% B, 50% C.

lulose have shown a 2°C change per base pair in 0.9–1 *M* salt[4,5]. The higher incremental temperature found here is probably due to the lower salt concentrations used but may suggest an influence of the stationary phase silica on the hybrid melting temperature.

For aqueous-based chromatography, a practical limit is imposed by the fact that water freezes at 0°C. As Fig. 3 shows, at either salt concentration used, the column could potentially be used to separate hybrids of lengths down to about a trimer at temperatures above 0°C, and that raising salt concentration can be used to extend the range of useful temperatures somewhat. Columns could thus be made to specifically bind even the hexamers typical of many restriction endonuclease cleavage sites.

Salt gradients can also be used for highly selective elution, as shown in Fig. 4. For this experiment, the sample was loaded at 35°C which is close to the upper limit of temperature at which hybridization with A_{12}, the smallest oligomer present in the sample, can still occur (see Fig. 3). Under these conditions, not all of the oligoadenine applied is retained by the column as indicated by the absorption eluting early in the chromatogram. This is in contrast to the results obtained in Fig. 2 where the sample was loaded at 8°C. However, the oligoadenines that are retained by the column are selectively eluted by the salt gradient used, and hybrid lengths differing by a single nucleotide base are readily resolved. Since many HPLC systems are not outfitted with programmable temperature controllers, salt gradient elution could be more widely used with existing instruments.

DISCUSSION

Polynucleotide affinity chromatography at low pressures has proven to be a valuable technique for over 20 years. Most commonly it has been used where low resolution is inconsequential such as in the purification of polyadenylated mRNA. For

these uses, the mRNA is loaded onto an oligo-(dT) or oligo-(uracil) column in a high-ionic-strength buffer and eluted by an abrupt change to low ionic strengths. For such uses, resolving mRNAs which differ in a single base pair is not necessary or desirable.

Although the coupling chemistry used should work with any nucleotide sequence, we have used oligo-(dT) columns as a model to investigate the limits and potential of the method. The results show that resolution based upon a single base difference can be obtained by either temperature-dependent or salt-dependent elution. When injection occurs at temperatures well below the hybrid melting temperature, all of the DNA injected was able to hybridize with the column in 1 min or less (Fig. 2); however, this is not necessarily the case for higher-temperature injections (Fig. 4). Lengths of 3–4 are the minimum which will hybridize at temperatures above 0°C, but increasing the salt concentration in the mobile phase increases the temperature of melting (Fig. 3) and this, or the inclusion of trimethylamine in the mobile phase[6], could probably be used to extend the method to shorter-length hybrids. However, even with a length limit as high as six bases, columns specific to average length restriction endonuclease cleavage sites could be made. However, since at random, any sequence length L will occur once in every 4^L nucleotides, such short sequence columns would not be very selective.

In these model studies, we have not tried to optimize either speed or column capacity. We have used very long gradients to carefully observe where elution occurs. With a little effort, the column and equipment could be changed to obtain more rapid temperature equilibration. Higher salt concentrations or trimethylamine can be used to increase the temperature at which loaded samples will rapidly hybridize with the column. These and other improvements would speed the separations obtained.

Capacity can also be increased. The Macrosphere-WCX silica used has about 500 μmol of free carboxylate per g of silica while the columns we have prepared contain at most 72 absorbance units of 5′-aminoethyl-$(dT)_{18}$ per g. This amount represents only about 0.5 μmol of DNA per g of silica, and thus little of the potential coupling capacity of the silica was used in the experiments presented here. Coupling of larger amounts of DNA is thus probably feasible but not necessarily desirable. Most experiments in molecular biology typically require smaller amounts of DNA than the capacity of the columns already made.

The high resolution possible with polynucleotide HPAC may allow different uses than the preparative role the low-pressure version of the technique has typically served. Analytical and clinical uses of polynucleotide HPAC may also be important. Polynucleotide hybridization is certainly capable of the required selectivity as demonstrated by the common use of Southern[7] and "Northern" hybridizations to specifically detect a single nucleotide sequence in the presence of other polynucleotides. However, detection may need to be improved. For example, a simple calculation shows that the peak of A_{12} in Fig. 2 resulted from about 0.2 nmol of oligomer chain or about 10^{14} chains. A peak one hundredth the size would easily be detectable and thus about 10^{12} chains of A_{12} should be detectable. This would be considered to be sensitive detection for most chromatographic methods but probably would not be sensitive enough for work with low-copy-number polynucleotides obtained from tissue. Radioisotope labeling with ^{32}P can, however, be used to increase sensitivity in these cases, and polymerase chain reaction technology[9] could be used to amplify low-abundance polynucleotides.

Polynucleotide HPAC is thus a sensitive, selective, high-resolution chromatographic technique which has remained relatively unexplored but is potentially useful.

ACKNOWLEDGEMENTS

This work was supported by grants from the NIH (GM 43609) and NSF (DMB 8996229). We thank William Foster of Alltech/Applied Science for the kind gift of the Macrosphere-WCX cartridges and Professor Steve Larsen for synthesizing the 5′-aminoethyl-$(dT)_{18}$ and for much helpful discussion. One of us (T.A.G.) is a recipient of the Alltech Fellowship in Chromatography, the support of which is gratefully acknowledged.

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Journal of Chromatography, 508 (1990) 289–299
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 286

Modification of silica with glucose for the separation of proteins by high-performance liquid chromatography

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(First received March 9th, 1989; revised manuscript received January 12th, 1990)

SUMMARY

The synthesis and testing of a mild stationary phase for protein separation are described. Using either glucose or epoxyglucose coupled to aminopropylsilica it is possible to achieve shielding of the silica surface with preservation of the pore structure. The stability of the coating and the interaction with standard proteins were studied. Basic proteins are not retained on either product. The glucosesilica is unstable in aqueous buffers, but the epoxyglucosesilica is not affected, even at high pH.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a well accepted technique for the purification and separation of proteins (see, *e.g.*, ref. 1). There is a large number of mobile and stationary phases in use[2], which reflects the diversity of proteins, the complexity of protein mixtures and the different applications[3]. It may also be regarded as indicative of the dissatisfaction with present packings.

One of the main concerns when separating proteins is the risk of denaturation. Some general guidelines can be given, *e.g.*, aqueous solutions buffered at a suitable pH and the elimination of strong protein–surface interactions. The latter is achieved with a "hydrophilic" surface. Preservation of the biological activity of a particular protein may require more specific conditions, *e.g.*, a low temperature, a water concentration between certain limits, the presence of special reagents to prevent oxidation and proteolysis[4]. These conditions are especially important with preparative separations.

Polysaccharides have been succesfully used for decades as stationary phases for the liquid chromatography of proteins[5]. Owing to the virtual absence of adsorption effects, size exclusion can be effected with these materials. Interactive chromatography is possible after suitable derivatization, and this derivatization chemistry has been well described[6]. Polysaccharide particles were strengthened for the use in HPLC by cross-linking them intensively[7]. However the chromatographic performance of these materials is poor and the rigidity is still insufficient.

Silica has been the support material of choice for stationary phases in HPLC[8]. It

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can be synthesized as uniform spherical porous particles of small diameter for optimum resolution. The pore diameter can be varied from below 100 to over 1000 Å. Well known disadvantages are the active surface and dissolution at high pH. A polymer coating on the surface can prevent these effects to some extent[9].

In a further effort to synthesize a better phase for mild protein separations, we decided to combine the advantages of polysaccharides and silica. This has been tried before but the results were not completely satisfactory[10–12]. Protein recoveries were low and the stability was poor.

Because significant reductions in the accessible surface area of the silica support can occur after coating procedures owing to narrowing and blocking of the pores[13], we attempted to bind the small molecule glucose onto the surface and to study the size-exclusion behaviour. Such a material would be an ideal base for other separation systems.

EXPERIMENTAL

Chemicals

Silica supports were obtained from Merck (Darmstadt, F.R.G.) (LiChrospher Si 1000, Si 500, Si 300, Si 100, 10 μm) and Shandon (Runcorn, U.K.) (Hypersil, 5 μm; WP300, 10 μm). Triethoxyaminopropylsilane was supplied by Janssen (Beerse, Belgium). The dextrans used in the size-exclusion experiments were from Pharmacia (Uppsala, Sweden). Methyl 2,3-anhydro-4,6-O-benzylidine-α-D-allopyranoside (referred to as epoxyglucose reagent in the following), sodium cyanoborohydride, [^{14}C]glucose and the proteins were obtained from Sigma (St. Louis, MO, U.S.A.) and phenyl mercuriacetate (as an antimicrobial agent) from BDH (Poole, U.K.). Other chemicals and solvents were of analytical-reagent grade and used as received unless stated otherwise.

Equipment

HPLC was performed with constant-flow pumps (Spectroflow 400; Kratos, Ramsey, NJ, U.S.A.), an injection valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.), a UV detector (Spectroflow 757; Kratos) and a recorder (BD 40; Kipp and Zonen, Delft, The Netherlands). Gradients were run with two pumps and a gradient programmer (Spectroflow 450; Kratos).

A liquid scintillation system (Tri-Carb 300 CD; Packard, Downers Grove, IL, U.S.A.) with scintillator cocktail (Scintillator 299; Packard) was used for radioactivity measurements.

Packing procedure

Silicas were slurry packed into stainless-steel columns, using methanol as the slurry and driving liquid, with a maximum packing pressure of 500 bar. Before packing fines were removed by uspending the silica in methanol, allowing it to settle and discarding the top layer of turbid methanol.

Preparation of aminopropylsilica

A suspension of activated silica (activated in a vacuum oven at 120°C for 16 h) in a 4% (v/v) solution of triethoxyaminopropylsilane in dry, freshly distilled toluene

(dried over molecular sieves in a Soxhlet apparatus) was refluxed for 16 h. The product was isolated by suction filtration, washed with 100 ml each of toluene, acetone and diethyl ether, then dried for 5 h in a vacuum oven at 120°C.

Picric acid assay

The assay for amino content is a modification of a method described by Alpert and Regnier[14]. An 13-g amount of moist picric acid is dissolved in 100 ml of dichloromethane and the aqueous layer is discarded. An accurately weighed amount of aminopropylsilica (50–100 mg) is suspended in 5 ml of this solution. After a few hours the silica is washed on a glass filter with dichloromethane until the washing solution is colourless. Next, the silica is transferred to another glass filter and the bound picric acid is washed off with a solution of 5% triethylamine in dichloromethane and collected. After appropriate dilution the absorbance is measured at 358 nm (ε = 14 500).

Titration of amino groups

Direct titration of amino groups on silica was carried out with hydrochloric acid using an indicator. To 10 ml of an aqueous solution of sodium chloride (4 *M*) a few drops of a 0.1% indicator solution of bromocresol green–methyl red (5:1) in methanol are added. With a small drop of 0.1 *M* hydrochloric acid the indicator is converted to the acid form. Next, a weighed amount of modified silica (*ca.* 400 mg) is added and the suspension is degassed and titrated with 0.1 *M* hydrochloric acid.

Preparation of glucosesilica

To 0.5 g of aminopropylsilica in 25 ml of methanol are added 0.5 g of glucose (a 10-fold excess), 5 mg of ammonium chloride and 200 mg of sodium cyanoborohydride. After refluxing for 16 h the product is washed with 100 ml each of methanol, water, acetone and diethyl ether.

In order to determine the yield, a reaction on a five times smaller scale was carried out and 10 μCi of [^{14}C]glucose were added. After washing, the product was suspended in 50 ml 1.5 *M* sodium hydroxide solution and heated to 50°C to dissolve the silica particles. An aliquot of this solution was counted after addition of scintillator cocktail. With the periodate treatment (see Results and Discussion) the product was suspended in 50 ml 0.1 *M* sodium periodate for 16 h, then washed, dissolved and counted as described.

Preparation of epoxyglucosesilica

To aminopropylsilica (0.5 g) in 25 ml of dichloroethane and 1 ml of triethylamine are added 200 mg (3 equiv.) of methyl 2,3-anhydro-4,6-O-benzylidine-α-D-allopyranoside. The suspension is heated on a water-bath (60°C) for 2 or 16 h, with occasional swirling, then washed with dichloroethane, acetone, water, acetone and diethyl ether. The benzaldehyde protecting group can be removed by heating in dilute sulphuric acid (0.0025 *M*) on a water-bath (60°C) for several hours.

RESULTS AND DISCUSSION

Aminopropylsilica

To attach glucose on the silica surface, the latter has to contain a suitable functional group. This was achieved by silanizing the silica with aminopropylsilane[15]. The product was analysed with a picric acid assay and a titration (Table I). The determination of a functional group was preferred to, *e.g.*, elemental analysis, because the former probably corresponds better to a measurement of the glucose reaction product. Although there is a significant difference in the results of the two methods, these figures are believed to be indicative of the coverage obtained. The coverage of the silica surface can be estimated by relating the values to surface areas as supplied by the manufacturer[16]. These figures correspond well with those reported by others[17].

TABLE I

COVERAGE OF SILICA WITH AMINO GROUPS AFTER SILANIZATION

Surface area data from manufacturer. Picric acid assay and titration as described. NH_2 is the mean of the picric acid assay and titration.

Silica type	*Surface area* (m^2/g)	*Picric acid assay* (*mmol/g*)	*Titration* (*mmol/g*)	NH_2 ($\mu mol/m^2$)
Si 1000	30	0.10	0.13	3.8
Si 500	60	0.20	0.22	3.5
Si 300	250	0.63	0.60	2.5
Si 100	250	0.68	0.82	3.0
Hypersil (5 μm)	170	0.44	0.56	2.9

The absence of water in the reaction mixture should promote the formation of a monolayer. This was checked by varying the concentration of the reagent and measuring the number of amino groups, again with the two methods mentioned (Fig. 1). The relationship suggests a levelling out at about 0.3 mmol/g. A maximum coverage of 4 μmol/m^2, as is often found in such silica surface modifications, corresponds to 0.24 mmol/g for this material of 60 m^2/g. Hence, in the prepared material there is probably some polymerized reagent present. However, the degree of polymerization will be small and the surface probably resembles that of a monolayer.

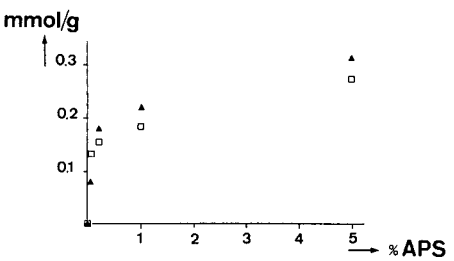

Fig. 1. Load of amino groups on silica using increasing concentrations of the silanizing agent. LiChrospher Si 500 (1 g) in dry toluene (50 ml); vol.-% triethoxyaminopropylsilane. ▲ = Titration; □ = picric acid assay.

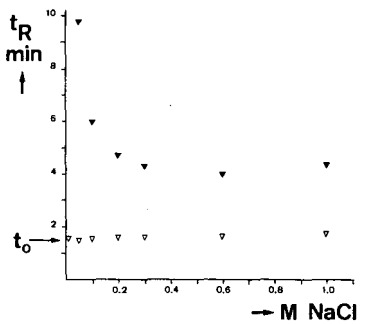

Fig. 2. Retention times (t_R) of lysozyme on aminopropylsilica prepared from Hypersil (WP 300). ▽ = Just after packing; ▼ = after 1.2 l of eluent. Column, 120 × 3 mm I.D.; mobile phase, 0.01 *M* Na_2HPO_4–NaH_2PO_4 (pH 7.0) + NaCl; flow-rate, 0.5 ml/min; lysozyme, 1 mg/ml; injection, 20 µl. Acetone used as t_0 (dead volume) marker.

Using a buffer of pH 7, the retention of lysozyme (p*I* 11) increases rapidly in a column packed with this material, indicating contact between the protein and the native silica surface. The ion-exchange nature of the interaction is demonstrated in Fig. 2. Lysozyme can be eluted easily from freshly prepared aminopropylsilica. After a certain amount of mobile phase, however, addition of salt is necessary for elution. With increasing salt concentration the retention decreases. With the 0.05 *M* buffer in the same column, the retention time of ovalbumin (OVA) decreased from 7 min at 2.6 l to 2 min after 7 l of eluent, while whereas bovine serum albumin (BSA) did not elute. A chromatogram of this used material is shown in Fig. 3.

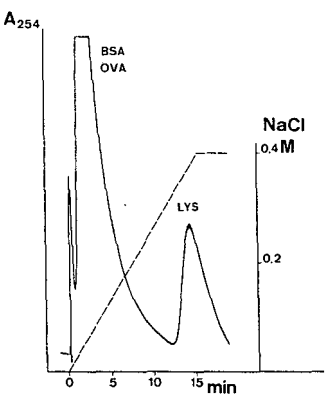

Fig. 3. Chromatogram of standard proteins on aminopropylsilica prepared from LiChrospher Si 300. Column, 150 × 2 mm I.D.; mobile phase, A = 0.05 *M* NaH_2PO_4–Na_2HPO_4 (pH 7.0), B = A + 0.4 *M* NaCl, gradient from A to B in 15 min; flow-rate, 1 ml/min; proteins, BSA 10 mg/ml, ovalbumin 10 mg/ml, lysozyme 1 mg/ml; injection, 20 µl. Absorbance at 254 nm, 0.1 a.u.f.s.

Glucosesilica

In a first attempt glucose was coupled to the aminopropylsilica. This reaction has been described by Kiselev *et al.*[10]. Here we used different reaction conditions, with methanol instead of aqueous buffer and addition of cyanoborohydride to reduce the

TABLE II

LOAD OF GLUCOSE ON SILICA WITH RADIOACTIVITY MEASUREMENTS

Amount of glucose in reaction solution, 443 μmol + about 10 μCi of [^{14}C]glucose. Silica: aminopropyl-modified Hypersil (5 μm); NH_2 concentration, 500 μmol/g (with picric acid assay and titration); surface area, 170 m^2/g. Periodate treatment: theoretically only C-1 and C-2 counted, *i.e.*, one third of the glucose load.

Glucose solution (cpm)[a]	*Glucosesilica (cpm)*	*Aminopropyl-silica (mg)*	*Glucose load (μmol/m²)*	*Periodate treatment*		
				Periodate-treated glucosesilica (cpm)	*Aminopropyl-silica (mg)*	*Glucose load (μmol/m²)*
748 350	58 930	73	2.8	8967	64	1.5
	52 034	72	2.5	11 240	80	1.5

[a] cpm = Counts per minute.

Schiff base. Using radioactively labelled glucose we observed a coverage of 2.8 μmol/m^2 glucose (Table II). However, this radioactivity measurement does not discriminate between chemically bound and adsorbed glucose. The adsorbed glucose cannot be eliminated simply by more extensive washing, as this causes hydrolysis of bound material (see above). Therefore, we applied another method for the determination of the glucose moieties, using a periodate treatment preceding the radioactivity counting. The periodate hydrolyses diol functionalities, leaving only C-1 and C-2 of the glucose molecule fixed to the amino group. The degradation products containing the other carbon atoms can be assumed to elute easily in the washing steps. Assuming uniform carbon labelling, a significantly lower bonding of 1.5 μmol/m^2 was found.

Lysozyme, chromatographed on freshly prepared glucose-modified material, merges with the peaks of BSA and OVA (Fig. 4). However, all three proteins still show some retention and peak tailing. These results demonstrate that shielding of the silica surface and aminopropyl indeed occurs, but is not perfect.

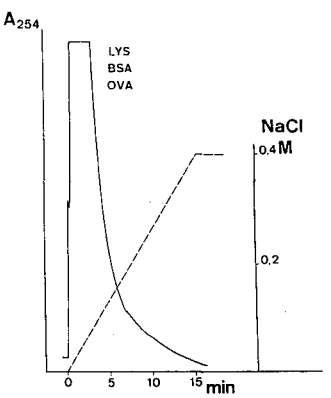

Fig. 4. Chromatogram of standard proteins on glucoseaminopropylsilica prepared from LiChrospher Si 300. Conditions as in Fig. 2.

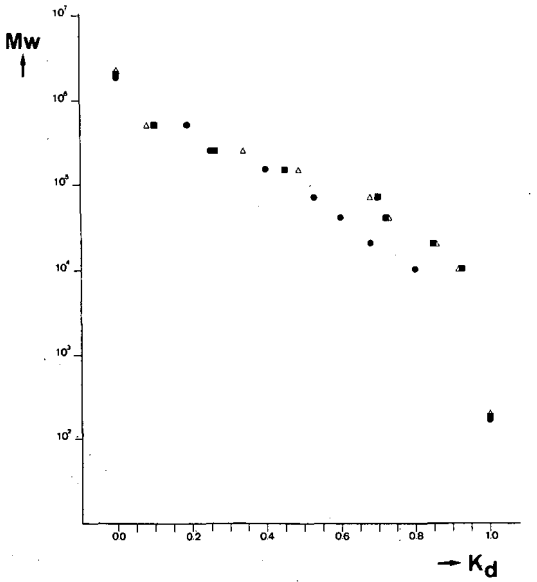

Fig. 5. Size-exclusion behaviour of dextrans. Silica (Hypersil WP 300) (■), glucosesilica (△) and epoxyglucosesilica (●), both prepared from Hypersil WP 300. Column, 450 × 3 mm I.D.; mobile phase, water; flow-rate, 0.2 ml/min. The distribution coefficient K_D is $(V_e - V_0)/(V_m - V_0)$, where V_e is the elution volume of the dextran. The interstitial volume V_0 corresponds to the elution volume of dextran 2 000 000; the total permeation volume V_m corresponds to the elution volume of glucose. Mw = Molecular weight.

The preservation of the pore structure was checked with a size-exclusion experiment (Fig. 5). No significant loss of pore volume is apparent.

The same column was used to study the interaction of the surface with standard proteins. As expected, during a 1-day experiment, the retention volumes shifted, indicating the instability of the stationary phase. Therefore, no in-depth study was done.

Epoxyglucosesilica

In another approach we applied a reaction between the amino moiety on the silica and an epoxy group present in a (protected) glucose derivative. Aminopropylsilica was reacted with methyl 2,3-anhydro-4,6-O-benzylidine-allopyranoside[18] (Fig. 6). The protecting benzaldehyde can be easily released by acid hydrolysis, after surface coating. We attempted to determine the surface coverage by means of this hydrolysis and subsequent determination of benzaldehyde (with the DNPH reaction[19]). However, the results corresponded to only 10–20% conversion of the amino groups. We assume that these results are much too low, in view of the observed shielding effect (see below). It is likely that part of the benzaldehyde is already lost in the coupling reaction. Indeed, a strong smell of benzaldehyde is observed on performing the reaction.

A chromatogram is shown in Fig. 7. Lysozyme is nearly unretained whereas BSA and OVA are retained. Compared with Fig. 2, a complete reversal of elution order is observed. It can be assumed that the retentions of the negatively charged OVA and

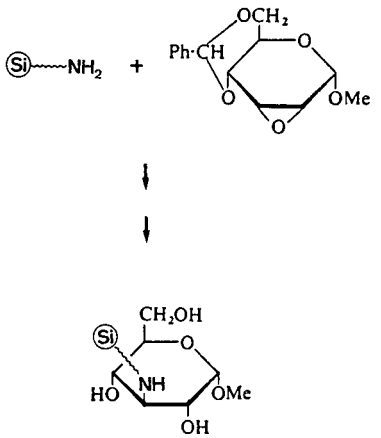

Fig. 6. Reaction of aminopropylsilica with epoxyglucose reagent. Me = Methyl; Ph = phenyl.

BSA are now caused predominantly by anion exchange on the remaining amino groups.

The stability of the layer was tested by monitoring the retention of lysozyme[12]. Although this is less direct a method than, *e.g.*, elemental analysis, it could give an indication of the loss of the glucose layer as bare silica retains lysozyme considerably. It appears that the 2-h reaction product is less stable than the 16-h product (Fig. 8). One explanation for the stability of the 16-h product could be a cross-linking reaction, presumably via the C-4 and C-6 atoms after the migration or release of benzaldehyde[20], or a polymerization reaction.

The size-exclusion behaviour of dextrans shows a slight change when compared with bare silica (Fig. 5). More specific measurements are necessary to study a possible small loss of pore volume.

Owing to the greater stability compared with the glucosesilica, the interaction of this stationary phase with standard proteins could be studied more thoroughly (Fig. 9).

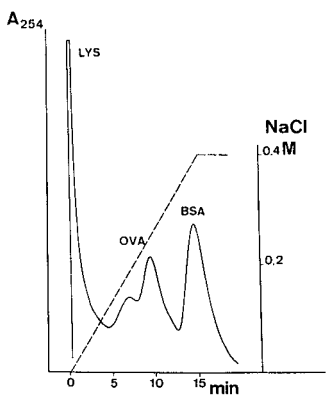

Fig. 7. Chromatogram of standard proteins on epoxyglucosesilica prepared from LiChrospher Si 300. Conditions as in Fig. 2.

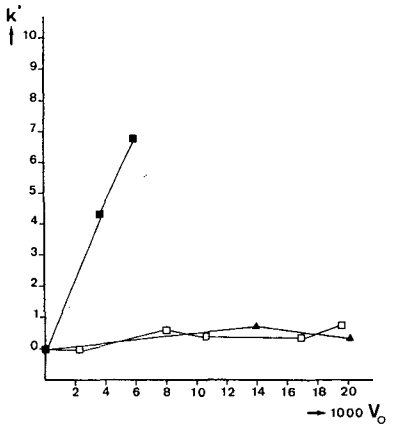

Fig. 8. Stability of epoxyglucosesilica, prepared from Hypersil WP 300, as estimated via the retention of lysozyme. (■) 2-h reaction product; (□, ▲) 16-h reaction product. Column, 60 × 3 mm I.D.; mobile phase, (■, □) 0.05 *M* NaH_2PO_4–Na_2HPO_4 (pH 7.0) + 0.001% phenylmercuriacetate (antimicrobial agent); (▲) same except pH 9.0; flow-rate, 1 ml/min. 20 000 V_0 (= 10 l) corresponding to 1 week of continuous use. Lysozyme, 1 mg/ml; injection, 20 μl. Uracil used as t_0 marker.

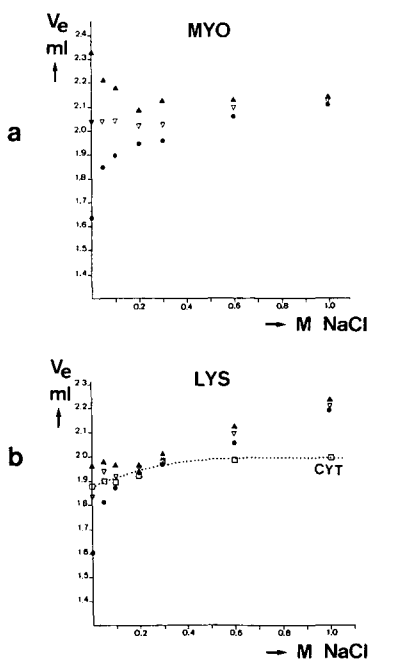

Fig. 9. Retention volumes of standard proteins on epoxyglucosesilica prepared from Hypersil (WP 300). Column, 450 × 3 mm I.D.; mobile phase, 0.01 *M* Na_2HPO_4–NaH_2PO_4, (●) pH 5, (▽) pH 7, (▲) pH 8, + NaCl (for cytochrome *c* only pH 7); flow-rate, 0.2 ml/min. Proteins: myoglobin, lysozyme, cytochrome *c*, 2 mg/ml. Injection, 20 μl.

The influence of increasing ionic strength on the retention of myoglobin (p*I* 7.3) is different for the three pH values: at pH 8 it decreases, at pH 7 it is nearly constant and at pH 5 it increases. This behaviour is opposite to that commonly observed with diol stationary phases[21]. It can be explained by assuming an anion-exchange interaction in this pH range. The behaviour of lysozyme, which is positively charged at all three pH values, also measured as a function of pH and ionic strength (Fig. 9b), indeed precludes the presence of any cation-exchange interaction. This would lead to stronger retention at low ionic strength, which is the opposite of what is observed. At high sodium chloride concentration the retention volume is larger than the estimated void volume of 2.0 ml. The hydrophobic nature of this retention, virtually independent of pH, is confirmed by the results obtained with cytochrome *c*. This small protein has a low hydrophobicity[22] and a constant retention volume at high ionic strength is indeed observed.

The shielding of the silica surface and the remarkable stability of the epoxyglucose silica identify this method as an interesting chemical approach; a small difunctional molecule reacted with aminopropylsilica can cover the acidic surface and give long-term stability at high pH (Fig. 8).

CONCLUSION

There are many chemical approaches to the coating of silica with a hydrophilic layer, two of which have been described here. In both instances a glucose molecule was bound to aminopropylsilica. The main objectives were good shielding of the silica surface and stability in aqueous mobile phases. Although the glucosesilica has a reasonable degree of surface load, it proved to be very unstable. With the epoxyglucosesilica there is good shielding of the acidic silica and very good stability even at high pH. However, the layer is not inert, and ionic and hydrophobic interactions remain. Pore volumes seem to be preserved in both instances.

Although these products on their own do not represent the ideal inert stationary phase, they demonstrate that with the right chemistry stable hydrophilic glucose layers should be attainable. Further attempts in this direction are now being made in our laboratory.

ACKNOWLEDGEMENT

We thank Professor Dr. H. Das for advice on radioactivity measurements.

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Journal of Chromatography, 508 (1990) 301–308
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 339

Direct and reverse flow-through heterogeneous scintillation counting of radiolabelled amino acids using post-column solvent segmentation

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(First received November 16th, 1989; revised manuscript received February 5th, 1990)

SUMMARY

A method for flow-through heterogeneous scintillation counting of ^{14}C-labelled amino acids is described. It is based on post-column solvent segmentation of the aqueous column eluate with hexane before the eluate enters the flow cell of the radioactivity monitor. Using yttrium silicate scintillator granules of 60–80 μm diameter, counting efficiencies of 0.71 (^{14}C) and 0.01 (^{3}H) are obtained. Segmentation of the column eluate allows the temporary storage of the column eluate in a capillary storage loop without additional band broadening and, after the separation is completed, re-introduction of the segmented stream through the radioactivity monitor at reduced flow-rate so as to increase the sensitivity of flow-through radioactivity detection.

INTRODUCTION

Since flow-through radioactivity detectors became commercially available, attempts have been made to overcome the inherent drawback of flow-through radioactivity counting of high-performance liquid chromatographic (HPLC) column eluates, *i.e.*, the coupling of a fast separation with a slow counting step[1–4]. Our group developed a method for flow-through liquid scintillation counting (LSC) of aqueous HPLC column eluates based on the use of a water-immiscible liquid scintillator[3,4]. Before entering the radioactivity detector, the column eluate is extracted on-line with the liquid scintillator (direct counting mode). In addition, the creation of a segmented pattern allows the storage of the complete chromatogram in a capillary storage loop while suppressing extra-column band broadening. When the separation is complete, the contents of the storage loop can be re-introduced into the radioactivity detector at low flow-rates (reverse counting mode) to increase the counting time and, hence, the

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sensitivity of flow-through counting relative to the direct counting mode. In this way, counting times of over 10 min can be applied in favourable cases[4]. In order to reduce the analysis time of the reverse counting mode, regions of interest (RoIs) can be defined. Only during these RoIs is the flow-rate decreased while the remainder of the segmented stream is transported through the detector at higher, *i.e.*, conventional flow-rates.

With this principle, the counting efficiency of low- to medium-energy beta emitters was found to be determined primarily by the mobile phase composition and the extraction yield of the analytes from the aqueous into the organic (scintillator) plugs. The proposed method is not applicable in situations where the analytes cannot be extracted efficiently. This applies to *e.g.*, amino acids, peptides, sugars, nucleic acids and catecholamines.

Since in heterogeneous (solid) scintillation counting (HSC) the counting efficiency is determined by the type and packing density of the scintillator granules only[5], it is of interest to study the combined use of post-column segmentation and flow-through HSC. This alternative could widen the range of application of reverse counting to include non-extractable analytes. In principle, any water-immiscible solvent can then be used for segmentation of the aqueous column eluate.

The approach was first studied by van Nieuwkerk *et al.*[6] in the determination of radiolabelled amino acids. They used a cylindrical, 18-μm yttium silicate-packed glass cell. Although transport of purely aqueous or hexane streams posed no problems, it was found that transporting hexane-segmented aqueous streams through the cell led to a significant increase in the pressure drop across the cell and, finally, cell breakage, probably caused by clogging of the packed cell with particulate matter present in the aqueous phase.

In this study, solvent segmentation was combined with reverse flow-through HSC for the determination of radiolabelled polar analytes, using a commercially available radioactivity monitor equipped with an yttrium silicate-packed cell. Before use, particulate matter was removed from all aqueous solvent mixtures by filtration over a 0.2-μm membrane filter. Relatively large (60–80-μm) scintillator granules were used.

EXPERIMENTAL

Apparatus

A schematic diagram of the set-up used is given in Fig. 1. The column liquid chromatographic system consisted of a Model 114M solvent-delivery module (Beckman, Fullerton, CA, U.S.A.), a Model 7126 six-port injection valve (Rheodyne, Cotati, CA, U.S.A.), a cartridge column holder (Brownlee Labs., Santa Clara, CA, U.S.A.) and a Uvikon variable-wavelength detector, equipped with a Model LCD 725 8-μl flow cell (Kontron, Zürich, Switzerland). A Model P-500 syringe pump (Pharmacia, Uppsala, Sweden) was used to deliver the post-column solvent. Mixing of the two streams took place in a 0.25 mm I.D. T-piece (Valco, Houston, TX, U.S.A.). The combined stream was led through a Ramona-5-LS HPLC radioactivity detector, equipped with a 0.50-ml cell (empty volume after packing), constructed from spirally wound PTFE capillary and packed with 60–80-μm yttrium silicate granules (Raytest, Straubenhardt, F.R.G.). After radioactivity detection, the segmented

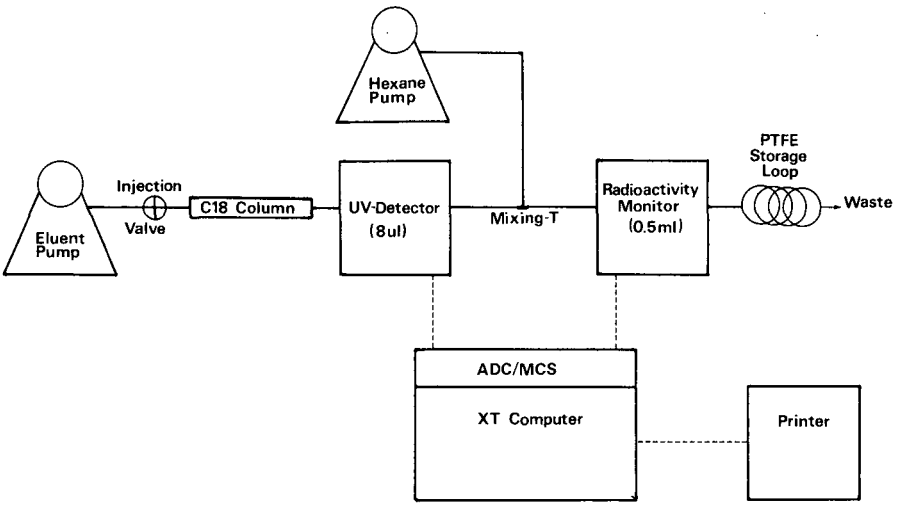

Fig. 1. Schematic diagram of the set-up used for HPLC with flow-through direct heterogeneous scintillation counting (HSC).

stream was stored in a PTFE capillary storage loop (0.8 mm I.D., wall thickness 0.4 mm).

Data acquisition and processing of the analogue (UV) and digital (radioactivity) signal was based on a commercially available software package (Radio-Chroma-to-Graphic-System, version 10.8-MCS, Raytest). This package consists of a triple trace standard program (IM 2313), run-time peak search/fraction collector control (IM 2303), auto-injector control (IM 2304), quench correction (IM 2310) and manual or fully automatic evaluation (IM 2310). It runs on a Tulip PC compact 2 computer with hard disk (Tulip Computers, 's-Hertogenbosch, The Netherlands), equipped with a ADC/multiscaler card for simultaneous data acquisition of one analogue input (configured for up to 100 mV) and two digital (TTL-pulse) inputs. Hard copies were provided by a Model LC-10 multi-front printer (Star Micronics, Nakayoshida, Japan).

Flow-through counting efficiencies, E, were calculated according to

$$E = C_n(F_e + F_s)/[V_d DPM(s)] \quad (1)$$

where C_n is the net peak area (in counts), F_e and F_s are the flow-rates of the mobile phase and make-up flow, respectively (in ml/min), V_d is the void volume of the packed flow cell of the radioactivity monitor (0.5 ml) and $DPM(s)$ is the absolute activity in the peak (in disintegrations per minute).

Absolute activities in the peaks were determined by the sample channels ratio method. For this, peaks eluting from the HPLC system were collected in 20-ml counting vials and, after addition of 15 ml of a water-miscible liquid scintillator, counted on a Model PW 4701 liquid scintillator counter (Philips, Apeldoorn, The Netherlands). ^3H and ^{14}C calibration standards were obtained from Amersham (Amersham, Bucks, U.K.).

Chromatography

Separations were performed using a 5-μm C_{18}-bonded Spheri-5 column (220 × 4.6 mm I.D.) (Pierce, Rockford, IL, U.S.A.). An injection volume of 23 μl was used throughout this study. Mobile phases were made from HPLC/Spectro-grade methanol (Alltech, Deerfield, IL, U.S.A.) and water, buffered to pH 4.2 with HPLC-grade sodium acetate (trihydrate) (Fisons, Loughborough, U.K.) and glacial acetic acid (J. T. Baker, Deventer, The Netherlands). Sodium 1-hexanesulphonate was used as an additive to the mobile phase (Alltech Europe, Eke, Belgium). Before use, water was deionized using a Nanopure II system (Barnstead, Boston, MA, U.S.A.) and subsequently filtered over a 0.2-μm RC 57 membrane filter (Schleicher and Schüll, Dassel, F.R.G.). Before use, mobile phases were degassed using an ultrasonic water-bath.

Chemicals

L[U-^{14}C]Amino acids and tritiated water were obtained from Amersham. Unlabelled amino acid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and technical-grade hexane (mixed isomers) from J. T. Baker. Pico-fluor-40 (Packard, Groningen, The Netherlands) was used as a water-miscible liquid scintillator for absolute activity determinations.

RESULTS AND DISCUSSION

In a study of the potential of a particular reverse counting mode, the experimental results must self-evidently be compared with those obtained by direct measurements. Both modes of operation are, therefore, discussed below.

Direct measurements

A solution containing five ^{14}C-labelled amino acids and spiked with unlabelled phenylalanine was separated by HPLC with UV and flow-through HSC detection. Phenylalanine is the only amino acid which displays good UV absorption and is commercially available as the ^{14}C-labelled compound. In order to suppress additional band broadening in the radioactivity monitor arising from the relatively large (0.50 ml) cell volume, a make-up flow had to be added after the UV detector. To this end, after UV detection a water-miscible [methanol–water (1:9, v/v)] or, alternatively, an immiscible solvent (hexane) was added to the column eluate. The results are shown in Fig. 2. As can be seen from the phenylalanine signals, the make-up flow allows one to obtain band widths in the radioactivity monitor comparable to those observed in the reference detector.

The counting efficiency, calculated from the ^{14}C peak areas, the absolute activities in the peaks and the mean residence time in the detector according to eqn. 1 varied from 0.70 to 0.72. For tritiated water, a counting efficiency of 0.01 was calculated. The typical pressure drop across the cell was about 0.4 MPa at mobile phase and hexane flow-rates of 1.0 ml/min each. No significant increase was observed during a 2-week period of intensive use. Apart from the relatively large (60–80 μm) scintillator granules, this could be due to the effective removal of particulate matter from the mobile phase before use.

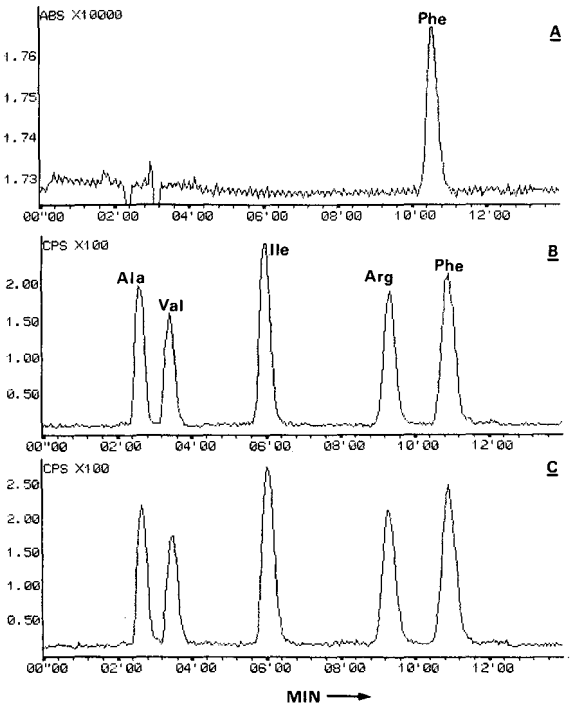

Fig. 2. HPLC of a mixture of radiolabelled amino acids spiked with phenylalanine, with (A) UV detection (220 nm) and (B,C) direct flow-through heterogeneous scintillation counting. Sample, [^{14}C]Ala (350 Bq), [^{14}C]Val (306 Bq), [^{14}C]Ile (544 Bq), [^{14}C]Arg (438 Bq) and [^{14}C]Phe (575 Bq); column, Pierce, 5-μm C_{18} Spheri-5 (220 × 4.5 mm I.D.); mobile phase, methanol–0.05 *M* sodium acetate buffer, pH 4.2 (1:9, v/v) at 1.0 ml/min. In B, a make-up flow of methanol–water (1:9, v/v) was added to the HPLC–UV effluent at 1.0 ml/min via a 0.25 mm I.D. mixing tee. In C, the make-up flow was hexane at 1.0 ml/min.

Reverse measurements

In previous work[6], it was demonstrated that for the storage of segmented aqueous column eluates containing non-extracted analytes, a PTFE storage loop has to be used in order to prevent band broadening via diffusion of the analytes through the aqeous film formed on the inner wall of a stainless-steel tubular capillary. Owing to their low mechanical strength and porosity, PTFE capillaries of 0.4 mm wall thickness have limited pressure resistance, which may hamper their utilization in combination with the transport of segmented streams through packed cells. Fortunately, the conditions encountered in this study posed no problems and flow-rates of up to 1.0 ml/min could easily be employed. In a critical situation, the use of a PTFE-coated stainless-steel capillary may be a good alternative.

In Fig. 3, reverse radiograms of a sample recorded at different flow-rates through the detector are shown; hexane was used as the make-up and segmentation liquid. The additional band broadening can be estimated by comparing the ^{14}C peak widths (in ml) at 10% of the peak height in the direct (not shown) and corresponding reverse radiograms. The ratio of the band widths is a convenient measure for the band

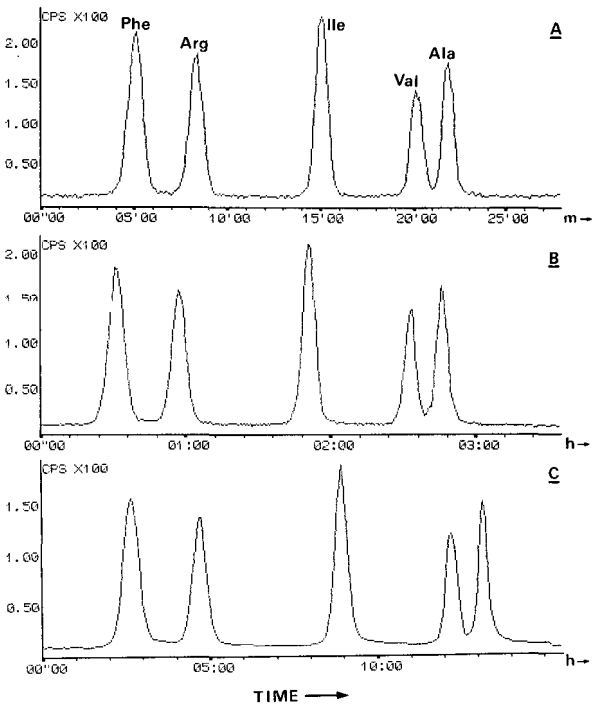

Fig. 3. Reverse radiograms of a mixture of ^{14}C-labelled amino acids, recorded for different counting times, T_d. During the three corresponding direct measurements (data not shown), the HPLC column eluate was segmented with hexane at 1.0 ml/min at the UV detector outlet and stored in a 0.8 mm I.D. PTFE capillary storage loop. After storage of the complete chromatogram, the eluant and hexane pumps were stopped and the outlet of the storage loop was connected to the outlet of the HPLC column. The contents of the loop were then transported through the cell using the eluent pump. The transport flow-rates were (A) 1.0, (B) 0.123 and (C) 0.026 ml/min, corresponding to counting times of 0.5, 4.06 and 19.23 min, respectively. for the HPLC conditions during the direct measurements, see Fig. 2C. Note the different time axes in A (minutes) and B and C (hours).

broadening. In Fig. 4, they are plotted as a function of the counting time. With one exception (alanine), which cannot easily be explained, the amino acids display band broadening ratios between 1.0 (no broadening observable) and 1.2 over the whole range of counting times studied, *i.e.*, for T_d = 0.15–20 min. Obviously, additional band broadening is kept at acceptable levels and certainly is not a function of the counting time.

Table I shows that plots of the net peak area, C_n, *versus* the flow-through counting time of the detector cell, T_d, in the reverse measurements show good linearity for all test solutes over the range T_d = 0.25–4.17 min. The latter counting time corresponds to a flow-rate of 0.12 ml/min through the radioactivity monitor. Note that the slopes of the [^{14}C]amino acid calibration graphs (net peak areas *versus* counting time) differ owing to the different amounts of amino acid in the sample injected onto the column (see Table I).

Deviations from linearity were observed for longer counting times. They are

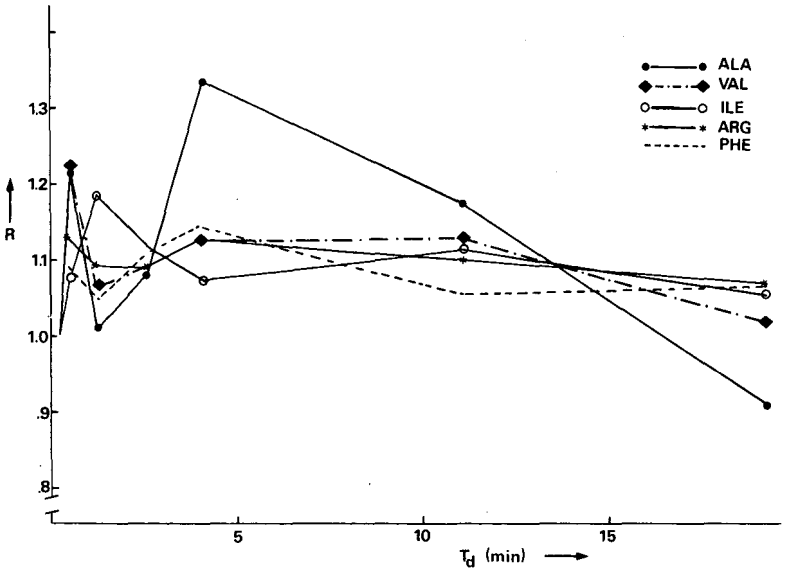

Fig. 4. Band broadening in reverse radiograms relative to that in the corresponding direct radiograms as a function of the counting time, T_d. On the ordinate the ratio, R, of the band widths (measured at 10% of the peak height) from reverse and direct radiograms is given. For HPLC conditions, see Fig. 2C.

best explained from the reduced accuracy in the determination of T_d at the very low flow-rates used for these measurements.

Accordingly, under the conditions mentioned above, a gain factor of about 17 in counting time and, hence, sensitivity is obtained in the reverse counting mode in comparison with the direct counting mode.

CONCLUSIONS

Heterogeneous scintillation counting can be combined with segmentation of aqueous HPLC column eluates to allow both direct and reverse flow-through radioactivity determinations. Additional band broadening is easily kept within acceptable

TABLE I

LINEAR REGRESSION ANALYSIS ON NET PEAK AREAS, C_n (IN COUNTS), *VERSUS* FLOW-THROUGH COUNTING TIME, T_d (IN min)

$C_n = AT_d + B$; T_d ranges from 0.25 to 4.17 min ($n = 5$).

[^{14}C]Amino acid	*Amount injected (Bq)*	*A*	*B*	*R*
Alanine	350	12862	489	0.9998
Valine	306	10509	879	0.9990
Isoleucine	544	18945	1409	0.9992
Arginine	438	15836	1054	0.9996
Phenylalanine	575	19985	1299	0.9996

limits, and flow-through counting times of over 4 min can be used in the reverse mode, which is about a 17-fold increase over direct measurements. As a result, sensitivities comparable to those in off-line liquid scintillation counting can be obtained, yet avoiding most of the disadvantages inherent in the latter technique, such as the loss of resolution in the radiograms, the time-consuming procedure involved in preparing sample vials and data analysis and the high volume of radioactive waste produced. In principle, the total analysis time can be reduced by using flow programming in the reverse mode, as was demonstrated for flow-through liquid scintillation counting[4]. As flow programming of the HPLC pump during a run was not possible with the present set-up, this aspect was not explored.

The limitations of the present principle are inherent in systems using solid scintillator-packed flow cells and are well known from the literature[7]. First, although the yttrium silicate-packed cell has a satisfactory counting efficiency for ^{14}C (E = 0.71), the counting efficiency for ^{3}H was low (E = 0.01) A better performance may be obtained with other types of solid scintillators or by decreasing the scintillator particle size. Second, radiolabelled material may be adsorbed on the scintillator surface and increases in the background count rate can occur after injecting less well defined radioactive samples. In this study, this was observed after injecting an aqueous [^{14}C]leucine sample that had been stored for over 4 years at 4°C. On the other hand, even in this instance, the background count rate could be restored to its original (low) level by flushing the cell with dilute nitric acid. In other words, the introduction of the segmentation/storage principle extends the application range of heterogeneous scintillation counting, without creating additional problems.

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Journal of Chromatography, 508 (1990) 309–317
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Contributions to the calculation of retention data in ion-pair chromatography

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SUMMARY

The retention behaviour of organic bases (*m*- and *o*-aminophenol, dopamine) was investigated in the pH range 2,5–6,8 using aqueous phosphate buffer and constant ionic strength. The capacity factors were determined on two ODS-silica columns in the absence and presence of sodium octylsulphonate. Using the calculated values of the hydrophobic capacities of the columns, a logarithmic relationship is proposed between the capacity factor of the non-protonated base in the absence and presence of the ion-pairing reagent and the actual hydrophobic capacity of the column. For the retention ratios of the protonated base species, the electrostatic theory introduced by Stahlberg proved to be acceptable. At low pH the protonation of the octylsulphonate ion is also considered. The calculated and experimentally obtained capacity factors show acceptable agreement with each other.

INTRODUCTION

With the introduction of modern high-performance liquid column chromatography and the use of chemically bonded alkyl groups on silica as (reverse) stationary phases, an immense development of liquid chromatographic procedures began. During the development of the stationary phases, many efforts were made by the producers to develop products with guaranteed quality for long-term use. The most important point was reproducibility of the products, *i.e.*, the columns filled with the phases should have the same properties, and the second was the preservation of the original properties during the long-term use of the columns.

In spite of many efforts, if one wishes to change the column in a given separation, one has to consider the properties of the columns, even when the particle size, surface area, and nature of the alkyl groups are identical in both columns.

Several papers have been published on the theory of ion-pair chromatography in the last decade. Many models were introduced, but among them three main features can be distinguished. One is based on the formal description of the ion-pair formation reaction taking place between the ion-pairing reagent adsorbed on the stationary phase and the solute ion in the solution. This was treated in detail by Melander *et al.*[1].

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The second type is concerned with the more rigorous description of the equilibria taking place between the two phases, and considering also the limited amounts and the different binding abilities of the (polar and non-polar) binding sites of the stationary phase[2–6]. The third type model is different to the first two, and was introduced by Stahlberg and co-workers[7,8]. In this model the electrostatic equation of Gouy and Chapman was used to describe the interaction between the ions present in the two phases.

In this paper we describe the calculations and considerations made in order to come closer to the solution of the problems that arise on changing columns in a given chromatographic separation.

EXPERIMENTAL

The experiments were carried out with a chromatographic system consisting of eluent reservoirs, Model 6000 A pumps (Waters Assoc.), Model 7010 six-port injection valves (Rheodyne), thermostated columns[9] and a Type LC-55 UV–VIS spectrophotometric detector (Perkin-Elmer).

Two octadecylsilica columns were used: ODS-Hypersil (Shandon-Southern), 150 mm × 4.6 mm I.D., film thickness 5 μm; 1.52 g; C = 8.8%; S_{BET} = 173 m^2/g; V_0 = 1.8 ml; density ρ = 0.817; and ODS-Supelcosil (Supelco), 120 mm × 4.6 mm I.D., film thickness 5 μm; 1.3 g; C = 9.97%; S_{BET} = 155 m^2/g; V_0 = 1.5 ml; density ρ = 0.838. The Supelcosil column was new whereas the Hypersil column was well used.

The aqueous mobile phase contained phosphate buffer (C_{PO_4} = 0.02 M) of various pH (2.5–6.6) and sodium bromide to keep the sodium ion concentration at C_{Na} = 0.08 M. Octylsulphonic acid was used as the ion-pair-forming agent.

To ensure similar conditions for the stationary phases [similar coverage by the ion-pairing reagent (IP)], the concentrations in the mobile (C_{IP}) and stationary phases ($\bar{C}_{IP}$) were as follows: for the Hypersil column C_{IP} = 0.005 M and $\bar{C}_{IP}$ = 0.078 mmol/g and for the Supelcosil column C_{IP} = 0.0025 M and $\bar{C}_{IP}$ = 0.088 mmol/g. Changes in these concentrations in the pH range investigated were not significant.

The solutes investigated and the logarithm of the protonation constants were as follows: *m*-aminophenol (MAP), log K = 4.25; *o*-aminophenol (OAP), log K = 4.8; and dopamine [DOP; 2-(3,4-dihydroxyphenyl)ethylamine], log K = 8.9.

The capacity factors of the solutes were determined on both columns at different pH values, in the absence and presence of the ion-pairing reagent. The values obtained are presented in Figs. 1 and 2. The estimated capacity factors for the unprotonated and protonated bases are given in Table I.

The non-polar, hydrophobic capacity (q^0) was calculated from the carbon contents and surface areas of the stationary phases using the method of Berendsen *et al.*[10]. The polar capacity (q^x) was determined by breakthrough experiments, using 10^{-3} M hydroxide solution after acid treatment (with nitric acid) and washing of the column. The values found were as follows: Hypersil, q^0 = 360 and q^x = 350 μmol/g, and Supelcosil, q^0 = 510 and q^x = 190 μmol/g. Because the Hypersil column was well used, 300 μmol/g was used instead of the calculated 360 μmol/g in further calculations.

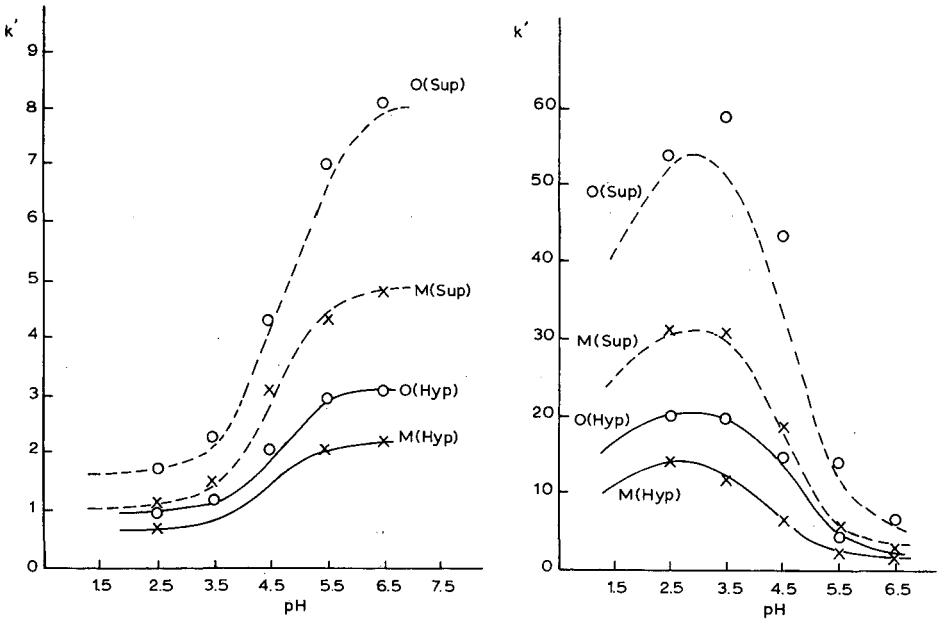

Fig. 1. Capacity factors of *o*-aminophenol (O) and *m*-aminophenol (M) at various pH values of the mobile phase using Hypersil and Supelcosil columns. The symbols (○ and ×) denote the values found experimentally and the full and dashed lines are the calculated values. Log K_M = 4.5; log K_0 = 4.7; log K_{IP} = 1.0; C_{IP} = 0.0 M.

Fig. 2. Capacity factors of *o*-aminophenol (O) and *m*-aminophenol (M) at various pH values in the presence of octylsulphonate ion-pair forming ion, using Hypersil and Supelcosil columns. Symbols and lines as in Fig. 1. C_{IP} = 0.005 *M* for the Hypersil column and 0.0025 *M* for the Supelcosil column.

The phase ratios used in the calculations were

$$\beta^{0}_{Hyp} = \frac{0.300}{1.8/1.52} = 0.25$$

$$\beta^{x}_{Hyp} = \frac{0.350}{1.8/1.52} = 0.30$$

TABLE I

CAPACITY FACTORS OF UNPROTONATED AND PROTONATED BASES

Capacity factor	*In absence of IP*		*In presence of IP*	
	Hypersil	*Supelcosil*	*Hypersil*	*Supelcosil*
$k'_{B(MAP)}$	2.14	4.68	1.58	2.82
$k'_{BH(MAP)}$	0.65	1.0	14.0	36.0
$k'_{B(OAP)}$	3.1	8.0	2.0	5.0
$k'_{BH(OAP)}$	0.9	1.5	21	66
$k'_{BH(DOP)}$	1.5	4.6	19	62

$$\beta^{0}_{Sup} = \frac{0.510}{1.5/1.3} = 0.43$$

$$\beta^{x}_{Sup} = \frac{0.190}{1.5/1.3} = 0.17$$

where 0 and x refer to the hydrophobic and polar phase ratio, respctively.

RESULTS AND DISCUSSION

As can be seen from the data in Table I, the capacity factors for the all species are higher for the Supelcosil than for the Hypersil column. The difference is greater for the more hydrophobic species (MAP < OAP < DOP) both in the absence and presence of the ion-pairing agent.

Adsorption of the solutes in the absence of the ion-pairing reagent

The distribution ratios were calculated using the equation

$$d = k'/\beta \tag{1}$$

where k' is the capacity factor. For the non-protonated bases, $d_{B(MAP)} = 8.56$ for Hypersil and 10.88 for Supelcosil and $d_{B(OAP)} = 12.4$ for Hypersil and 18.60 for Supelcosil.

Assuming the following reaction[3,6]:

$$B + A \overset{K_B}{\rightleftharpoons} BA \tag{2a}$$

where A is the adsorption site on the stationary phase

$$K_B = \frac{(B)}{[B]\,q^0} \tag{2b}$$

where parentheses represent the concentration in the stationary phase and square brackets that in the mobile phase. We did not obtain identical K_B values for the two columns: $K_{B(MAP)} = 28.5$ for Hypersil and 21.8 for Supelcosil and $K_{B(OAP)}$ 41.3 for Hypersil and 37.2 for Supelcosil. Hence the adsorption strength is not linearly dependent on the hydrophobic capacity.

To establish whether the protonated base (cations) are bound by the polar or by the hydrophobic area of the column, indirect calculations were used to obtain the polar and non-polar contributions:

$$k'_{BH(Hyp)} = d^{x}_{BH}\beta^{x}_{Hyp} + d^{0}_{BH}\beta^{0}_{Hyp} \tag{3}$$

$$k'_{BH(Sup)} = d^{x}_{BH}\beta^{x}_{Sup} + d^{0}_{BH}\beta^{0}_{Sup} \tag{4}$$

As $\beta^{x}_{Hyp} > \beta^{x}_{Sup}$ and $\beta^{0}_{Hyp} < \beta^{0}_{Sup}$, it was expected that the d^{x}_{BH} *and* d^{0}_{BH} values found would give an indication of the sorption mechanism.

It was found in all instances that the polar contribution was almost zero compared with the hydrophobic contribution. Hence we suggest that the absorption of the protonated base takes place in the form of the ion pair formed with phosphate ions, ($BH \cdot H_2PO_4$), *i.e.*,

$$BH^+ + \overline{NaH_2PO_4} \rightleftharpoons \overline{BH \cdot H_2PO_4} + Na^+ \tag{5}$$

where the bars indicate compounds present in the stationary phase.

$$K_{BH} = \frac{(BH)[Na^+]}{[BH^+]q^0} = d_{BH} \cdot \frac{[Na^+]}{q^0} \tag{6}$$

The calculated K_{BH} values for the two bases and for the two columns were as follows: $K_{BH(MAP)} = 0.69$ for Hypersil and 0.37 for Supelcosil and $K_{BH(OAP)} = 0.96$ for Hypersil and 0.55 for Supelcosil. The K_{BH} values obtained were different for the different phases. Hence we conclude that the adsorption strength does not depend linearly on q^0.

Adsorption of the solutes in the presence of the ion-pairing reagent

The adsorption of the ion-pairing reagent may take place according to the following equation:

$$IP^- + \overline{NaH_2PO_4} \overset{K_{IP}}{\rightleftharpoons} \overline{Na \cdot IP} + H_2PO_4^- \tag{7}$$

$$K_{IP} = \frac{(IP)[H_2PO_4^-]}{[IP^-]q^0} \tag{8}$$

for the two columns the calculated values were found to be

$K_{IP(H)} = (0.078 \cdot 0.02)/(0.005 \cdot 0.3) = 1.04$ and $K_{IP(S)} = (0.088 \cdot 0.02)/(0.0025 \cdot 0.5) = 1.41$. Hence the extent of adsorption cannot be described simply by eqn. 7.

The adsorption of the non-protonated base in the presence of ion-pair forming species was always lower than that in its absence. It is true that the hydrophobic surface is partly occupied by the ion-pairing reagent, but a considerable part of the surface is still free. Therefore, the assumption of the displacement of ion-pairing ions by the solutes is not necessary.

However, we may attribute this lowering effect of the adsorbed ion-pairing reagent to the decreased hydrophobic capacity if we assume that the adsorption strength of the base depends on the accessible surface area not linearly but logarithmically, *i.e.*,

$$\log k'_B = \gamma q^0 + \log k_0 \tag{9}$$

As we know the k'_B values of the compounds in the absence of the ion-pairing reagent for Hypersil ($q = 0.3$) and for Supelcosil ($q = 0.51$), γ and k_0 can be calculated and also the log k'_B values corresponding to the actual capacities, which were left free in presence of the ion-pairing reagent (in this instance it was assumed that with the ion-pairing reagent one alkyl group was occupied). γ is characteristic for a given compound.

Calculated γ and k'_B values in the presence of the ion-pairing reagent were found as follows: for MAP, $\gamma_M = 1.7$ and log $k_0 = -0.18$, for $q^0 = 0.3$–0.078 $k'_{B(IP)} = 1.57$ (Hypersil) and for $q^0 = 0.5$–0.088, $k'_{B(IP)} = 3.3$ (Supelcosil); and for OAP, $\gamma_0 = 2.05$ and log $k_0 = -0.125$, for $q^0 = 0.3$–0.078, $k'_{B(IP)} = 2.13$ (Hypersil) and for $q^0 = 0.5$–0.088, $k'_{B(IP)} = 5.2$ (Supelcosil). The experimental values for k'_B are 1.58 and 2.82, respectively, for MAP, and 2.0 and 5.0, respectively, for OAP (see Table I).

The protonated base, having a positive loading, *i.e.*, being a cation, forms a hydrophobic ion-pair compound with the octylsulphonate ion (ion-pairing reagent) and therefore the capacity factor will be high.

According to the classical theories, used widely in the literature[1], an ion-exchange process takes place between the cations (sodium) of the ion-pairing reagent ion adsorbed on the stationary phase and the protonated base cation. Thus,

$$BH^+ + \overline{IP^-Na^+} \overset{K_x}{\rightleftharpoons} \overline{IP \cdot BH} + Na^+ \tag{10}$$

At equilibrium:

$$K_x = \frac{(BH)[Na^+]}{[BH^+](IP)} = d_{BH}\frac{[Na^+]}{(IP)} \tag{11}$$

As the total amount of the ion-pair forming reagent is much higher than that of the protonated base cation, the ion-exchange equilibrium ratio, denoted by K_x, may be assumed to be constant. If we take the known concentrations in the experiments carried out with the two different columns, the calculated K_x values, however, were not found to be identical: MAP, 57.4 and 76.1; OAP, 86.1 and 139.5; and DOP, 78 and 131 for Hypersil and Supelcosil, respectively. If the ion-exchange mechanism were to be valid, the value of K_x would have been independent of the column used.

Reasonable values for the description of the retention of the protonated base cations can be obtained if the electrostatic theory and the equations introduced by Stahlberg and coworkers[7,8] is used. According to the theory, the capacity factor is expressed by the following equation:

$$k'_{BH} = \beta^0 \exp\left(-\frac{\Delta G^0_{AH} - z_{BH}F\Delta\psi}{RT}\right) \tag{12}$$

where ΔG^0_{AH} is the chemical energy term, $-z_{BH}F\Delta\psi$ is the electrostatic energy sterm, z is the charge of the ion adsorbed ($+1$ for BH^+), F is the Faraday constant, R the gas constant, T temperature and ψ the electrostatic potential.

The ratio of the capacity factors in the presence and absence of the ion-pairing reagent (which is responsible for the electrostatic potential at the stationary phase) is simple:

$$\frac{k'_{BH(IP)}}{k'_{BH(0)}} = \exp\left(-z_{BH}\Delta\psi \cdot \frac{F}{RT}\right) \tag{13}$$

Taking the capacity factors found experimentally and using eqn. 13, the following values were found for $-z_{BH}\Delta\psi_0 F/RT(= X)$: for Hypersil [(IP) = 0.078], MAP 3.07, OAP 3.15 and DOP 2.54; and for Supelcosil [(IP) = 0.088], MAP 3.58, OAP 3.78 and DOP 2.60. The values obtained for MAP and OAP are similar, but those for DOP are different, as the structure and size of the ion are different. The ratios of the X values found for the two columns, 3.58/3.07 = 1.17, 3.78/3.15 = 1.20 and 2.60/2.54 = 1.02, nearly correspond to the ratio of the concentrations of the ion-pair forming reagent (octylsulphonate) on the two stationary phases: 0.088/0.078 = 1.13. The concentration of the ion-pairing reagent on the stationary phase under the given conditions is directly responsible for the electrostatic interaction.

CONCLUSIONS

In the absence of the ion-pair forming reagent the retention of the non-protonated and protonated base depends on the hydrophobic capacity of the column. The relationship between k' and q^0 is not linear but logarithmic.

The retention of the non-protonated base is lower in the presence of the ion-pair forming reagent than in its absence, and the difference ($\Delta \log k'$) corresponds to the surface capacity occupied by the ion-pairing reagent. No competition between the base and the ion-pairing reagent is considered.

The increased retention of the protonated base cations caused by ion-pair formation cannot be described with ion exchange, but can be described using the electrostatic theory, considering the concentration of the ion-pairing reagent on the stationary phase.

For the calculation of capacity factors from one column to another, if the experimental conditions are the same, the following equations can be proposed:

For the unprotonated base:

$$\log k'_2 - \log k'_1 = \gamma(q_2^0 - q_1^0) \tag{14}$$

$$\log k'_{(0)} - \log k'_{(IP)} = \gamma(IP) \tag{15}$$

where γ is characteristic of the solute and k_2, q_2^0 and k_1, q_1 refer to two columns of similar size and stationary phase;

For the protonated base:

$$\log k'_{BH(IP)} - \log k'_{BH(0)} = X' \tag{16}$$

$$\log k'_2 - \log k'_1 = X' \cdot \frac{(IP)_2}{(IP)_1} \tag{17}$$

where $X' = 0.43\,X$

If we know k'_B and k'_{BH}, the pH dependence of the k' values in absence of the ion-pairing reagent can be calculated in the usual way[1]:

$$k'_{B/BH} = k'_B\varphi_B + k'_{BH}\varphi_{BH} \tag{18}$$

In the presence of the ion-pairing reagent, the equation is similar but the protonation of the ion-pairing reagent is also considered, *i.e.*,

$$k'_{B/BH(IP)} = k_B\varphi_B + k_{BH}\varphi_{BH}\varphi_{IP} \tag{19}$$

The φ values are the molar fractions of the corresponding species:

$$\varphi_B = \frac{1}{1 + [H^+]K_B}; \quad \varphi_{BH} = \frac{[H^+]K_B}{1 + [H^+]K_B} \tag{20}$$

$$\varphi_{IP} = \frac{1}{1 + [H^+]K_{IP}} \tag{21}$$

where K_B and K_{IP} are the protonation constants of the base and of the ion-pairing ion, respectively.

The calculated and experimentally found values are shown in Figs. 1 and 2. As can be seen, the values are in good agreement with each other.

It must be stressed that the all above considerations and conclusions are valid only for the simple systems investigated and one must be very cautious in generalizing the results obtained in the calculation of capacity factors of solutes that have larger molecules or more complicated structures (with more polar groups, etc.).

SYMBOLS

A	adsorption site on the stationary phase
B, BH^+	non-protonated and protonated base species, respectively
[B], [BH^+], [IP]	concentrations of the species B, BH^+ and IP in the mobile phase
(B), (BH), (IP)	concentrations of the species B, BH^+ and IP in the stationary phase
C	carbon content of the column packing material (%)
C_{IP}	concentration of the ion-pair forming reagent in the eluent (mol/l)
$\bar{C}_{IP}$	concentration of the ion-pair forming reagent in the stationary phase (mmol/g)
d	distribution ratio (the subscript refers to the species
DAP	dopamine
F	Faraday constant
ΔG^0_{BH}	free energy change of the adsorption of the spcies BII^+
Hyp	Hypersil
IP	ion-pair forming reagent
k'	capacity factor (the subscript refers to the species, the number to the columns)
k_0	parameter of the logarithmic eqn. 9
K	concentration equilibrium "constant" (the subscript refers to the reaction)

MAP	*m*-aminophenol
OAP	*o*-aminophenol
q^0	hydrophobic capacity of the stationary phase (μmol/g)
q^x	polar capacity of the stationary phase (μmol/g)
R	gas constant
S_{BET}	specific surface area of the column packing material obtained by the BET method (m^2/g)
Sup	Supelcosil
T	temperature (K)
V_0	dead volume of the column (ml)
z_{BH}	charge number of the species BH^+
X	exponent term of eqn. 13
β^0	phase ratio (hydrophobic)
β^x	phase ratio (polar)
γ	parameter of the logarithmic eqn. 9 (the subscript refers to the species)
ρ	density of the column packing (g/ml)
φ	molar fraction of a species; the subscript refers to the species
$\Delta\psi$	electrostatic surface potential change at the adsorption

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Journal of Chromatography, 508 (1990) 319–331
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 318

Analysis of poly(styrene–co-methyl acrylate) and poly(styrene–co-butyl acrylate) by high-performance liquid chromatography

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(First received October 16th, 1989; revised manuscript received January 30th, 1990)

SUMMARY

Poly(styrene–co-methyl acrylate) and poly(styrene–co-butyl acrylate) were separated according to their chemical composition by gradient elution. The chromatographic separation on silica was optimized for a gradient ranging from *n*-heptane as a non-solvent to dichloromethane containing a small amount of methanol as a strong solvent. The influence of different stationary phases, the chemical composition and molecular mass on the separation of the copolymers was investigated. From the results of different chromatographic and turbidimetric experiments it is concluded that the copolymer separation is controlled by both precipitation and adsorption mechanisms. The contribution of adsorption processes to the separation is only advantageous when normal-phase gradients are applied.

INTRODUCTION

In research on copolymers, liquid chromatographic cross-fractionation (CCF) is an important tool in the analysis of the molecular mass chemical composition distribution (MMCCD) of such components[1]. The fractionation of copolymers according to molecular mass (MM) is usually performed by size-exclusion chromatography (SEC), followed by a normal- or reversed-phase gradient high-performance liquid chromatographic (HPLC) separation for the determination of the chemical composition distribution (CCD).

The gradient HPLC analysis of copolymers may be complicated by the starting and final eluents and the shape of the gradient, possible interferences from the sta-

0021-9673/90/$03.50

tionary phases and the influence of the MM of the copolymers. In these analyses of copolymers, the gradient usually starts with a non-solvent, and subsequently the solvating power of the eluent is increased by the gradient.

For the separation of styrene–methyl acrylate (SMA) copolymers according to chemical composition, a gradient high-speed LC separation on silica as the stationary phase has been reported[2]. No interference of the MM on the separation was observed. For the separation of poly(styrene–co-acrylonitrile)[3–8] no influence of the applied stationary phases was reported, indicating that this separation is controlled by a precipitation mechanism rather than by an adsorption process. On the other hand, the separations of poly(styrene–co-methyl methacrylate (PSMMA)[6,9–17] and poly (styrene–co-ethyl methacrylate)[1,4,18] appeared to be influenced by the used normal-phase (NP) and reversed-phase (RP) stationary phases. Moreover, for NP packings Glöckner and Van den Berg [6,9] showed that for the elution of a specific PSMMA sample with a typical gradient an increased percentage of the applied solvent, tetrahydrofuran (THF), was necessary in comparison with the results of turbidimetric titrations. This indicates that adsorption may also play a role in this separation process.

The detection of the copolymers was performed using UV absorption at 254 or 259 nm. At these wavelengths absorption is due to the aromatic styrene monomer parts of the polymers. This implies that the absorption coefficient increases with increase in the styrene content of a typical copolymer. On the other hand, the acrylonitrile or (meth)acrylate homopolymers are difficult to detect with this detection method.

In this study we developed and optimized an HPLC gradient system on silica as a stationary phase for PSMA, which also allowed the UV detection of the MA homopolymer. The investigated eluents were dichloromethane (DCM), dichloroethane (DCE) and distilled THF without stabilizer. As recommended by Snyder[19], a few percent of methanol was added to DCM and DCE in order to increase their eluting strength to a value comparable to that of THF.

We also investigated the influence of a number of stationary phases, the chemical composition and the MM on the separation of the copolymers under study. Turbidimetric titrations were performed and compared with the chromatographic data to study the influence of the different stationary phases. Also, a similar separation system for poly(styrene–co-butyl acrylate) (PSBA), a copolymer in which the two monomeric units differ less in polarity compared with PSMA, was developed.

EXPERIMENTAL

Chemicals

The applied solvents *n*-heptane (extra pure), DCM (analytical-reagent or HPLC grade), methanol (HPLC grade) and THF with stabilizer (analytical-reagent grade) were obtained from Merck (Darmstadt, F.R.G.) and DCE (analytical-reagent grade) from Fluka (Buchs, Switzerland). Before use, the solvents were filtered through a 0.45-μm HVLP membrane filter (Millipore, Bedford, MA, U.S.A.).

Samples

The polymer and copolymer samples with a narrow CCD were synthesized by

low-conversion solution polymerization in toluene at 62°C with 2,2'-azobis(isobutyronitrile) as an initiator under argon. The reaction mixture was poured into *n*-heptane after *ca.* 10% conversion. Subsequently the precipitated (co)polymer was dried under vacuum at 50°C. In Table I the (co)polymer samples are listed, including the number-average chemical composition and the weight-average molecular mass (M_w). The molecular masses of the samples were determined by SEC and the chemical composition by proton nuclear magnetic resonance spectroscopy.

Gradient HPLC

The eluent gradients were performed with a Model 720 system controller and two Model 510 HPLC pumps (Millipore–Waters, Milford, MA, U.S.A.). In all instances the linear gradients from time $t = 0$ to 18 min ranged from 10% to 100% of a typical solvent (a mixture of methanol with DCM or DCE) in the non-solvent (*n*-heptane). The eluent flow-rate was 0.4 ml/min. The samples were injected with a Waters Assoc. Model 710 Intelligent Sample Processor (WISP). UV detection was performed with a Waters Assoc. Model 490 multi-wavelength Detector at 235 and 260 nm. The chromatographic separations were carried out on a Chromsep 5-μm

TABLE I

SYNTHESIZED LOW-CONVERSION SOLUTION (CO)POLYMER SAMPLES

Sample	*Composition (% styrene)*	M_w *(kg/mol)*
PS	100	43
PSMA 1	83	23
PSMA 2	83	22
PSMA 3	81	63
PSMA 4	77	20
PSMA 5	76	66
PSMA 6	67	72
PSMA 7	67	105
PSMA 8	64	20
PSMA 9	57	74
PSMA 10	49	32
PSMA 11	46	75
PSMA 12	46	23
PSMA 13	33	65
PSMA 14	31	113
PSMA 15	29	58
PSMA 16	27	30
PSMA 17	12	110
PMA	0	111
PBA	0	255
PSBA 1	12	177
PSBA 2	24	138
PSBA 3	35	103
PSBA 4	41	83
PSBA 5	44	99
PSBA 6	61	83
PSBA 7	69	–
PSBA 8	78	59

TABLE II

STATIONARY PHASES APPLIED IN THE GRADIENT HPLC OF SMA COPOLYMERS

Stationary phase	*Particle size (μm)*
Nucleosil Si-50	5
Nucleosil CN	5
Nucleosil NH_2	5
LiChrosorb diol	5
LiChrosorb RP-18	5
LiChrosorb RP-8	5
Glass beads	0–20

silica cartridge column (10 cm × 3.0 mm I.D.) (Chrompack, Middelburg, The Netherlands).

Laboratory-packed columns (10 cm × 4.0 mm I.D.) (Knauer, Berlin, F.R.G.) were used for the investigation of the different stationary phases, listed in Table II. The eluent flow-rate was 0.8 ml/min. In these experiments methanol was also applied as a non-solvent. To study the influence of temperature on the separation of the copolymer samples, the column temperature was controlled with a water-jacket thermostatic bath at different temperatures ranging from 20 to 45°C.

Size-exclusion chromatography

The HPLC equipment used for the SEC analyses and fractionations of the (co)polymers consisted of a Waters Assoc. Model 510 HPLC pump, a WISP Model 712, a Model 440 absorbance detector operating at 254 nm, a Model 410 differential refractometer, a Waters Assoc. automated switching valve and a Cygnet fraction collector (Isco, Lincoln, NE, U.S.A.). The separations were performed on three Waters Assoc. μStyragel columns (25 cm × 7 mm I.D.; average particle size 10 μm; nominal pore sizes of the packings 10^2, 10^3 and 10^4 nm). THF under helium was used as the eluent at a flow-rate of 0.6 ml/min. Analyses of the (co)polymers were performed with 20-μl injections of a solution of a typical polymer in THF (1 g/l) whereas for the fractionations 1000 μl of the same solutions were injected. The processing of the chromatographic data was performed on two SP 4100 computing integrators (Spectra-Physics, Santa Clara, CA, U.S.A.) equipped with a Kerr 4100 D minifile (Spectra-Physics).

Turbidimetric titrations

Turbidimetric titrations were performed by dissolving 25 mg of a typical polymer in 25 ml of solvent. Subsequently, this solution was titrated with small portions of a non-solvent (*n*-heptane or methanol) at ambient temperature with continuous stirring. Precipitation was monitored by measuring the absorption at 500 nm with an HP 8451 A diode-array spectrophotometer (Hewlett-Packard, San Diego, CA, U.S.A.). During the titration the solution was circulated through a bypass, connected to a 4 × 1 × 1 cm glass measuring cuvette in order to detect the absorption properties of the suspension. The precipitation points were calculated by the intersection of the tangent at the deflection point with the horizontal axis in a plot of the concentration-corrected absorption *versus* the percentage of non-solvent added.

RESULTS AND DISCUSSION

In order to allow the UV detection of PMA and PBA, solvents with a sufficiently low UV cut-off must be used. Of the three strong solvents investigated, DCM was the most suitable eluent with respect to a constant low-background UV absorption. The application of distilled THF was avoided as much as possible for safety and storage reasons. The lowest possible detection wavelength appeared to be 235 nm with a basic absorption of 0.3 absorbance.

For the optimum separation of PSMA on the Chromspher silica stationary phase, the influence of the following parameters was studied: eluent flow-rate, gradient speed, temperature (Fig. 1) and percentage of methanol in the strong solvents (Fig. 2). Without taking into account the width of the CCD of the copolymers, the separation efficiency was determined by calculating the average peak width at half-height for the separated copolymer standards expressed as a styrene percentage. The optimum eluent flow-rate and gradient shape were 0.4 ml/min and 5%/min, respectively. The data in Fig. 1 show the dependence of the retention times of the copolymers on temperature. From this it can be concluded that for accurate measurements the column temperature must be strictly controlled.

For an optimum separation efficiency the methanol fraction in the strong solvent should be as low as possible, but sufficient to elute the strongest adsorbing (co)polymer of the sample. It turned out that for PSMA and PSBA on the silica column the methanol concentration in DCM should be 4% and 1.2%, respectively.

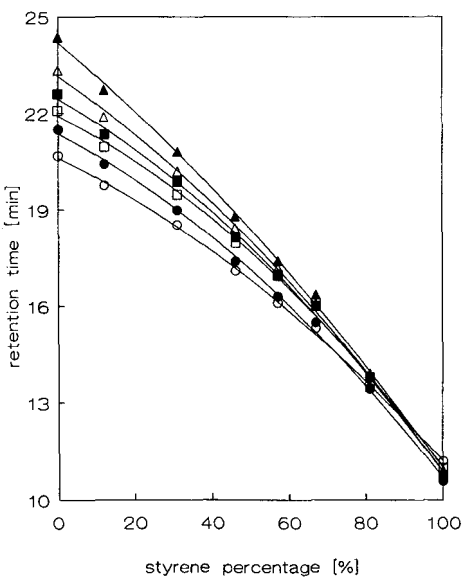

Fig. 1. Retention time as a function of the styrene percentage in PSMA at different temperatures, fitted with second-degree polynomials. Samples, PMA, PSMA 3, 7, 9, 11, 14 and 17, PS. Solvent, DCM + 4% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; ○ = 20.2°C; ● = 25.1°C; □ = 30.2°C; ■ = 34.5°C; △ = 39.7°C; ▲ = 45.1°C.

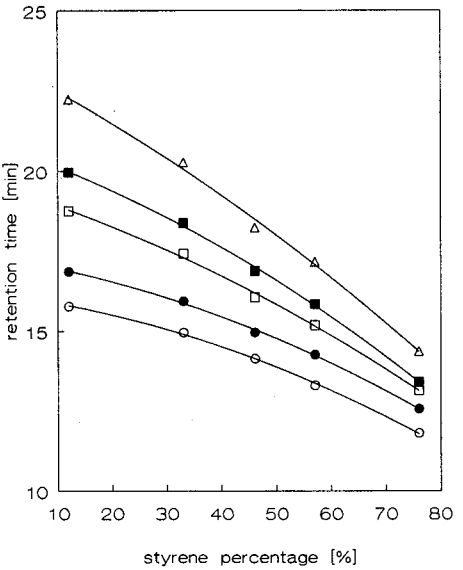

Fig. 2. Retention time as a function of the styrene percentage in PSMA at different compositions of the eluent, fitted with second-degree polynomials. Samples, PSMA 5, 9, 11, 13 and 17. Solvent, DCM + methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, ambient. Methanol (%): ○ = 20; ● = 10; □ = 5; ■ = 4; △ = 3.

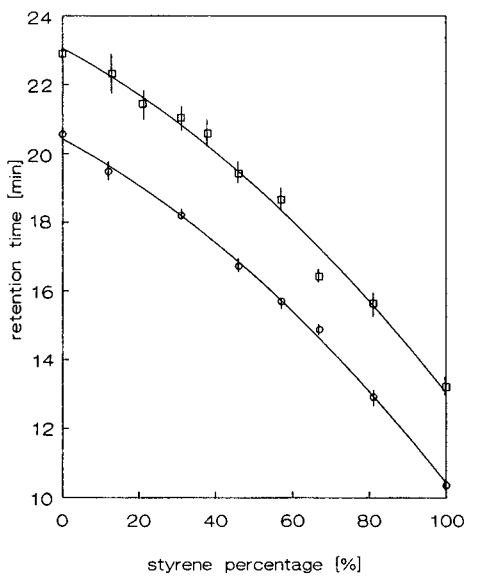

Fig. 3. Retention time as a function of the styrene percentage in PSMA and PSBA fitted with second-degree polynomials, with an indication of the peak width at half-height of the standards used. Samples, PSMA mixture, PBA, PSBA 1–8, PS. Solvents, DCM + 4% methanol for PSMA, DCM + 1.2% methanol for PSBA; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, ambient. ○ = PSMA; □ = PSBA.

For both PSMA and PSBA a similar relationship between the percentage of styrene in the copolymer and the retention time was observed (Fig. 3). Fig. 3 also indicates a decreased separation efficiency for PSBA, owing to a smaller difference in polarity between the two monomer components. An increase in the column temperature improved the separation efficiency of PSMA owing to an increased adsorption of PMA. On the other hand, the effect of increasing the column temperature may also be obtained, to a certain extent, by decreasing the methanol content of the eluent. The disadvantage of the latter possibility is the increased elution time of PS.

Several polymers (Table III) were fractionated according to molecular mass by SEC in order to investigate the influence of the molecular mass on gradient HPLC. The fractions were analysed with three different HPLC systems (Figs. 4–6). In these figures the influence of the molecular mass is shown in plots of the retention time *versus* $(MM)^{-1/2}$. According to Glöckner[3] such a plot results in a straight line when the separation is dependent on the solubility. If the molecular mass influences the retention time it decreases linearly with $(MM)^{-1/2}$. However, under the optimum conditions for the separation of PSMA this influence is negligible, simplifying the interpretation of the data. The peak broadening of the copolymer standards at lower MM is mainly caused by the broader CCD at lower molecular masses, controlled by the copolymerization kinetics[20]. The peak width increases with decreasing MM and reaches unacceptable values below an MM of 10 kg/mol for PSMA. At the same time the ratio of the UV absorption ratios (peak area or height) at 260 nm to 235 nm decreases strongly at the same low molecular masses. These two observations are shown in Fig. 7.

For accurate quantitative measurements, the relationship between the relative absorption coefficient and the composition of a specific copolymer must be known. To determine this relationship, a mixture of six SMA copolymers and both the homopolymers was prepared (PS, PSMA 3, 7, 9, 11, 14 and 17 and PMA) with exactly known relative amounts. This mixture was separated and the peak areas were measured. Fig. 8 shows the absorption coefficients of PSMA relative to polystyrene at 235 nm. The correlation coefficients of the two absorption curves are 0.995 at 260 nm and

TABLE III

MOLECULAR MASSES (kg/mol) OF SEC-FRACTIONS

$M_w \approx M_n$ (M_w = weight-average molecular weight; M_n = number-average molecular weight).

Sample	*Fraction No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
PSMA 1	60	35	25	13	8.0	2.1		
PSMA 8	70	34	11	5.0	2.5	1.2		
PSMA 12	70	40	25	1.5	9.0	4.0		
PSMA 16	80	50	30	20	14	6.0		
PMA	300	140	70	42	24	16	11	2.1
Mixture (PS, PSMA 3,7, 9,11, 14 and 17, PMA)	220	120	82	59	40	25	13	
PSBA 2	330	210	150	110	82	53	23	
PSBA 6	200	130	92	68	48	31	16	

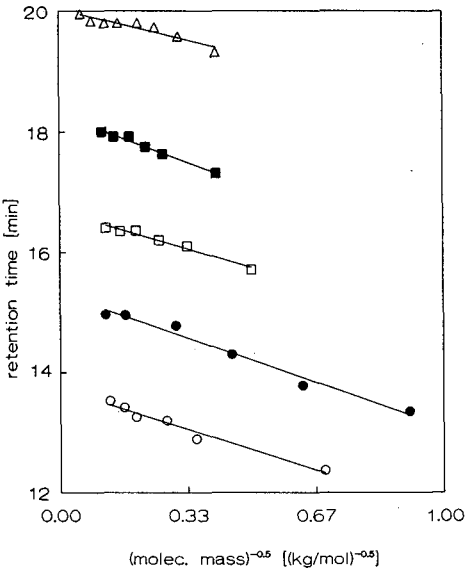

Fig. 4. Retention time as a function of $(MM)^{-1/2}$ of PSMA, fitted with first-degree polynomials. Solvent, DCE + 10% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, ambient. ○ = PSMA 1; ● = PSMA 8; □ = PSMA 12; ■ = PSMA 16; △ = PMA.

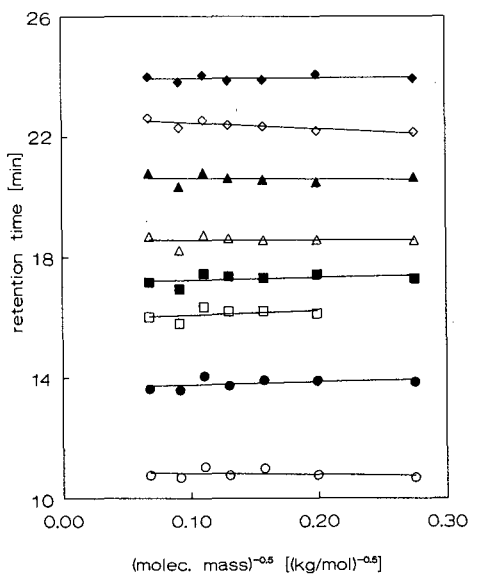

Fig. 5. Retention time as a function of $(MM)^{-1/2}$ of PSMA, fitted with first-degree polynomials. Solvents, DCM + 4% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, 30°C. ○ = PS; ● = PSMA 3; □ = PSMA 7; ■ = PSMA 9; △ = PSMA 11; ▲ PSMA 14; ◇ = PSMA 17; ◆ = PMA.

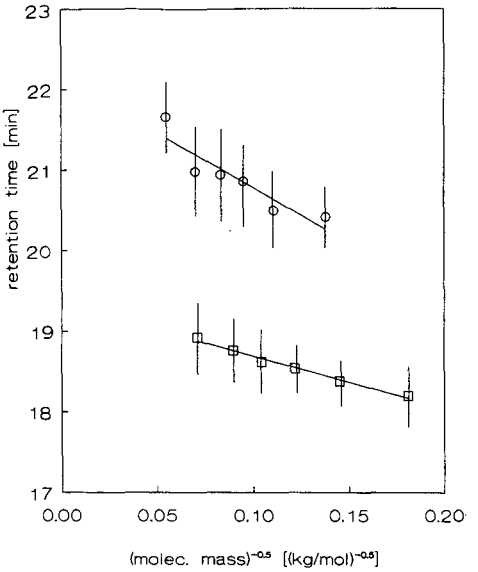

Fig. 6. Retention time as a function of $(MM)^{-1/2}$ of PSBA, fitted with first-degree polynomials, including an indication of the peak width at half-height. Solvent, DCM + 1.2% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, ambient. ○ = PSBA 2; □ = PSBA 6.

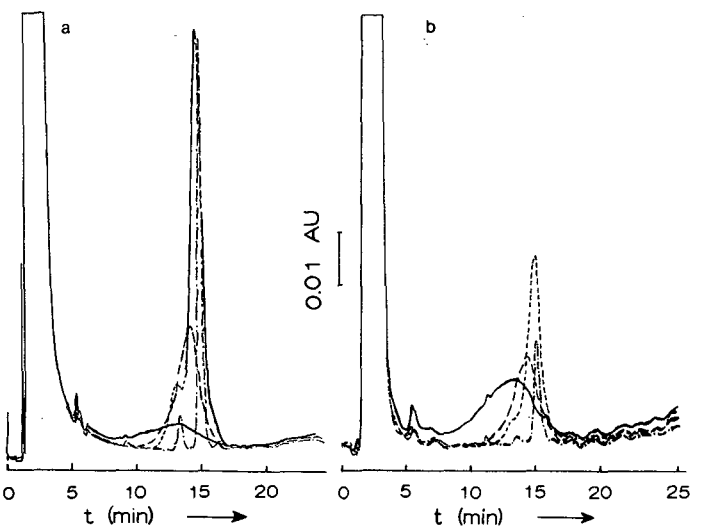

Fig. 7. Chromatograms of different SEC fractions of PSMA 8. Solvent, DCE + 10% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate 0.4 ml/min; column, Chromspher silica, 100 × 3 mm I.D.; temperature, ambient. (a) 260 nm; (b) 235 nm. Molecular mass: —— = 1.2 kg/mol; – – – = 5 kg/mol; - - - - = 11 kg/mol – · – · – = 70 kg/mol.

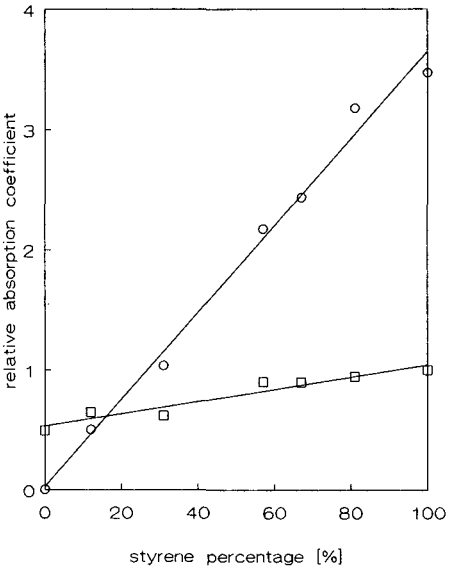

Fig. 8. Relative adsorption coefficient of PSMA as a function of the styrene percentage. Sample: mixture. ○ = 260 nm; □ = 235 nm.

0.96 at 235 nm. Application of the UV absorption at 235 nm proved to be impossible as a quantitative detection method because of two drawbacks: insufficient linearity of the UV absorption at 235 nm with respect to the chemical composition; and the decreasing ratio of the absorption ratio at 260 to 235 nm at decreasing molecular mass. These effects can be explained by the contribution of polymer end-groups being higher at a lower molecular mass. From this it can be concluded that copolymers with a low styrene fraction require another detection system in order to obtain accurate quantitative data.

For the study of the six different column packings in the NP mode, with a gradient from *n*-heptane to DCM with 5% methanol, the retention times of the sample peaks were expressed as the *n*-heptane content of the eluent leaving the column at the same time. It should be mentioned that the HPLC gradient was optimized for silica and was subsequently applied to the other stationary phases. Fig. 9 shows the curves (second degree polynomial fitting) of the percentage of *n*-heptane *versus* the percentage of styrene in PSMA, for the chromatographic and turbidimetric data. The same experiments were performed with three different stationary phases in the RP mode with a gradient from methanol to DCM (Fig. 10). The efficiency of the NP and RP separations is shown in Table IV, expressed as the average peak width at half-height for the PSMA 3, 5, 6, 9 and 11 samples.

For an NP gradient, applied with NP packings, it is shown that in addition to precipitation, the adsorption mechanism also plays a role in this particular chromatographic process. This can be observed by comparing the chromatographic and the corresponding precipitation data. The NP gradient analysis on C_{18}-modified silica and the titration experiments gave similar results. Also, an extremely low separation efficiency on this C_{18}-modified silica was observed. From this it can be concluded that

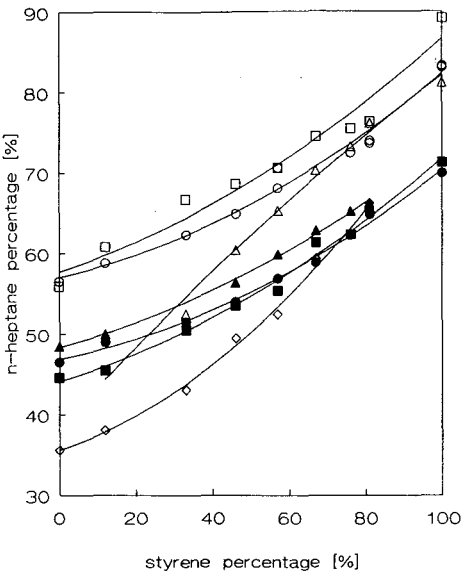

Fig. 9. Solvent composition at precipitation points and the eluent composition at elution times expressed in % *n*-heptane as a function of the styrene percentage of PSMA. Curves fitted with second-degree polynomials. Samples, PS, PSMA 3, 5, 6, 9, 11, 13 and 17, PMA. Solvent, DCM + 5% methanol; non-solvent, *n*-heptane; gradient, linear from 10% solvent in non-solvent to 100% solvent, 0–18 min; flow-rate, 0.8 ml/min; column, 100 × 4 mm I.D., stationary phases as indicated; temperature, ambient. ○ = Titration; ● = CN; □ = RP-18; ■ = diol; △ = glass; ▲ = NH_2; ◇ = Si.

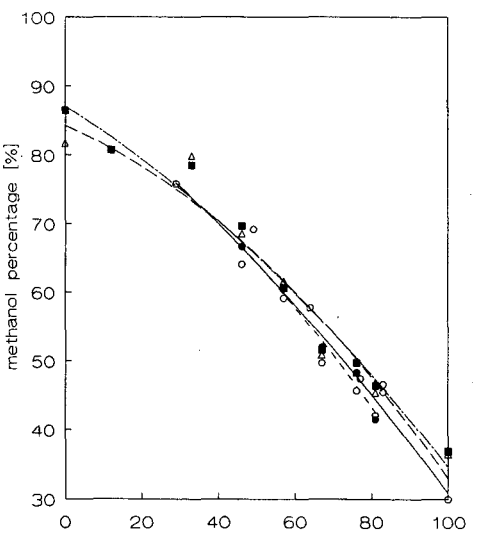

Fig. 10. Solvent composition at precipitation points and the eluent composition at elution times on different stationary phases expressed in % methanol as a function of the styrene percentage in PSMA. Curves are fitted with second-degree polynomials. HPLC samples, PS, PSMA 3, 5, 6, 9, 11, 13 and 27, PMA; titration samples, PS, PSMA 1–5, 7–11 and 15; non-solvent, methanol; solvent, DCM; gradient, linear from 100% non-solvent to 100% solvent, 0–18 min; flow-rate, 0.8 ml/min; column, 100 × 4 mm I.D., stationary phases as indicated; temperature, ambient. ○——○ = titration; ●---● = RP-8; △– – –△ = RP-18; ■– - - - -■ = CN.

TABLE IV

EFFICIENCY OF SEPARATION OF PSMA ON THE DIFFERENT STATIONARY PHASES IN THE NP OR RP MODE, EXPRESSED AS THE AVERAGE PEAK WIDTH AT HALF-HEIGHT, IN % STYRENE

Stationary phase	*Gradient*	*Separation efficiency*
Si	NP	4.5
CN	NP	4.5
NH_2	NP	4.7
Diol	NP	6.3
Glass	NP	15
RP-18	NP	22
RP-18	RP	5.5
RP-8	RP	8.5
CN	RP	12

in this instance the separation is controlled by a precipitation mechanism and the contribution of adsorption is negligible. On the other hand, this implies that the adsorption process observed for NP packings contributes to acceptable separation efficiencies and higher retention times on the polar silicas.

For the RP gradient experiments the corresponding elution and precipitation data indicate that the separation is achieved by the precipitation mechanism. However, to obtain a satisfactory separation with an RP gradient it is necessary to apply RP packings, because on NP packings the separation is disturbed by adsorption processes.

CONCLUSIONS

The use of DCM with a suitable amount of methanol as a strong solvent mixture has proved to be successful in combination with *n*-heptane as a non-solvent for the separation by gradient HPLC of SMA and SBA copolymers according to their chemical composition. This eluent combination can be applied with both bare and polar modified silica. In addition to the separation of poly(styrene–acrylates), the proposed eluent system is also suitable for, *e.g.*, poly(styrene–methacrylates), PSAN and other copolymers with comparable solubility and adsorption properties. For each monomer combination the eluent system only has to be optimized according to the methanol content of the strong solvent.

Further studies will include the application of other detection systems to facilitate the quantitative analysis of copolymers with a low styrene content and the application of gradient HPLC to the characterization of emulsion copolymers and other copolymer systems.

ACKNOWLEDGEMENTS

The authors gratefully thank Mr. W. Kingma for performing some of the SEC analyses and fractionations, Mr. K. C. Houthooft for experimental assistance and

Mrs. D. Tjallema for accurate preparation of the manuscript. This investigation was partly supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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Journal of Chromatography, 508 (1990) 333–339
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 336

Separation of carboxylic acid enantiomers by gas chromatography after rapid derivatization with (*R*)- or (*S*)-1-phenylethylamine after activation by ethyl chloroformate

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(First received November 21st, 1989; revised manuscript received February 5th, 1990)

SUMMARY

The application of the chloroformate activation method for the formation of diastereometric of chiral carboxylic acids is described. This rapid procedure was extended to the gas chromatographic analysis of carboxylic acids with the chiral carbon in the 3-position with respect to the carboxylic group, mandelic and tropic acid and a carboxylic acid containing carbamate. The purities of (*R*)- and (*S*)-1-phenylethylamine chiral reagents were investigated using (*R*)-O-methylmandelic acid of high optical purity. The identities of the derivatives were studied using mass spectrometry. With the hydroxy acids not only is the expected amide formed but also the hydroxy group is converted into carbonate.

INTRODUCTION

Carboxylic acids with chiral carbons can be converted into a large number of different kinds of diastereomeric derivatives[1–3]. Amides are frequently used owing to their chemical stability and the rigidity of the amide bond. Normally the derivatization process consists of two steps, namely activation of the acid to an energy-rich intermediate and then reaction with an appropriate chiral amine. The first step frequently involves the use of noxious reagents and the second step is often time consuming. Combined with evaporation steps, such procedures can be cumbersome. In liquid chromatography, with UV or fluorescence detection, the responses of the diastereoisomers are not necessarily identical[1]. This should not be a problem when using capillary gas chromatography with flame ionization detection.

As many analytical amide-formation reactions take 0.5–1.5 h, excluding evaporation, Björkman[4] developed a method that reduced the overall derivatization time to 3 min. The method is based on the formation of a mixed anhydride with ethyl chloroformate, which is then reacted with L-leucinamide. The method was applied to the determination by liquid chromatography of the enantiomers of indoprofen[4] and ketoprofen[5] isolated from plasma. An advantage with L-leucinamide, in addition to its high enantiomeric purity[4,6], is its poor UV-absorbing properties. Papers have

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appeared using this method, sometimes with minor modifications, for the determination of anti-inflammatory drugs of the 2-arylpropionic acid type[7–12].

In this work we report the application of this rapid method to the analysis of chiral carboxylic acids by capillary gas chromatography with flame ionization detection. In the acids investigated the asymmetric carbon is in both the 2- and 3-positions with respect to the carboxylic group. The derivatization reaction has also been extended to some interesting 2-hydroxy acids such as tropic acid and mandelic acid, and to 2-(phenylaminocarbonyloxy)propionic acid.

EXPERIMENTAL

Instrumentation and chromatographic conditions

A Varian 3700 gas chromatograph equipped with a flame ionization detector was used. The injector and detector temperatures were both 300°C. The carrier gas was nitrogen with an inlet pressure of 80 kPa (120 kPa for the carbamate). The split flow-rate was 20 ml/min. The make-up gas to the detector had a flow-rate of 20 ml/min. An SE-54 fused-silica capillary column was used (25 m × 0.32 mm I.D., film thickness 0.25 μm). The chromatograms were recorded with a Hewlett-Packard 3390A integrator.

Mass spectra were recorded using the same type of column and gas chromatograph connected to a Varian-MAT 44S instrument. Helium was used as the carrier gas, the transfer line kept at 250°C and the ionization energy was 70 eV.

Reagents and chemicals

(*R*)(+)-1-Phenylethylamine (98+%) and (S)(−)-1-phenylethylamine (97–98%) were obtained from EGA-Chemie (Steinheim/Albuch, F.R.G.), and triethylamine from Eastman Kodak (Rochester, NY, U.S.A.), ethyl chloroformate (zur Synthese) from Merck (Darmstadt, F.R.G.), were redistilled. (*R*/*S*)-Indoprofen and (*S*(+)-naproxen were purchased from Sigma (St. Louis, MO, U.S.A.), (*R*/*S*)-ibuprofen from Astra (Södertälje, Sweden), (*R*/*S*)-ketoprofen from Bayer (Leverkusen, F.R.G.), (*R*/*S*)-2-phenylbutanoic acid from Fluka (Buchs, Switzerland), (*R*/*S*)-tropic acid from Sigma and (*R*/*S*)-mandelic acid from Riedel-De Haën (Seelze, Hannover, F.R.G.). (*R*)-O-Methylmandelic acid, (*R*/*S*)-1-indanylacetic acid, (*R*/*S*)-1-tetralylacetic acid and (*S*)-2-(phenylaminocarbonyloxy)propionic acid [(*S*)-PACOPA] were obtained from Jansen (Beerse, Belgium) and the metoprolol metabolite H 104/83 (4-methoxyethylphenoxymethyllactic acid) came from the Department of Organic Chemistry, Hässle (Mölndal, Sweden). Some of the structures are given in Fig. 1.

Solvents and other reagents were of analytical-reagent grade.

Derivatization

The acids were dissolved in acetonitrile and a volume (ranging from 150 to 400 μl) containing 1–3 mg of the acid was mixed with 200 μl of 50 m*M* triethylamine in acetonitrile. To the mixture were added, at intervals of 30 s, 100 μl of ethyl chloroformate (60 m*M*) in acetonitrile and 200 μl of (*S*)(−) or (*R*)(+)-1-phenylethylamine–triethylamine (0.5 *M* each) in methanol. After 2 min, 0.5 ml of hydrochloric acid (0.25 *M*) was added to stop the reaction. The derivative was then extracted with 3 ml of ethyl acetate and 2 μl were injected into the gas chromatograph.

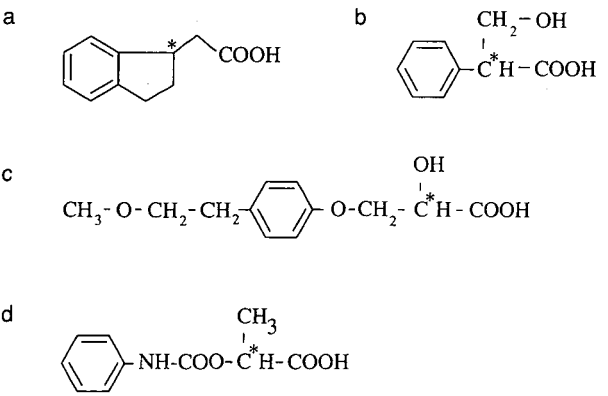

Fig. 1. Structures of (a) 1-indanylacetic acid, (b) tropic acid, (c) lactic acid metabolite of metoprolol (H 104/83) and (d) 2-phenylaminocarbonyloxy) propanoic acid (PACOPA). The asterisks indicate the asymmetric carbon atoms.

The derivatives of (*R*/*S*)-2-phenylbutanoic acid and (*R*/*S*)-mandelic acid, made for testing the possible stereoselectivity of the second step of the reaction, were prepared as above, but the reactions were stopped with 0.25 *M* hydrochloric acid 5, 10, 15, 20, 30 and 45 s and 1, 1.5, 2, 3 and 5 min after the addition of the chiral amine solution.

RESULTS AND DISCUSSION

Identification of derivatives

The amide derivative of (*R*/*S*)-1-indanylacetic acid that was formed in the reaction (Fig. 2) was identified by mass spectrometry. We obtained the expected molecular ion and fragment ions due to the indan ring. The base peak is at *m*/*z* 105 (phenethyl). In the total ion current chromatogram recorded we also observed a large peak whose mass spectrum corresponded to the ethyl carbamate of phenylethylamine.

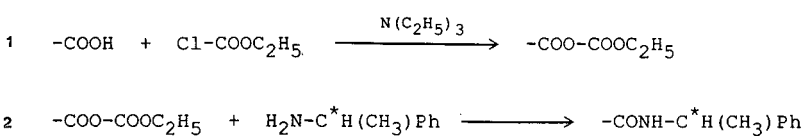

Fig. 2. Reaction scheme for the rapid formation of amide from a carboxylic acid with 1-phenylethylamine after activation with ethyl chloroformate in the presence of triethylamine. (1) Activation; (2) amidation.

The metoprolol metabolite derivative was also subjected to analysis by mass spectrometry (Fig. 3a). Based on this spectrum, the structure in Fig. 3b is proposed. Eliminated neutral fragments are carbon dioxide and ethanol (Figure 3b). This reaction to a carbonate has not been observed when using buffer (pH 8 and higher) in the derivatization of cathecolamines with methyl chloroformate followed by gas chroma-

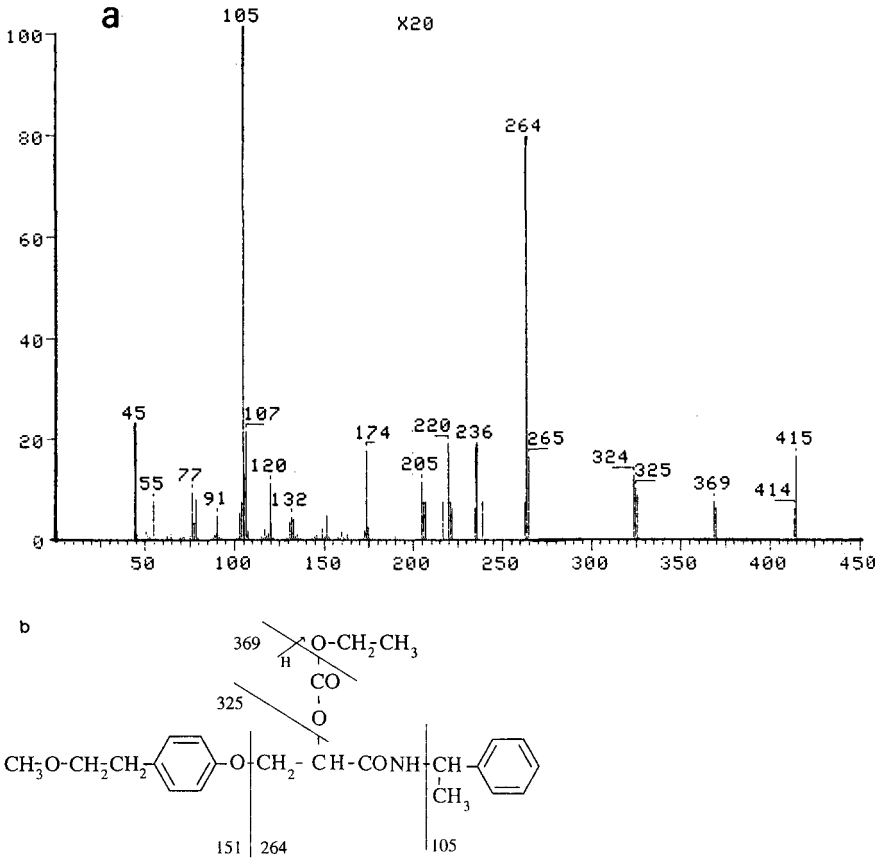

Fig. 3. (a) Mass spectrum of the carbonate/amide derivative of the metoprolol metabolite H 104/83, 1st peak. (b) Proposed structure of the carbonate/amide derivative of the metoprolol metabolite H 104/83 and some fragmentation routes. MV = 415.

tographic analysis[13]. Hence using this derivatization procedure it is not necessary to block the alcohol group by *e.g.*, trimethylsilylation. Simultaneous derivatization of hydroxy acids has been reported with isocyanates[14] but requires prolonged heating.

The mass spectra of the diastereomers of (*S*)-PACOPA gave a molecular ion at *m/z* 312 that can be cleaved to phenyl isocyanate and an alcohol[15]. The charge is predominantely retained on the isocyanate ion, which is the base peak (*m/z* 119). Some anilinium ion is observed at *m/z* 93, which can be explained by the molecular ion expelling carbon dioxide and an allenic ion at *m/z* 175 is formed[15].

Separation of (R)- and (S)-1-phenylethylamides on SE-54

The separation factors for the derivatives formed are given in Table I. For the (*S*)-naproxen and the (*R*)-O-methyl mandelic acid derivatives the second peak is the *R,R* (or *S,S*) derivative. In general, symmetrical peaks with baseline separation were observed except for indoprofen, whose peaks showed some tailing. As expected, the

TABLE I

SEPARATION OF 1-PHENYLETHYLAMINE DERIVATIVES ON AN SE-54 CAPILLARY COLUMN

Compound	*Separation factor*		*Capacity factor of peak 2 (230°C)*
	230°C	*250°C*	
1-(Indanyl)acetic acid (see Fig. 1)	1.034	1.023	4.65
1-(Tetralyl)acetic acid	1.040	1.031	7.07
2-Phenylbutanoic acid	1.075	–	1.97
Indoprofen, 2-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid	–	1.044	3.65[b]
Ibuprofen, 4-(isobutylphenyl)propionic acid	1.091[a]	1.081	3.90
Ketoprofen, 2-(3-benzoylphenyl)propionic acid	1.120[a]	1.104[a]	23.53
Naproxen, (*S*)-2-(6-methoxy-2-naphthyl)propionic acid	1.132[a]	1.113[a]	16.46
Tropic acid (see Fig. 1)	1.085	–	2.96
H 104/83 (see Fig. 1)	–	1.066	11.10[b]
Mandelic acid, 1-hydroxy-1-phenylacetic acid	1.039	–	4.44
(*R*)-O-Methylmandelic acid	1.080	–	2.13

[a] Baseline separation.
[b] 250°C.

two β-chiral acids gave lower separation factors than their α-chiral analogues (Table I). The closer the chiral centres are to each other, combined with bulky substituents, the easier is the separation[16].

The hydroxy acids investigated have lower separation factors than most of the 2-arylpropionic acids (Table I). The chromatogram in Fig. 4 shows the separation of the enantiomers of the derivatized metoprolol metabolite. This compound required a longer time to be eluted. The better separation factor of the O-methyl ether of mandel-

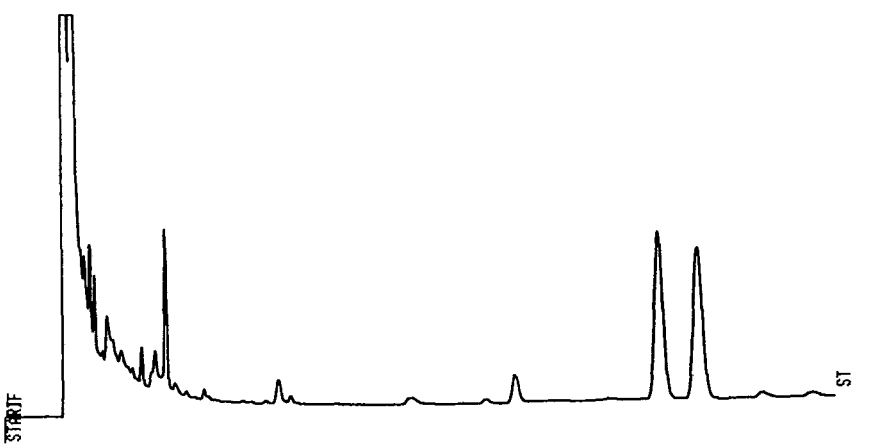

Fig. 4. Gas chromatogram of the mixed derivative in Fig. 3b. Column temperature, 250°C. Retention time for peak 2, 12 min.

ic acid shows that the carbonate group of the hydroxy acids reduces the difference in volatility between the diastereoisomers in the gas chromatographic system. Also for derivatized (*S*)-PACOPA the separation factor is sufficient, 1.054 (220°C), for baseline separation.

Enantiomeric purity of the 1-phenylethylamines

We chose (*R*)(+)- and (*S*)(−)-1-phenylethylamine as the chiral derivatizing agents because they have given good results in chromatographic analysis[17–22]. L-Leucinamide[4,5] is too polar to be suitable for gas chromatographic analysis.

The purity of the chiral amines is only indicated on the label, and rarely discussed in any of the references. The (*R*)-O-methylmandelic acid available, which was >99.8% pure[23] and has been used for the determination of the enantiomers of tocainide[24], was derivatized with (*R*)(+)- and (*S*)(−)-1-phenylethylamine. If we assume that the (*R*)-O-methylmandelic acid is 100% pure and we use the peak areas to measure the purity of the chiral amines, then (*R*)(+)-1-phenylethylamine was 98.34% pure [relative standard deviation (R.S.D.) = 0.03, $n = 6$] and (*S*)(−)-1-phenylethylamine 97.98% (R.S.D. = 0.10, $n = 6$). The remainder is due to the antipode, and any traces of (*S*)-O-methylmandelic acid present (<0.2%).

Enantiomeric purity of (S)-PACOPA

This compound can be used for classical resolution by recrystallization of amines and can be prepared from readily available and cheap starting materials. After derivatization with both (*R*)- and (*S*)-phenylethylamine, the chromatograms showed the presence of 80% as *S,R* and 74% as *S,S*, respectively. The discrepancy might be due to racemization under the alkaline derivatization conditions used, as the enantiomeric contamination of the amines is <2%.

Possible stereoselectivity of the second step in the derivatization reaction

The derivatization reaction of (*R/S*)-2-phenylbutyric acid was stopped with hydrochloric acid at times ranging from 5 s to 5 min after the addition of the chiral amine. The samples were then chromatographed at 210°C and the peak-area ratio was calculated. The same procedure was also performed with mandelic acid (220°C). No change in the peak-area ratios with increasing time was observed, so it is concluded that the reaction is not stereoselective to any significant extent.

ACKNOWLEDGEMENTS

We thank Magnar Ervik, Bo Lamm, Sam Larsson and Jörgen Vessman for their kind interest and valuable help in this work.

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Journal of Chromatography, 508 (1990) 341–348
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 324

Use of two-dimensional gas chromatography in the direct enantiomer separation of chiral essential oil components

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(First received October 17th, 1989; revised manuscript received February 2nd, 1990)

SUMMARY

The enantiomeric excess of a component of an essential oil can be determined on-line with normal gas chromatographic analysis by applying two-dimensional gas chromatography with a second column coated with a chiral stationary phase. The enantiomeric excess for the examples reported was evaluated by complexation gas chromatography, which was demonstrated to give successful enantiomer separations without derivatization of several monoterpenoids and compounds peculiar to the essential oil field.

INTRODUCTION

The enantiomer resolution of a component in a complex mixture, such as an essential oil, is of importance both from a quality control point of view, where it can contribute to the evaluation of the quality and origin of the oil itself, and in scientific studies to evaluate, for instance, the biosynthetic pathway of a component, the geographic origin of an essential oil or the production of artefacts. A two-dimensional gas chromatographic (TDGC) system, provided with a second column coated with a chiral stationary phase, allows us to determine the enantiomeric excess of a component of an essential oil on-line with normal GC analysis, while avoiding the isolation of the component itself. Wang *et al.*[1] first used a two-column GC system provided with valveless switching for the determination of amino acid enantiomeric excess in 1983. In 1984, Schomburg *et al.*[2] described the chiral resolution of the four racemic menthol isomers and the two α-ionol isomers in the form of their isopropylurethane derivatives by TDGC using XE-60–(*S*)-valines–(*S*)-α-phenylethylamide as the stationary phase in the second column. Unfortunately, when isopropylurethane or other derivatives are used, the information on the total oil normally obtained from the first column is lost; in other words, the gas chromatographic pattern of the total oil is no longer available simultaneously with that of the enantiomeric resolution of the essential oil component in question.

In the last few years, new chiral stationary phases have been developed which permit racemate analysis without requiring derivatization. In particular, two different

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types of stationary phases were shown to be successful in the GC separation of several enantiomeric compounds of natural origin as such: the variously modified α-,β- and γ-cyclodextrins (used as such or diluted in normal GC stationary phases), which were introduced among others by Koscielsky and co-workers[3,4], Juvancz *et al.*[5], Venema and Tolsma[6], Koenig *et al.*[7] and Nowotny *et al.*[8], and the Ni^{II}, Co^{II} and Mn^{II} derivatives of bis-3-heptafluorobutanoyl camphorate, which operate on the better known principle of complexation chromatography developed by Schurig and co-workers[9,10].

Recently, Mosandl[11] applied TDGC to the direct enantiomer separation of underivatized chiral γ-lactones from food and beverages by using a heptakis(3-O-acetyl-2,6-di-O-pentyl)-β-cyclodextrin as a chiral stationary phase for the second column.

The aim of this paper is to show the possibilities of TDGC provided with a column for complexation chromatography as a second column in the enantiomer separation of essential oil components. The reliability and selectivity of the proposed technique are demonstrated by assessing the enantiomeric purity of menthol and menthones from peppermint essential oil and of linalyl acetate from lavender essential oil.

EXPERIMENTAL

Reagents

The order of elution of the compounds under analysis from the Chira-Metal (1–4) column was established analysing optically pure compounds. Racemic menthol, menthones and linalyl acetate and (−)-menthol, (−)-menthone, (−)-isomenthone and (−)-linalyl acetate were supplied by Aldrich (Milan, Italy).

Essential oil preparation

Peppermint and lavender essential oils were obtained by submitting 10 g of dried plant material to steam distillation in the modified Marcusson micro-apparatus developed in the authors' laboratory[12]. Commercially available peppermint and lavender oils were also analysed.

TDGC system

TDGC analyses were carried out by applying the MUSIC system (Chrompack, Middelburg, The Netherlands) to a two-oven GC system obtained by coupling a Carlo Erba 2900 with a Carlo Erba 4160 GC unit. The transfer line between the first and second ovens was constructed in the authors' laboratory; it was heated and temperature controlled through the second oven injector heating system. MUSIC is a two-dimensional chromatographic system based on the principle of flow switching proposed about 20 years ago by Deans[13] and provided with a cold trap between the first and second column (*i.e.*, the analytical column). The working principle of MUSIC can be briefly summarized as follows. During the heart-cut step, the effluent from the first column is directed into a 1 m × 220 μm I.D. fused-silica capillary and trapped by expanding liquid carbon dioxide (or liquid nitrogen). When the transfer is complete, the cold trap is ballistically heated in a few seconds to a temperature high enough to vaporize the sample instantaneously. The trapped fraction is then directed into a 220-μm analytical column. Further details of the operation of MUSIC have

been reported elsewhere[14,15]. The operating conditions for MUSIC were trap-base temperature 220°C, trap temperature on reinjection 220°C and pressure at the switching point 0.92 atm.

TDGC analysis

A 20 m × 530 μm I.D. fused-silica open tubular (FSOT) column coated with OV-1 (film thickness 1 μm) and a 12 m × 220 μm I.D. SE-54 FSOT column containing nickel(II) bis[3-heptafluorobutanoyl-(1*R*)-camphorate] [Chira-Metal (1–4), CC&CC, Kirchentellinsfurt, F.R.G.] were installed in the first and second ovens, respectively. The column temperature in the first oven was programmed from 50 to 220°C at 3°C/min. The following conditions were used: injector temperature, 240°C; detector temperature, 250°C; transfer-line temperature, 230°C; carrier gas, helium; and flow-rate at the head of the first column, 10 ml/min.

Peppermint oil. A 1-μl volume of peppermint essential oil solution diluted 1:15 000 with hexane was injected into the first column. The heart-cut temperatures were *ca.* 95°C for menthone and 98°C for menthol. The analysis on the second column was carried out isothermally at 110°C.

Lavender oil. A 1-μl volume of lavender essential oil solution diluted 1:15 000 with hexane was injected into the first column. The heart-cut temperature was 105°C. The analysis on the second column was carried out isothermally at 100°C.

RESULTS AND DISCUSSION

The GC resolution of a racemate cannot easily be planned theoretically because, unfortunately, a stationary phase of general use for GC enantiomer analysis has not yet been developed and a suitable stationary phase for the separation of a particular racemate must therefore be found experimentally. Complexation chromatography was demonstrated to be very effective in enantiomer analysis without derivatization for several monoterpenoids and compounds peculiar to the essential oil field[16]. The fundamental principles of complexation chromatography were recently reviewed by Schurig[17]. The low maximum allowable operating temperature (110°C, and only for short periods) of complexation chromatographic stationary phases currently available limits their applicability to the essential oil field as it makes the enantiomeric analysis of several monoterpenoids and of most sesquiterpenoids generally impossible[16]. For this reason, a double-oven GC system was assembled in order to operate with the two columns independently heated, and to protect the column coated with the thermolabile complexation chromatographic stationary phase, although MUSIC could successfully operate with a single-oven GC unit (of course provided with two flame ionization detectors).

The optical activity of a compound of natural origin is generally peculiar and related to its biogenetic pathway. Therefore, an unadulterated essential oil is characterized by a definite and plant-specific distribution of its chiral components. Peppermint essential oil, for instance, is characterized by the presence of (−)-menthone and (−)-menthol[18] and lavender essential oil by (−)-linalyl acetate. The enantiomeric excess of menthone and menthol was investigated in a freshly distilled peppermint essential oil and in some commercially available oils; the gas chromatographic pattern of the freshly distilled oil is shown in Fig. 1. Menthol and methone/isomenthone

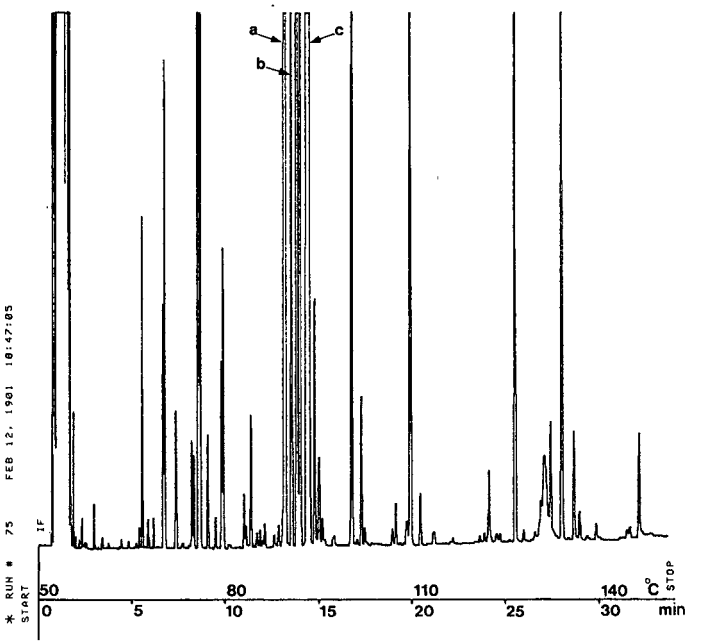

Fig. 1. Gas chromatographic pattern of a freshly distilled peppermint oil: (a) menthone; (b) isomenthone; (c) menthol.

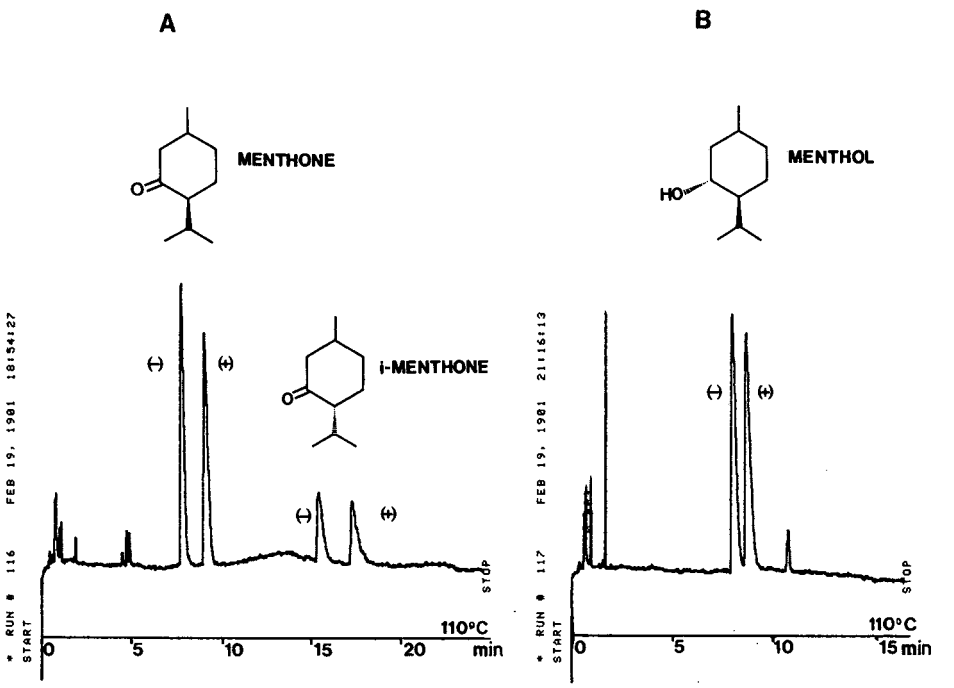

Fig. 2. Enantiomer analyses by TDGC of the menthone/isomenthone (A) and menthol (B) racemates heart-cut from standard solutions.

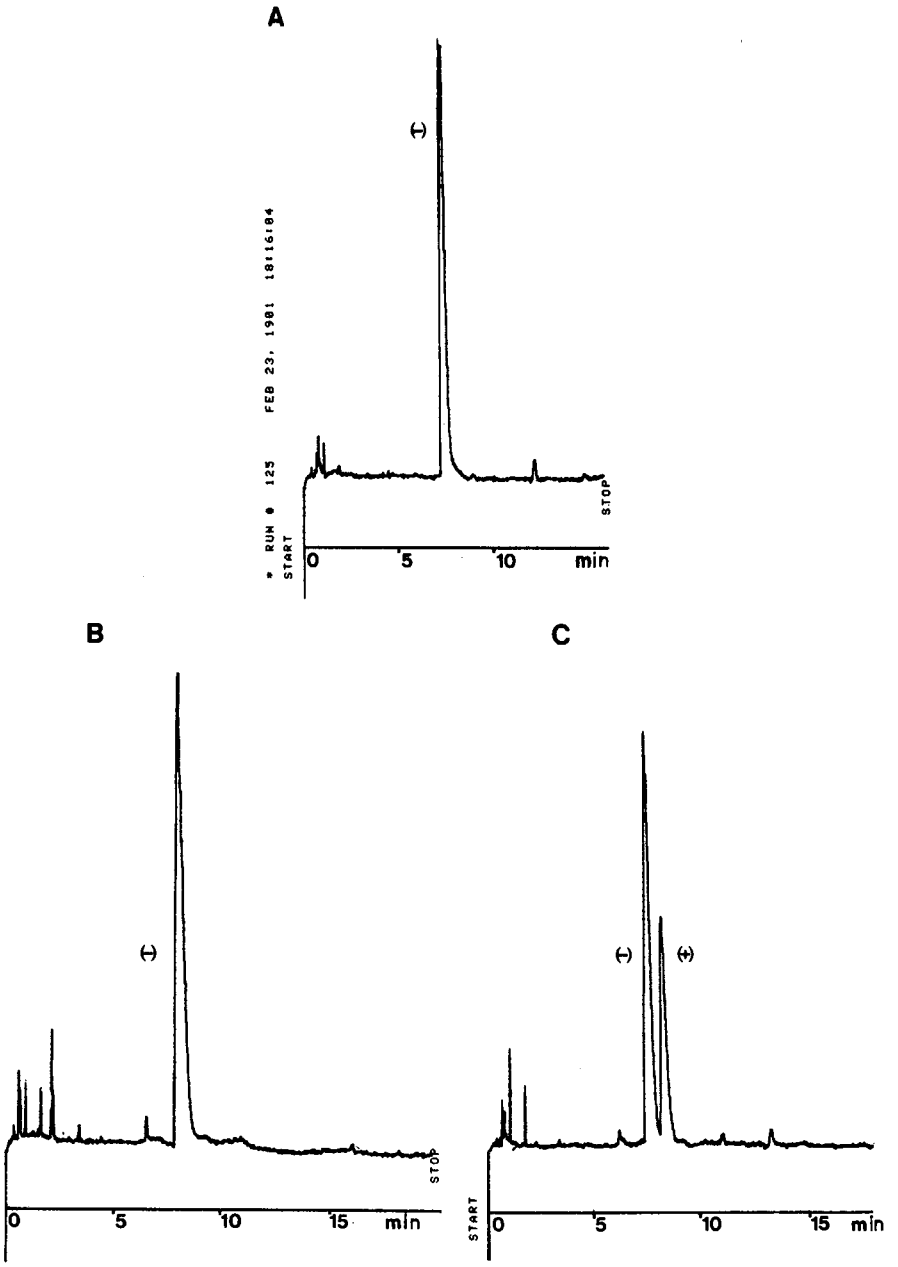

Fig. 3. Analysis by TDGC of menthone (A) and menthol (B) enantiomeric excess in the peppermint oil in Fig. 1 and (C) menthol in a commercially available oil.

racemates were first heart-cut from the standard solutions (Fig. 2); these analyses were each repeated five times to control their qualitative and quantitative reproducibility. The menthol and menthone stereoisomer ratios in the above oil and in one of the commercial oils, probably spiked with menthol racemate, are reported in Fig. 3.

Similar results were obtained in the enantiomer recognition of linalyl acetate from a freshly distilled lavender oil (Fig. 4) and from some commercially available oils. Fig. 5 shows the heart-cut of the standard solution of linalyl acetate racemate, the linalyl acetate stereoisomer ratio in the lavender oil in Fig. 4 and that in a commercial oil probably spiked with linalyl acetate racemate.

The results reported here are clearly encouraging and demonstrate how the direct enantiomer analysis by TDGC of a component in an essential oil is rapid, easy and effective. Further studies of the use of direct TDGC enantiomer analysis for characterizing optically active components of essential oils using various stationary phases are in progress.

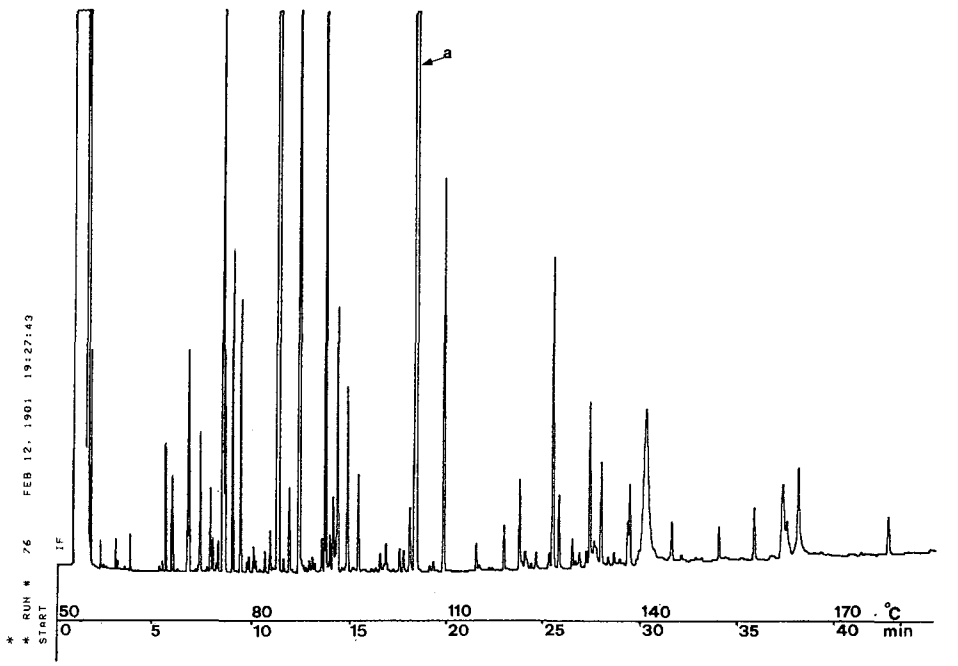

Fig. 4. Gas chromatographic pattern of a freshly distilled lavender oil: (a) linalyl acetate.

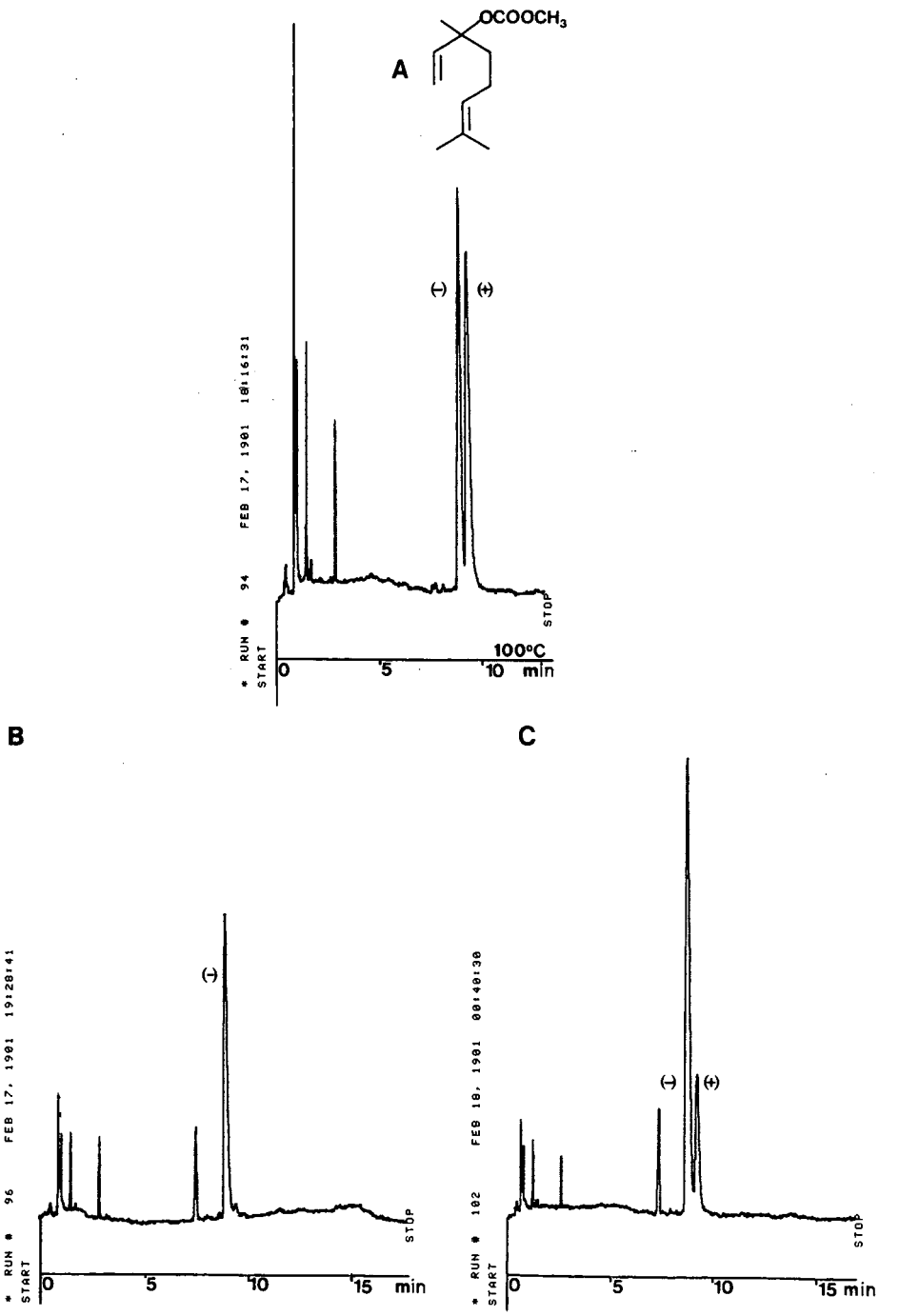

Fig. 5. Analyses by TDGC of the enantiomeric excess of linalyl acetate (A) in a racemate standard solution, (B) in the lavender oil in Fig. 4 and (C) in a commercially available oil.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministero della Pubblica Istruzione (60% and 40% research funds) and the Assessorato Agricoltura Foreste e Ambiente Naturale della Regione Autonoma Valle d'Aosta for financial support to the laboratory. The authors thanks Professor F. V. Schurig for helpful discussions and advice.

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Journal of Chromatography, 508 (1990) 349–356
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 326

Pentafluorophenyldiazoalkanes as novel derivatization reagents for the determination of sensitive carboxylic acids by gas chromatography–negative-ion mass spectrometry

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SUMMARY

A convenient access to analytically useful pentafluorobenzyl esters of sensitive carboxylic acids is described. The novel derivatization procedure takes advantage of the chemoselectivity of diazo compounds towards acids and utilizes pentafluorophenyldiazoalkanes as derivatization reagents. The superiority of the diazo reagent over pentafluorobenzyl bromide is demonstrated with the efficient derivatization of sensitive carboxylic acids which decompose extensively when subjected to the routine procedure. The α-methylpentafluorobenzyl (MPFB) esters of mono- and dicarboxylic acids thus formed exhibit excellent gas chromatographic properties. The scope and limitation of the use of diastereomeric MPFB esters is also discussed.

INTRODUCTION

Pentafluorophenyl derivatives of various functional groups are widely used in analytical gas chromatography (GC)[1–3], owing to their excellent GC properties and electron-capturing capabilities. Further, it has been demonstrated that the selectivity and sensitivity of the analytical methods may be greatly improved by use of a mass spectrometric detector which registers selected negative ions generated in the ion source by chemical ionization and electron capture[4–7].

Carboxylic acids (**1**), and in particular eicosanoids[8,9], are readily derivatized to their corresponding 2,3,4,5,6-pentafluorobenzyl ester derivatives **4a** by use of 2,3,4,5,6-pentafluorobenzylbromide (**2a**, Fig. 1). After ionization, the ester **4a** is subjected to a unique tailor-made fragmentation (Fig. 2) to yield the neutral 2,3,4,5,6-pentafluorobenzyl radical (M = 181) and the carboxylate anion $[M-181]^-$, the latter being recorded by means of negative-ion chemical ionization mass spectrometry (NICI-MS) at the femtomole to attomole level. So far the required carboxylic ester derivatives **4** have been prepared exclusively from the analytes **1** and bromide **2a** in the presence of a non-nucleophilic base (*e.g.*, N,N-diisopropyl-

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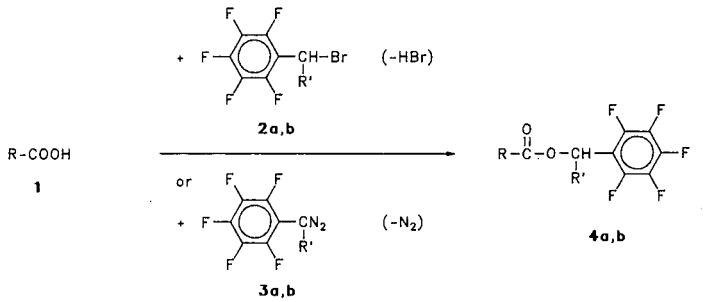

Fig. 1. Derivatization of carboxylic acids (**1**) with pentafluorobenzylbromides **2a** and **2b** and pentafluorophenyldiazoalkanes **3a** and **3b**. Series **a**, R′ = H; series **b**, R′ = CH_3.

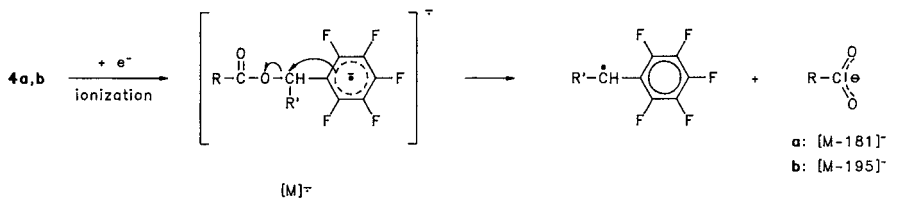

Fig. 2. Mass spectrometric (NICI-MS) fragmentation of esters **4a** and **4b**. Series **a**, R′ = H; series **b**, R′ = CH_3.

ethylamine, "Hünig's base"). This reaction, however, seems to be critical with respect to the selectivity of the ester formation and recovery of the product. In general, large amounts of unwanted side-products are formed and excess or decomposed reagent has to be removed which otherwise would cause elevated chemical noise levels[10,11].

In order to circumvent these problems volatile and more selective reagents for analytical pentafluorobenzyl ester formation from **1** are required. In this paper, an efficient solution to the problem of selective esterification of sensitive and/or nucleophilic acids and dicarboxylic acids is presented. The method consists of the smooth and convenient conversion of a carboxylic acid function into 2,3,4,5,6-pentafluorobenzyl esters (PFB esters, **4a**) or 1-(pentafluorophenyl)ethyl esters (α-methyl-2,3,4,5,6-pentafluorobenzyl esters, MPFB esters, **4b**) using the new reagents (pentafluorophenyl)diazomethane (**3a**) or 1-(pentafluorophenyl)diazoethane (**3b**)[12], respectively.

EXPERIMENTAL

Chemicals

The following acids and reagents were obtained from Fluka (Neu-Ulm, F.R.G.) and Aldrich (Steinheim, F.R.G.) and used as received: erucic acid (*cis*-13-docosaenoic acid), behenic acid (docosanoic acid), suberic acid (octanedioic acid), thiodiglycolic acid, (±)-2-phenylpropionic acid, 2,3,4,5,6-pentafluorobenzylbromide (**2a**), (±)-1-(pentafluorophenyl)ethanol and bis(trimethylsilyl)trifluoroacetamide (BSTFA). Thiodiglycolic acid sulphoxide (dicarboxymethyl sulphoxide)[13], dithiodiglycolic acid

(dicarboxymethyl disulphide)[13], (±)-3-(carboxymethylthio)lactic acid[13] and (±)-[13,17,17,18,18,19,19-$^2H_7$]-2,3,4,5,20-pentanor-19-carboxy-15-dehydro-13,14-dihydroprostaglandin E_1 (d_7-PGE-MM)[14] were prepared as described.

The homogeneity of the reagents and derivatives was checked by thin-layer chromatography (TLC) on 0.2 mm precoated silica gel plates (silica gel 60 F_{254}) (E. Merck, Darmstadt, F.R.G.) using the mobile phase ethylacetate–*n*-hexane (1:5, v/v) or/and ^{1}HNMR spectroscopy (80 MHz, δ values in ppm referred to internal TMS). The melting points reported are uncorrected.

(±)-1-(Pentafluorophenyl)ethyl bromide (**2b**)[15] was synthesized from (±)-1-(pentafluorophenyl)ethanol and PBr_3 in the presence of pyridine at −20°C and isolated in 65% yield after vacuum distillation as a colourless liquid, b.p. 73–76°C (17 mmHg). TLC: R_F 0.81. ^{1}H NMR ($CDCl_3$): 2.12 (d, J = 7.1 Hz, 3H, CH_3), 5.41 (q, J = 7.1 Hz, 1H, CHBr). Analytically pure pentafluorophenyldiazoalkanes **3a** and **3b** were prepared as described previously[12]. In the frozen state (−80°C) both compounds are stable for at least 5 years. Although no hazard has been observed, undiluted diazoalkane reagents should generally be handled with care[16].

Synthesis of reference compounds

2,3,4,5,6-Pentafluorobenzyl docosanoate. A mixture of docosanoic acid (190 mg, 0.56 mmol), 2,3,4,5,6-pentafluorobenzyl bromide (**2a**, 180 mg, 0.69 mmol) and N,N-diisopropylethylamine (150 mg, 1.2 mmol) in 2 ml of dry dimethylformamide (DMF) was stirred at 40°C for 16 h. After diluting with ethyl acetate (50 ml), the mixture was washed with 0.1 *M* HCl, water, half-saturated aqueous $NaHCO_3$ and water (3 × 5 ml each). The dried (Na_2SO_4) solution was evaporated under vacuum and the residue was dissolved in a minimum volume of ethyl acetate–*n*-hexane (1:5, v/v) and purified by column chromatography on silica gel 60 (20 g) (E. Merck) using the same solvent mixture as the mobile phase. Collection of the appropriate fractions, evaporation and drying under vacuum gave pure, crystalline ester in 97% yield (282 mg). An analytical sample was recrystallized from *n*-hexane, m.p. 68.5°C. TLC: R_F 0.77. Calculated for $C_{29}H_{45}F_5O_2$ (520.6), C 66.90, H 8.71; found, C 66.79, H 8.22%.

Related esters. In a similar manner, the following esters were prepared from the corresponding carboxylic acids and bromides **2a** and **2b**.

2,3,4,5,6-Pentafluorobenzyl *cis*-13-docosaenoate was obtained as a colourless oil (96% yield) which slowly crystallized on treatment with *n*-hexane (−40°C), m.p. 21–23°C. TLC: R_F 0.69. Calculated for $C_{29}H_{43}F_5O_2$ (518.6), C 67.16, H 8.36; found, C 67.02, H 8.32%. ^{1}H NMR ($CDCl_3$): 0.88 (bt, $J \approx 6$ Hz, 3H, CH_3), 1.26 (bs, 30 H), C2.02 (m, 4H, H-12/15), 2.33 (t, $J \approx 7$ Hz, 2H, H-2), 5.19 (t, J = 1.5 Hz, 2H, OCH_2), 5.36 (t, J = 4.6 Hz, 2H, H-13/14).

1-(Pentafluorophenyl)ethyl docosanoate was isolated in 75% yield after warming the reaction mixture at 40°C for 96 h; m.p. 55.5°C (from methanol). TLC: R_F 0.86. Calculated for $C_{30}H_{47}F_5O_2$ (534.7), C 67.39, H 8.86; found, C 67.32, H 9.11%. ^{1}H NMR ($CDCl_3$): 0.88 (bt, J = 4.9 Hz, 3H, H-22), 1.25 (bs, 38 H), 1.64 (d, J = 6.8 Hz, 3H, $OCHCH_3$), 2.32 (t, J = 7.3 Hz, 2H, H-2), 6.10 (q, J = 6.8 Hz, 1H, OCH).

2,3,4,5,6-Pentafluorobenzyl [13,14-$^2H_2$]docosanoate. Tris(triphenylphosphine)rhodium(I) chloride (Wilkinson's catalyst) (60 mg) was dissolved in 10 ml of ethyl acetate under an atmosphere of molecular deuterium gas (>99.7% ^{2}H). To the rapidly stirred homogeneous mixture was added a solution of 2,3,4,5,6-pentafluoro-

benzyl *cis*-13-docosaenoate (100 mg, 0.19 mmol) in 2 ml of ethyl acetate. After further stirring at room temperature for 14 h, excess, *n*-hexane was added and the mixture was filtered through silica. The crude product obtained after evaporation was recrystallized from hot methanol to give 82 mg (81%) of the deuterated ester, m.p. 68°C. Calculated for $C_{29}H_{43}{}^2H_2F_5O_2$(522.6), C 66.65, $^1H+{}^2H$ 9.06; found, C 66.66 $^1H+{}^2H$ 9.24%. MS [positive-ion, electron impact, direct inlet mode (PI-EI-DIP), 20 eV]: 522 (52%), 341 (100%), 323 (82%), 305 (17%), 241 (8%). High-resolution MS ($M^{+\cdot}$): calculated, 522.3465; found, 522.3462.

*1-(Pentafluorophenyl)ethyl [13,14-$^2H_2$]docosanoate.*The crude 1-(pentafluorophenyl)ethyl *cis*-13-docosaenoate (MW 532.6), which was obtained in 69% yield from the reaction of erucic acid with **2b**, was used without further purification in the subsequent deuteration step. After purification as described above, crystalline material was obtained, m.p. 55°C (from methanol), yield 61%. Calculated for $C_{30}H_{45}{}^2H_2F_5O_2$ (536.7), C 67.14, $^1H+{}^2H$ 9.20; found, C 67.34, $^1H+{}^2H$ 9.02%. ^1H NMR ($CDCl_3$): 0.88 (bt, $J \approx 6$ Hz, 3H, H-22), 1.25 (bs, 36 H), 1.64(d, $J = 6.8$ Hz, 3H, $OCHCH_3$), 2.32 (t, $J = 7.5$ Hz, 2H, H-2), 6.11 (q, $J = 6.8$ Hz, 1H, OCH).

Instrumentation

Gas chromatography–mass spectrometry (GC–MS) was performed with a Hewlett-Packard 5985 quadrupole mass spectrometer operating in the negative-ion chemical ionization (NICI) mode (130 eV, reactant gas methane) coupled with an HP 5840 A gas chromatograph (carrier gas helium with an inlet pressure of 50 kPa). An SE-52 fused-silica capillary column was used (25 m × 0.32 mm I.D., 0.25 μm film thickness) (Chromatographie Service, Langerwehe, F.R.G.), initial temperature 110°C held for 1 min and then increased at a rate of 30°C/min to 320°C. For GC–electron capture detection (ECD) a Hewlett-Packard 5890 gas chromatograph was used, with nitrogen as carrier gas (50 kPa) and make-up gas (55 ml/min) and a detector temperature of 320°C.

Positive-ion and high-resolution mass spectra were obtained in the direct inlet mode (DIP, EI, 20 and 70 eV) on a MAT 711 instrument.

Standard derivatization procedures

PFB esters[8]. The samples were reacted with 30 μl of a 35% (v/v) solution of PFB bromide (**2a**) in acetonitrile and 10 μl of N,N-diisopropylethylamine for 20 min at room temperature. The solvent and most of the excess of reagent were removed by evaporation under a stream of nitrogen. For GC–MS measurements the remaining residue was dissolved in dodecane whereas an additional purification step was included for studies in the GC–ECD configuration. Thus, water (200 μl) was added to the samples and the derivatives were extracted with *n*-hexane (2 × 500 μl). The combined extracts were applied to a Sep-Pak silica cartridge which had been preconditioned with *n*-hexane (5 ml). After washing with *n*-hexane (2 ml) followed by *n*-hexane–diethyl ether (10:1, v/v) (1 ml) and discarding the washings the esters were eluted with 8 ml of the latter solvent mixture.

MPFB esters[12]. A 50–60-fold excess of a 0.3 *M* solution of **3b** in benzene was added to the sample and the mixture was left at room temperature for 16 h. Excess of diazoalkane was either removed by evaporation or destroyed by addition of acetic acid (10 μl). Water (200 μl) was added and the esters were isolated by extraction with

n-hexane. After evaporation of the solvent, the samples were reconstituted in dodecane for measurement by GC–MS or GC–ECD.

Time course of derivatizations

A mixture of docosanoic acid (200 nmol) and 1-(pentafluorophenyl)ethyl [13,14$^2H_2$]docosanoate (200 nmol) was treated at both 22 and 40°C with 50 μl of a 0.3 *M* solution of **3b** in benzene. Aliquots (5 μl) were taken after 15 min, 30 min, 1 h, 2 h, 4 h and 16 h and the reaction was quenched by the addition of acetic acid. The samples were worked up and analysed by GC–MS as described.

Derivatization of d_7-PGE-MM

Analytical amounts (range 30 pmol–20 nmol) of d_7-PGE-MM were converted into the bismethoxime by treatment with 1.5 ml of a solution of O-methylhydroxylamine hydrochloride (100 mg/ml) in 1.5 *M* acetate (buffer pH 5) for 30 min at room temperature. The mixture was extracted with diethyl ether (3 × 2 ml) and, after evaporation of the combined extracts, the samples were derivatized with **2a** or **3b** as described above. Prior to injection the dried samples were dissolved in BSTFA.

Response of PFB and MPFB esters

A mixture of 10 nmol of the carboxylic acid and 10 nmol of suberic acid as internal standard were derivatized to the PFB or MPFB esters as described above. The samples were dissolved in 1 ml of dodecane.

The samples with (±)-3-(carboxymethylthio)lactic acid esters were dissolved in 100 μl of BSTFA and diluted 1:10 with dodecane.

(±)-d_7-PGE-MM (10 nmol) was converted into its methoxime as described above. Hexadecanedicarboxylic acid (10 nmol) as internal standard was then added, the sample divided into two equal parts and transformed into the corresponding PFB or MPFB esters. The samples were dissolved in 100 μl of BSTFA and diluted with dodecane to a final concentration of 10 pmol/μl.

RESULTS AND DISCUSSION

Whereas diazomethane is widely accepted as a reagent for derivatization of carboxylic acids, other diazoalkanes such as phenyldiazomethane[17] or (trideutero-)-diazoethane[18] have been used far less frequently for this purpose. However, none of these reagents offers any advantages with respect to GC–ECD or GC–NICI-MS techniques. Further, previous attempts to prepare electrophoric esters via derivatization with trifluorodiazoethane were unsuccessful because this reagent failed to react with carboxylic acids[19]. Although the novel diazo compounds **3a** and **3b** contain the electronegative pentafluorophenyl residue, their reactivity towards organic acids is still sufficient to give the corresponding esters[12].

In order to determine the optimum conditions for derivatization with the diazoalkanes **3a** and **3b**, the time course of derivatization was investigated for the reaction with docosanoic acid at two different temperatures. This long-chain fatty acid is expected to react very slowly and additionally should represent a good model for eicosanoids. For reaction with **3b** esterification is nearly complete after 16 h (Fig. 3). As expected, **3a** reacts much more slowly[12] and is therefore less convenient for routine derivatizations as it requires reaction times of several days (data not shown).

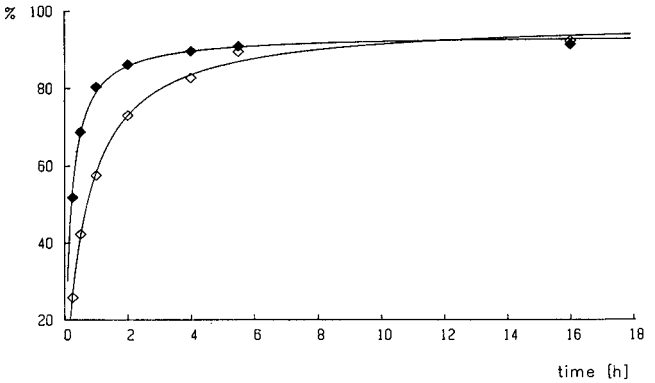

Fig. 3. Time course of derivatization of docosanoic acid with **3b**. ◇ = 25°C (average of two determinations); ◆ = 40°C.

Because the absolute responses of pure PFB and MPFB esters are identical (±5%) for carboxylic acids such as docosanoic or suberic acid (data not shown), the latter acid was employed as an internal standard to compare the two derivatization procedures. Several sensitive dicarboxylic acids which show partial decomposition when derivatized to the PFB ester in the usual manner were then converted to the PFB and MPFB esters using either **2a** or **3b**. The response of the main fragments was determined in relation to the corresponding suberic acid esters (Table I). In all cases the relative response of the MPFB ester, which essentially reflects a higher derivatization reaction yield, is better than that of the PFB ester. Thus, for sensitive carboxylic acids,

TABLE I

RELATIVE RESPONSES OF THE MAIN FRAGMENTS ($[M-181]^-$ OR $[M-195]^-$) OF THE PENTAFLUOROBENZYL (PFB) AND α-METHYLPENTAFLUOROBENZYL (MPFB) ESTERS OF DIFFERENT DICARBOXYLIC ACIDS IN RELATION TO THE RESPONSES OF THE SUBERIC ACID ESTERS (SET AT 100%)

Esters were prepared as described under Experimental.

Compound	*Relative area*		*Relative response, MPFB ester/PFB ester*
	PFB ester	*MPFB ester*	
Suberic acid	100.0	100.0	1.0
Thiodiglycolic acid	19.0	142.0	7.5
Thiodiglycolic acid sulphoxide	–[a]	0.5[b]	–
Dithiodiglycolic acid	11.8	16.7	1.4
(±)-3-(Carboxymethylthio)-lactic acid	4.5	53.0	11.8
(±)-d_7-PGE-MM[c]	35.9	173.0	4.8

[a] Not detectable.
[b] Main fragment $[M-1]^-$.
[c] Bismethoxime trimethylsilyl ether derivative; internal standard, hexadecanedicarboxylic acid.

3b seems to be an excellent alternative to the bromide **2a**, in particular as no additional purification step is necessary.

Fig. 4 shows the chromatogram of a mixture of bis-MPFB esters of aliphatic saturated dicarboxylic acids (C_6–C_{16}). The peak shape and separation of the bis-MPFB esters are identical with those of the bis-PFB esters. Although the derivatization reagent **3b** introduced two centres of chirality into the newly formed dicarboxylates, neither a separation of the diastereomers nor any peak broadening was observed in capillary GC. For chiral carboxylic acids, however, where the carboxy group is directly attached to the asymmetric centre, a separation of the diastereomers can be achieved (Table II).

The reagents **3b** may further be useful for a rapid check of the separability of chiral acids. If a separation of the diastereomeric esters could be achieved, then a conventional esterification of these acids with enantiomerically pure 2-(pentafluorophenyl)ethanol[20] should allow for the GC determination of their optical purity.

As observed for PFB esters, MPFB esters of the dicarboxylic acids studied exhibit a unique mass spectrometric fragmentation pattern and, with the exception of the corresponding derivative of thiodiglycolic acid sulphoxide (Table I), up to 90% of the total ion current accounts for the $[M-195]^-$ fragment and the natural abundance isotope masses. Because of the simple derivatization procedure described above and the excellent GC and MS properties of MPFB esters, a promising application of **3b** is

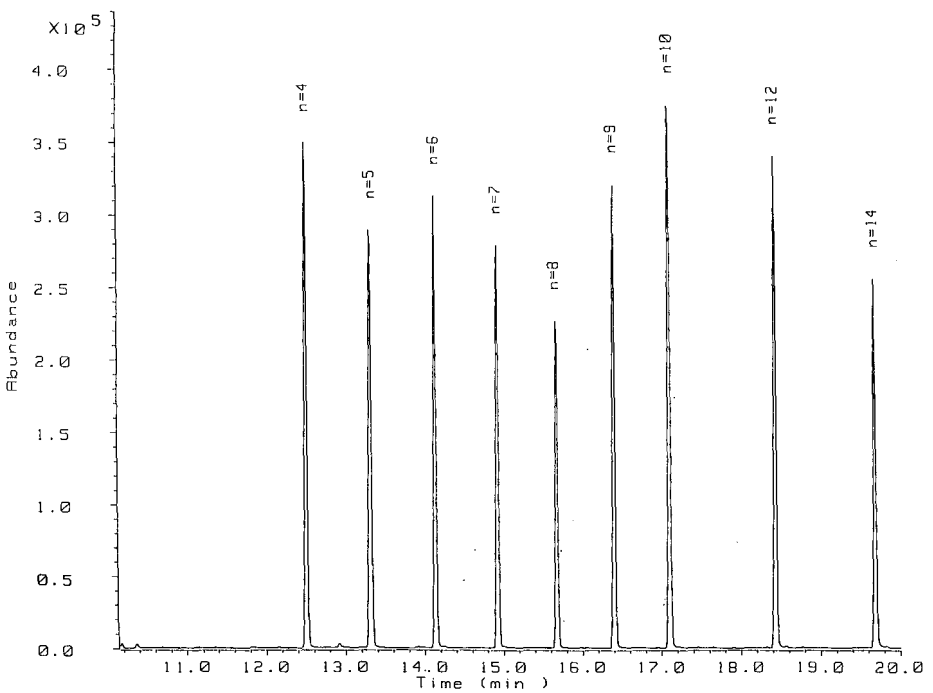

Fig. 4. $HOOC(CH_2)_nCOOH$ dicarboxylic acid mixture derivatized with **3b**. GC conditions: 25 m × 0.32 mm I.D. SE-52 fused-silica capillary column, temperature programmed at 10°C/min from 110 to 320°C.

TABLE II

SEPARATION FACTORS, α, FOR THE MPFB ESTERS OF SOME CHIRAL CARBOXYLIC ACIDS

Compound	α	*Temperature (°C)*[a]
(±)-2-Phenylpropionic acid	1.193	150
(+)-/(−)-Mandelic acid[b]	1.305	150
	1.246	170
(+)-/(−)-Mandelic acid *tert.*-butyl dimethylsilyl ether[b]	1.035	170
(±)-3-(Carboxymethylthio)-lactic acid	1.193	210

[a] Isothermal, GC conditions as indicated under Experimental.

[b] Equal amounts of both individual enantiomers of mandelic acid show identical responses of their diastereomeric esters.

expected in the trace analysis of organic acids. Preliminary results on the analysis of some of the sulphur-containing acids listed in Table I, in conjunction with stable isotope-labelled analogues as internal standards, indicate their successful identification and quantification in biological fluids in the lower femtomole range[21].

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Grant Me 792) and the Robert-Bosch Foundation (Stuttgart, F.R.G.).

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Journal of Chromatography, 508 (1990) 357–362
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 341

Determination of N-nitrosodimethylamine in fish products using gas chromatography with nitrogen–phosphorus detection

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(First received November 1st, 1989; revised manuscript received February 9th, 1990)

SUMMARY

A simple and rapid gas chromatographic method for the determination of N-nitrosodimethylamine (NDMA) in fish products is described. NDMA is extracted from a dried sample with methylene chloride, mixed with *n*-hexane and passed through a silica gel column. NDMA adsorbed on silica gel is eluted with methylene chloride–diethyl ether (7:3) and the eluate is passed through a Sep-Pak alumina A cartridge column, on which NDMA is adsorbed. NDMA is eluted from the cartridge with diethyl ether–methanol (2:1) and the solution is injected into a gas chromatograph with nitrogen–phosphorus detection. This method does not use solvent evaporation and concentration in the clean-up procedure, which eliminates the loss of volatile NDMA and artifactual formation of NDMA in the analytical procedure. The detection limit is 0.5–1 μg/kg and recoveries from salted pollack roe spiked at 40 and 4 μg/kg were 96.7% [relative standard deviation (R.S.D.) 3.6%] and 85.0% (R.S.D. 6.8%) respectively.

INTRODUCTION

There has been increasing concern over the occurrence of N-nitroso compounds in foods, because these compounds have been demonstrated to be carcinogenic in several species of animals and are therefore likely to be related to human cancer[1,2]. N-Nitrosodimethylamine (NDMA) causes liver damage in humans and rats[3] and is carcinogenic in rats[4]. Fish contains a high concentration of dimethylamine and its precursors[5,6], from which NDMA may be formed by reaction with various nitrosating agents (nitrous acid, nitrogen oxide and nitrous acidinium ion). Accordingly, fish and fish-derived foods must be routinely screened for NDMA content, especially in Japan where various types of fish products are used in the daily diet as protein sources.

A number of extraction and clean-up procedures for determining nitrosamines in foods have been described, including direct solvent extraction (followed by column chromatography)[7], solvent extraction on a dry Celite column[8,9], steam distilla-

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tion[10–12], low-temperature vacuum distillation[13] and high-temperature purge and trap methods[14–16]. These procedures are relatively cumbersome and time consuming, and include solvent evaporation and/or a high-temperature distillation step, which may cause losses[17] and artifactual formation of NDMA[8,18–20].

We have developed a simple and rapid extraction and clean-up method, in which a silica gel column and an alumina cartridge column clean-up are used successively. In this procedure, clean-up and concentration of the sample extract are carried out simultaneously, which avoids losses and artifactual formation of NDMA. Gas chromatography (GC) with a thermal energy analyser is generally the most accepted instrumental technique, because the latter is relatively specific for nitrosamines and is sufficiently sensitive enough. However, thermal energy analysers are expensive and are not commonly used in food quality control laboratories. We therefore used GC with nitrogen–phosphorus detection (NPD).

EXPERIMENTAL

Materials and chemicals

Pesticide-grade chemicals were used as received. *n*-Hexane, methanol, acetone and anhydrous sodium sulphate were obtained from Wako (Osaka, Japan). Alumina (neutral, activity 1; Woelm Pharma, Eschwege, F.R.G.) was used as received. Silica gel (No. 7734; Merck, Darmstadt, F.R.G.) was dried at 140°C for 3 h and stored in a desiccator. An alumina cartridge column (Sep-Pak alumina A) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Methylene chloride and diethyl ether (pesticide grade; Wako) were chromatographed on an alumina column just before use. N-Nitrosodimetylamine (NDMA) was of gas chromatography standard grade (Wako) and used as received.

Standard solutions

NDMA stock standard solution was prepared by dissolving 10 mg of NDMA in methanol and diluting to 10 ml with methanol. Analytical working standard and spiking solutions were prepared by diluting the stock solution with acetone.

Caution: NDMA is a potent carcinogen which should be handled with extreme caution in a ventilated hood. Inhalation of NDMA vapour and contact of NDMA with skin and clothing must be avoided.

Instrumentation

The glass column for clean-up was 300 mm × 15 mm I.D. with a coarse fritted disc and a PTFE stopcock. The GC column was a DB-WAX fused-silica open-tubular column (15 m × 0.53 mm I.D.) (J&W Scientific, Folsom, CA, U.S.A.). A Model 5710A gas chromatograph equipped with a nitrogen–phosphorus detector (Model 18789A, Hewlett-Packard, Avondale, PA, U.S.A.) was used. A direct injection inlet conversion kit (Model 2-3797, Supelco, Bellefonte, PA, U.S.A.) and make-up gas detector adaptor kit (Model 2-3648, Supelco) were used to connect the DB-WAX column to the Model 5710A chromatograph, which was designed for use of only packed columns. The make-up gas line was connected to the second injection port and the flow was regulated with the flow regulator. The GC conditions were as follows: injection temperature, 200°C; column temperature, 60°C (isothermal); detector

temperature, 300°C; carrier gas (nitrogen) flow-rate, 5.5 ml/min; make-up gas (nitrogen) flow-rate, 31.5 ml/min; hydrogen flow-rate, 3 ml/min; and air flow-rate, 100 ml/min.

In GC measurements, after injection and initial elution of solvent peaks, it was occasionally necessary to re-zero the baseline.

Extraction and clean-up

A 10.0-g amount of finely comminuted sample and anhydrous sodium sulphate (in a sufficient amount to dry the sample, 1–8 g) were placed in a glass mortar (I.D. 8 cm, depth 6 cm) and mixed thoroughly with a pestle to dryness. The mixture of sample and sodium sulphate was quantitatively transferred into a 100-ml centrifuge tube. The mortar was washed with 40 ml of methylene chloride and the washings were added to the centrifuge tube. If the water content of the sample was below 20%, 10.0 g of the comminuted sample were directly weighed into a 100-ml centrifuge tube and 40 ml of methylene chloride were added. The centrifuge tube was shaken for 10 min and centrifuged at 1000 *g* for 10 min. The upper extract was filtered through a sodium sulphate bed on a glass filter with suction. The residue was mixed with 10 ml of methylene chloride, centrifuged, and the extract was filtered as above. The extracts were combined and *ca.* 100 ml of *n*-hexane (twice the volume of the extract) were added and mixed thoroughly.

A silica gel column was prepared by dry packing and tapping 5 g of silica gel into a chromatographic glass column and 3 g sodium sulphate were placed on top. The *n*-hexane added extract was passed through the silica gel column and the eluate was discarded (fraction 1). Then the column was eluted with 30 ml of methylene chloride–diethyl ether (7:3) and the eluate was collected (fraction 2). Fraction 2 was passed through the Sep-Pak alumina A cartridge column and the eluate was discarded. Then the cartridge column was eluted with 2 ml of diethyl ether–methanol (2:1) and the eluate was collected in a 10-ml graduated test-tube with a ground-glass stopper. The volume was measured and this solution was ready for injection into the gas chromatograph with NPD. This sample solution was stored in a refrigerator until GC analysis.

Gas chromatographic analysis

The GC parameters were adjusted as described under *Instrumentation*. A 2-μl volume of each standard solution (5–50 μg/kg) was injected into the gas chromatograph and a calibration graph was constructed by plotting the peak area against the amount of NDMA. A 2-μl aliquot of sample solution was injected and NDMA was identified from the retention times of the peaks. The peak area was compared with the calibration graph and the amount of NDMA in the sample solution was calculated. The concentration of NDMA in the sample was calculated by dividing the amount of NDMA by the amount of sample (10 g).

RESULTS AND DISCUSSION

In most previous work methylene chloride was used as the extraction solvent for NDMA, and we also chose it because of its good solubility for nitrosamines. Water-containing samples must be dried for efficient extraction, so such samples were

thoroughly mixed with anhydrous sodium sulphate in a mortar before extraction. For example, salted pollack roe contains *ca.* 60% water and hence must be dried, but most smoked foods can be directly extracted after comminution.

Methylene chloride and diethyl ether must be purified by alumina column chromatography just before use, because aged solvents occasionally gave lower recoveries in our experiments. Probably NDMA was decomposed by hydrochloric acid or peroxide produced in the solvents on standing. An unknown substance in methylene chloride that was reported to enhance artifactual NDMA formation[20] was also removed by this chromatography.

We investigated the clean-up of NDMA extracted from samples by the use of silica gel and alumina column chromatography. In our experiments using NDMA standard, NDMA was not eluted from the silica gel column with *n*-hexane–methylene chloride mixtures (3:1 to 1:1). Therefore, the methylene chloride extract (*ca.* 50 ml) was mixed with *n*-hexane (100 ml) and the solution was passed through a silica gel column (5 g). NDMA adsorbed on the silica gel was then eluted with 30 ml of methylene chloride–diethyl ether (7:3).

West and Day[21] used alumina column clean-up for the determination of volatile nitrosamines in crops and soils, and eluted NDMA from 4% deactivated alumina with 1-chlorobutane or benzene. We tried a Sep-Pak alumina A cartridge column and found that it has an unexpectedly strong adsorbing ability for NDMA, and benzene, methylene chloride, diethyl ether and ethyl acetate could not elute NDMA from this column. Methanol or methanol-containing eluents were necessary for elution of NDMA from Sep-Pak alumina A. Therefore, when methylene chloride–diethyl ether eluate from the silica gel column was passed through the Sep-Pak alumina A column, NDMA was completely adsorbed, and could subsequently be eluted with 2 ml of diethyl ether–methanol (2:1).

Hence, we were able to clean-up and concentrate the sample extract solution (*ca.* 50 ml) to *ca.* 2 ml by two successive column chromatographic steps on silica gel (5 g) and Sep-Pak alumina A without the need for solvent evaporation. With this procedure, we could avoid losses of low-boiling NDMA and artifactual formation of NDMA in the analytical procedure. Crosby[17] stated that solvent evaporation is the single most critical step in nitrosamine analysis, and the artifactual formation of nitrosamines in the analytical procedure has always been a problem in nitrosamine analyses, various nitrosation inhibitors being used[17,18–20].

Fig. 1 shows gas chromatograms obtained with NPD of standard NDMA and extracts from salted pollack roe. There were no interfering peaks on the sample extract chromatograms, demonstrating that the sample extracts were effectively cleaned up by the two successive column chromatographic steps.

The detector response varied with the experimental conditions such as hydrogen and air flow-rates, activity of alkali source, collector voltage and detector temperature. Amounts of 3–5 pg of NDMA could be detected using the optimum conditions in our equipment, and ordinarily 10–20 pg of NDMA could be detected. Hence, the eluate from the Sep-Pak alumina A column (*ca.* 2 ml) can be injected directly into the gas chromatograph with a nitrogen–phosphorus detector. The detection limit in the sample is 0.5–1 μg/kg, and 0.3 μg/kg using the optimum conditions.

The recoveries of NDMA added to NDMA-free salted pollack roe at 40 and 4 μg/kg are given in Table I. These recoveries are higher than those obtained by a steam

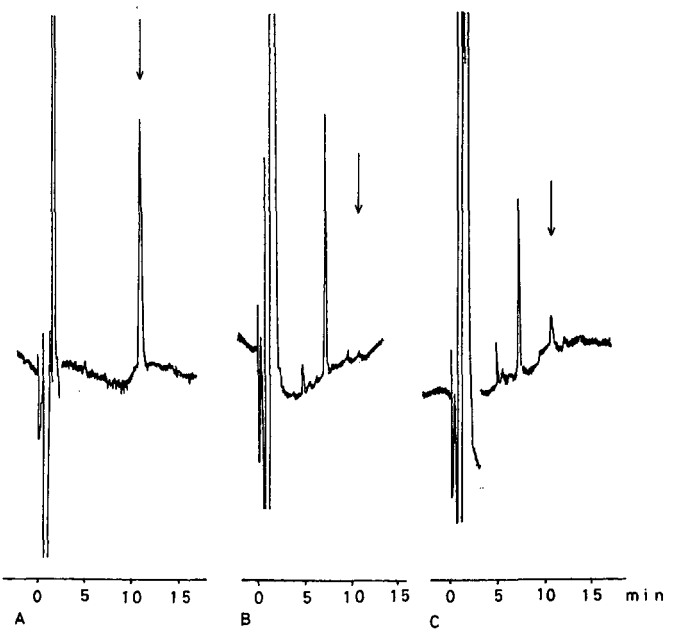

Fig. 1. Gas chromatograms. (A) Standard NDMA (0.7 ng); (B) extract from salted pollack roe (blank); (C) extract from salted pollack roe containing 0.7 μg/kg of NDMA. Arrows indicate retention time of NDMA.

distillation method (67–100%)[12] and a low-temperature vacuum distillation method (70–90%)[13], and compare well with those obtained with a Celite column (85–95%)[8] and a high-temperature purge and trap method (92%)[15]. In these reported methods, great care must be exercized to avoid unacceptable losses of volatile NDMA. The present results show that this procedure is applicable for rapidly screening fish products for NDMA in food quality control laboratories. Our method enables 10–12 samples to be extracted and cleaned up in a day, compared with six samples in a day using reported methods such as a Celite dry column[8,9] or steam distillation[10–12].

Several fish products obtained in local retail stores were analysed by this method and the results are given in Table II. These results are in the same range as reported

TABLE I

RECOVERY OF N-NITROSODIMETHYLAMINE ADDED TO SALTED POLLACK ROE

NDMA added (μg/kg)	*Recovery (%)*	*Average (%)*	*Relative standard deviation (%)*
40	96.8, 92.6, 96.5, 94.8, 103.0	96.7	3.6
4	74.7, 88.4, 88.4, 81.8, 91.7	85.0	6.8

TABLE II

N-NITROSODIMETHYLAMINE IN FISH PRODUCTS

Sample	*No. of samples analysed*	*No. of samples meeting NDMA level (μg/kg)*			
		ND[a]	*1–5*	*5–10*	*>10*
Salted pollack roe	47	43	4		
Smoked cuttlefish	11	2	3	3	3
Dried sardine	8	7		1	
Dried fish	6	6			

[a] Not detected (<1 μg/kg).

earlier[22]. Commercial salted pollack roe is manufactured by dipping raw pollack roe in salt solution containing sodium nitrite and ascorbic acid. The low content of NDMA in salted pollack roe in Table II, in spite of the high concentration of dimethylamine in the raw pollack roe (150–200 μg/kg)[5,6], indicates that the formation of NDMA was suppressed by the ascorbic acid present.

This method may also be applicable to the determination of other nitrosamines in foods and this application is now being investigated.

In conclusion, this method for the determination of NDMA in fish products is simple, rapid and accurate and has a low susceptibility to artifactual NDMA formation. NPD is now widely used in food quality control laboratories and consequently this method is suitable for routine screening of NDMA in fish products.

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Journal of Chromatography, 508 (1990) 363–374
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Chromatographic study of methylcyclopentadiene dimers and iso-dimers and determination of their boiling points

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(First received June 8th, 1989; revised manuscript received December 28th, 1989)

SUMMARY

The Kováts retention indices (I_R) of a family of mono- and polycycloolefins with one or more endo- or exocyclic double bonds on stationary phases of different polarity are related to their boiling points, molar volume, molar refractivity and first-order molecular connectivity. The boiling points of methylcyclopentadiene dimers and their iso-dimers containing an exocyclic double bond were estimated from the relationships between I_R (on squalane at 100°C), boiling point and molecular connectivity obtained from a group of cycloolefins selected at random.

INTRODUCTION

Close relationships to exist between the chromatographic behaviour of solutes and some of their physico-chemical properties, and in previous papers we have reported empirical relationships for several families of hydrocarbons[1–3]. Recently, Rohrbaugh and Jurs[4] have applied these relationships to the study of linear, branched and cyclic monoolefins. These relationships may be suitable for calculating approximate values of solute properties, such as the boiling point (t_b)[5,6] and the molar refractivity[2], although little use of this has been made so far. Nevertheless, the method seems to be interesting when one is dealing with compounds in complex mixtures which can only be resolved by gas chromatography (GC).

Kováts retention indices of a group of cycloolefins including dimethyldicyclopentadiene isomers were determined by GC on capillary columns loaded with stationary phases of polarity ranging beween 0 and 1550 (McReynolds' scale[7]), and the relationships between I_R and t_b, the molar refractivity, (M_R), the molar volume (V_M) and the first-order molecular connectivity (χ) were established. Boiling points of methylcyclopentadiene dimers and iso-dimers were estimated from these relationships.

EXPERIMENTAL

The mixture of methylcyclopentadiene (MCPD) dimer was supplied by Fluka (technical grade). Mixtures containing the iso-dimer compounds (peaks referred to as

0021-9673/90/$03.50

numbers 8 and 10) were prepared in the laboratory by reaction with hydrogen chloride at room temperature[8]. Other solutes were supplied by Fluka, Aldrich and Merck.

GC analyses were carried out with Hewlett-Packard 5830 and Perkin-Elmer 8320 gas chromatographs equipped with flame ionization detectors. The capillary columns and operating conditions used are summarized in Table I. Preparative GC was performed on a Perkin-Elmer F 21 gas chromatograph. The commercial mixture of MCPD dimers was fractionated using a series of three stainless-steel U-shaped columns (1 m × 8 mm I.D.) packed with 10% silicone E 301 on Chromosorb P NAW (60–80 mesh). The flow-rate of the carrier gas (nitrogen) was kept at 160 ml min^{-1}. Isothermal operation at 100°C was used for the fractionation with injector and detector temperatures of 150°C.

TABLE I

CAPILLARY COLUMNS AND EXPERIMENTAL CONDITIONS

Parameter	*Column*					
	1[a]	*2*[b]	*3*[b]	*4*[b]	*5*[a]	*6*[b]
Stationary phase	Squalane	OV-101	SE-54	OV-1701	Ucon LB 550X	OV-215
Length (m)	45	25	25	25	50	25
Inside diameter (mm)	0.50	0.23	0.22	0.22	0.25	0.22
Carrier gas (N_2)	1.30	1.00	1.50	1.00	1.00	0.85
Splitter (ml min^{-1})	1:91	1:150	1:53	1:120	1:92	1:194
Detector and injector temperature (°C)	170 275[c]	170 275[c]	170 275[c]	170 275[c]	170 275[c]	170 275[c]
McReynolds' polarity[d]	0	229	337	789	996	1545
Number of theoretical plates	78 000	95 000	95 000	118 000	76 500	94 000

[a] Stainless-steel capillary columns.

[b] Fused-silica capillary columns.

[c] For determinations of Kováts retention indices the injector and detector temperatures were kept at 275°C.

[d] Ref. 7.

Kováts retention indices of solutes were calculated by the "exact calculated method"[9], using a mixture of C_5–C_{15} *n*-alkanes as a standard.

The input values of the physico-chemical properties required for the correlation were obtained from different sources[10–14]. The first-order molecular connectivity was calculated from the molecular structure[15,16]. An HP 1000 computer was used for the calculations with a multilinear regression program.

RESULTS AND DISCUSSION

In order to assess the optimum experimental conditions for the chromatographic separation of the isomers and the accuracy of the separation of all components, the test mixture was run on six stationary phases of different polarity at 80 and 100°C[8]. The same seven chromatographic peaks with a molecular mass of 160 u were always

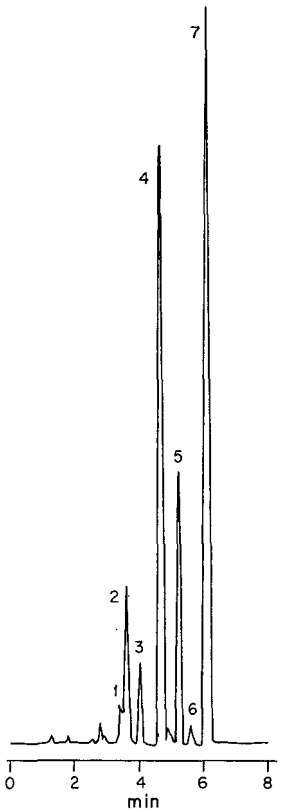


Fig. 1. Chromatogram of a commercial mixture of MCPD dimers on OV-215 at 80°C. The numbered peaks (1–7) belong to isomers of methylcyclopentadiene dimers. Analytical conditions as in Table I.

obtained. The dimers were retained differently by the tested stationary phases without the elution order being altered. Fig. 1, which is a chromatogram of MCPD dimers on OV-215 at 80°C, shows the complete separation of the seven components.

Table II shows the quantitative composition of the mixture obtained from

TABLE II

COMPOSITION (IN AREA %) OF THE COMMERCIAL MIXTURE OF METHYLCYCLOPENTADIENE DIMERS CHROMATOGRAPHED ON DIFFERENT STATIONARY PHASES AT 100°C

For chromatographic conditions see Table I.

Peak No.[a]	*Squalane*	*SE-54*	*OV-1701*	*Ucon LB 550X*	*OV-215*
1	2.33	2.42	1.74	1.88	1.72
2	7.09	7.42	8.17	7.08	8.40
3	3.98	4.09	4.06	4.85	4.02
4	28.85	28.73	29.53	28.41	28.27
5	14.97	14.81	13.76	14.98	15.01
6	1.13	1.25	1.29	1.23	1.25
7	41.65	41.30	41.45	41.56	41.33

[a] Peaks numbered relative to Fig. 1.

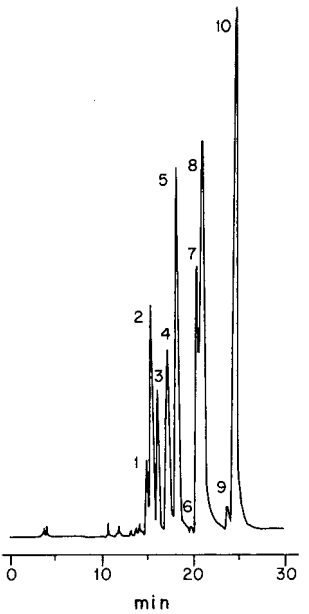
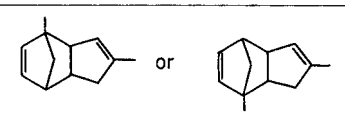
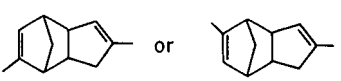
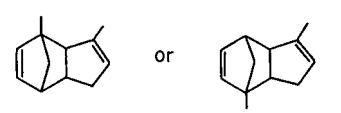
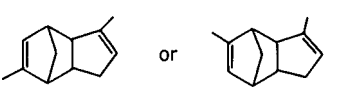
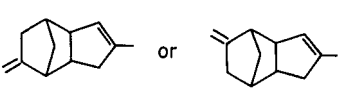
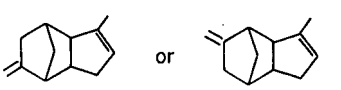


Fig. 2. Chromatogram of a mixture of MCPD dimers and their iso-dimers on squalane at 100°C. Peaks 1–7 as in Fig. 1; 8 and 10, isomers of 4 and 7 (iso-dimers). Analytical conditions as in Table I.

TABLE III

STRUCTURES OF ISOMERS OF METHYLCYCLOPENTADIENE DIMERS CONTAINED IN THE COMMERCIAL MIXTURE AND THEIR ISO-DIMERS

Peak No.	*Structure*	*Nomenclature*
2		*endo*-1,4-(or 4,7-)dimethyltricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene
4		*endo*-4,8-(or 4,9-)dimethyltricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene
5		*endo*-1,3-(or 3,7-)dimethyltricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene
7		*endo*-3,8-(or 3,9-)dimethyltricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene
8		*endo*-4-methyl-8-(or 9-)methylenetricyclo[5.2.1.$0^{2,6}$]dec-3-ene
10		*endo*-3-methyl-8-(or 9-)methylenetricyclo[5.2.1.02,6]dec-3-ene

chromatograms on different stationary phases at 100°C. The similarity of the results indicates that only seven isomers of MCPD dimers are present in the mixture, compounds 2, 4, 5 and 7 (see Fig. 1) being the major constituents.

The treatment of the mixture of isomers with an acidic solvent converted compounds 4 and 7 into two new compounds, 8 and 10 (iso-dimers), as shown in Fig. 2. This conversion could be an isomerization because the new compounds have slightly longer retention times than those of the dimers and the same molecular mass (160 u).

By preparative GC, isomer 7 was isolated as well as a mixture of 4 and 5, in which 4 is the main constituent (67%), and the conversion of compounds 4 and 7 into 8 and 10, respectively, was corroborated. The structures of the dimers and iso-dimers (Table III) were established mainly by relating the ^{1}H NMR signals of olefinic and methyl hydrogens to the changes caused by the isomerization[8]. The longer retention times of the iso-dimers are in accordance with the formation of a more polar exocyclic double bond.

Some physico-chemical properties of MCPD dimers and iso-dimers can be calculated from their experimental I_R values by using equations that relate I_R to these properties[1–6]. These equations were derived from eqn. 1 assuming that the solute vapour pressure, P^0, at the column temperature is proportional to its boiling point and that some solute physico-chemical (or structural) properties responsible for the solute–stationary phase interactions account for the activity coefficient of the solute in the stationary phase at infinite dilution, γ^∞.

$$V_g = \frac{273\ R}{\gamma^\infty P^0 M_1} \tag{1}$$

where V_g is the specific retention volume, R is the gas constant and M_1 is the molecular weight of the stationary phase.

In this way, the following equations were obtained for the calculation of Kováts retention indices:

$$I_R = I_R^0 + pP + qQ + \ldots = a + bt_b + pP + qQ + \ldots \tag{2}$$

where $P, Q, \ldots$, represent independent solute parameters which account for the different solute–stationary phase interaction mechanisms, such as dispersion, induction or orientation; the coefficients $p, q, \ldots$, describe the sensitivity of the retention process to the different interaction mechanisms; I_R^0, is the hypothetical retention index of a solute which forms ideal solutions with the phases ($\gamma^\infty = 1$). In such an ideal process, the solute chromatographic retention only depends on its vapour pressure, I_R^0 therefore being proportional to the boiling point. Hence $P, Q, \ldots$, are solute-independent parameters whereas t_b account for the chromatographic retention in an ideal process.

The Kováts retention indices of the dimers and iso-dimers on several stationary phases of different polarity at 80 and 100°C are given in Table IV.

In order to calculate the boiling points of these compounds, a large group of cycloolefins with similar structures was used to obtain eqns. 2 by regression analysis. The I_R values were determined using the same columns and operation conditions as used for MCPD dimers (Table I). The I_R values obtained are given in Table IV. The physico-chemical and structural properties accountable for the chromatographic

TABLE IV

I_R VALUES ON DIFFERENT STATIONARY PHASES AT 80°C AND 100°C

Compound[a]	I_R									
	Squalane		*OV-101*		*SE-54*		*OV-1701*		*OV-215*	
	80°C	*100°C*	*80°C*	*100°C*	*80°C*	*100°C*	*80°C*	*100°C*	*80°C*	*100°C*
1,3-Cyclopentadiene	510.5	534.7	541.5	545.3	549.5	552.5	–	–	604.2	606.8
Cyclopentene	547.7	562.3	559.7	561.4	566.5	569.5	–	–	574.3	579.6
2-Methyl-1,3-cyclopentadiene	627.5	630.0	641.9	644.0	649.6	653.8	678.9	–	711.2	722.4
1-Methyl-1,3-cyclopentadiene	627.5	630.0	641.9	644.0	652.8	653.8	685.5	–	711.2	722.4
Methylenecyclopentane*	–	–	653.5	657.2	–	–	–	–	681.7	683.8
1-Methylcyclopentene	646.3	651.0	653.5	654.4	659.9	663.1	–	–	670.1	674.4
Cyclohexene	676.8	680.5	683.2	688.1	693.0	698.0	711.1	718.6	708.6	712.9
2,5-Norbornadiene	686.3	691.1	706.9	710.8	718.3	722.8	745.3	751.6	759.9	776.5
2-Norbornene	714,6	721.2	725.7	731.3	735.2	741.1	754.5	760.8	766.1	775.5
3-Methylcyclohexene*	737.7	742.9	742.3	746.8	749.7	755.8	767.3	773.5	771.3	776.3
Methylenecyclohexane	737.8	743.0	744.5	749.8	754.1	759.3	772.9	780.4	779.0	789.2
4-Methylcyclohexene	739.9	744.6	744.3	748.1	751.2	757.5	771.3	777.7	771.4	780.4
1-Methylcyclohexene	768.5	771.5	771.2	775.5	780.4	785.3	–	–	793.0	797.4
Vinylcyclohexane*	821.4	826.7	823.2	828.8	831.9	838.5	850.5	857.7	861.9	865.9
4-Vinylcyclohexene	825.1	829.5	832.8	837.9	843.6	850.3	869.5	876.5	875.4	878.7
cis-Bicyclo[3.3.0]oct-2-ene	848.7	853.9	856.7	863.1	867.8	875.1	–	–	895.2	907.0

Ethylidenecyclohexane	861.8	865.9	868.8	873.4	878.0	899.7	—	—	895.6	914.0
5-Vinyl-2-norbornene	864.7	868.6	881.2	886.8	892.7	899.7	919.8	927.7	934.7	940.4
cis-5-Ethylidene-2-norbornene*	891.8	895.4	910.1	915.2	922.6	928.4	948.7	955.1	948.9	952.6
trans-5-Ethylidene-2-norbornene	900.6	906.5	916.5	920.7	928.5	934.8	955.6	—	958.0	962.2
exo-Dicyclopentadiene	996.6	1003.3	1006.8	1015.2	1022.4	1032.8	1005.7	1066.1	1075.5	1086.9
endo-Dicyclopentadiene	1003.3	1005.0	1016.4	1025.3	1032.4	1043.2	1065.5	1071.8	1082.2	1093.6
Bicyclo[4.3.0]nona-3,6(1)-diene	1031.5	1037.5	1038.8	1046.6	1056.6	1066.4	—	—	1136.6	1146.6
1,4-(or 4,7-)Dimethyl-DCPD[b]	1079.5	1086.7	1099.3	1107.6	1111.1	1121.4	1135.3	1147.9	1151.8	1162.0
4,8-(or 4,9-)Dimethyl-DCPD[b]	1100.2	1107.6	1124.5	1133.9	1136.8	1146.4	1161.6	1173.2	1176.2	1187.0
1,3-(or 3,7-)Dimethyl-DCPD[b]	1108.3	1115.8	1124.5	1133.9	1136.8	1148.5	1166.6	1180.2	1187.3	1199.5
cis-Cyclodecene*	1111.3	1121.4	1112.6	1122.5	1126.5	1139.1	1156.1	1168.6	1169.9	1181.3
3,8-(or 3,9-)Dimethyl-DCPD[b]	1130.4	1138.4	1149.6	1159.9	1163.5	1174.0	1191.3	1203.9	1200.3	1215.5
4-Methyl-8-(or 9-)methylene-DCP[c]	1134.7	1142.0	1156.0	1165.1	1173.5	1183.7	1203.7	1215.9	1210.7	1222.8
3-Methyl-8-(or 9-)methylene-DCP[c]	1161.6	1169.6	1176.2	1186.3	1176.2	1206.3	1227.6	1241.0	1236.6	1250.1
trans,cis,cis-1,5,9-cyclododecatriene	1270.4	1283.2	—	1296.7	1303.3	1317.2	—	—	1365.2	1382.0
α-Cedrene[d,*]	1379.1	1392.3	—	1392.6	1388.5	1403.3	—	—	1452.5	1471.8

[a] Standards for the simplified calculation of t_b are marked with asterisks.
[b] DCPD = tricyclo[5.2.1.$0^{2,6}$]deca-3,8-diene.
[c] DCP = tricyclo[5.2.1.$0^{2,6}$]dec-3-ene.
[d] (1*S*, 2*R*, 5*S*)-2,6,6,8-Tetramethyltricyclo[5.3.1.$0^{1,5}$]undec-8-ene.

TABLE V

PHYSICO-CHEMICAL PROPERTIES DATA OF STANDARD COMPOUNDS

Compound[a]	t_b (°C)	n	d (g ml^{-1})	M_R (ml mol^{-1})	V_M (ml mol^{-1})	χ	*Ref.*
1,3-Cyclopentadiene	40.0	1.4440	0.8021	21.890	82.409	1.8165	10
Cyclopentene	44.2	1.4225	0.772	22.440	88.250	2.1498	10
Methylenecyclopentane*	75.5	1.4360	0.780	27.538	105.320	2.5607	11
1-Methylcyclopentene	75.8	1.4320	0.778	27.362	105.281	2.5505	12
Cyclohexene	83.0	1.4465	0.8102	27.065	101.395	2.6498	10
2,5-Norbornadiene	89.5	1.4702	0.9064	28.378	101.677	2.8165	10
3-Methylcyclohexene*	104.0	1.4414	0.7990	31.809	120.363	3.0605	10
4-Methylcyclohexene	102.7	1.4414	0.7991	31.805	120.348	3.0437	10
1-Methylcyclohexene	110.0	1.4503	0.8102	31.917	118.699	3.0505	10
Vinylcyclohexane*	128.0	1.4420	0.804	36.266	137.065	3.5581	11, 13
4-Vinylcyclohexene	128.9	1.4639	0.8299	35.963	130.353	3.2079	10
cis-Bicyclo[3.3.0]oct-2-ene	134.0	1.437	0.889	31.600	121.586	3.6330	11
Ethylidenecyclohexane	136.0	1.4618	0.822	36.842	134.063	3.5731	12
cis-5-Ethylidene-2-norbornene*	147.3	1.4895	0.893	38.885	134.602	3.7330	13, 14
endo-Dicyclopentadiene	170.0	1.5120	1.0701	37.070	123.539	4.3000	10, 13
cis-Cyclodecene*	198.0	1.4854	0.873	45.215	157.651	4.6498	10, 12
1,4-(or 4,7-)Dimethyl-DCPD[b]	–	–	–	–	–	5.0563	–
4,8-(or 4,9-)Dimethyl-DCPD[b]	–	–	–	–	–	5.1109	–
1,3-(or 3,7-)Dimethyl-DCPD[b]	–	–	–	–	–	5.0662	–
3,8-(or 3,9-)Dimethyl-DCPD[b]	–	–	–	–	–	5.1210	–
4-Methyl-8-(or 9-)methylene-DCP[c]	–	–	–	–	–	5.1043	–
3-Methyl-8-(or 9-)methylene-DCP[c]	–	–	–	–	–	5.1143	–
trans,cis,cis-1,5,9-Cyclododecatriene	238.0	1.5140	0.907	53.865	178.919	4.9495	11
α-Cedrene[d,*]	262.5	1.5034	0.9342	64.709	218.754	5.8886	10, 12

[a–d] See Table IV.

retention[17] were available from different sources or could be calculated. They are given in Table V.

From the data in Tables IV and V, equations $I_R = f(t_b)$ were obtained in order to establish the extent of the deviation from the ideal chromatographic process. As

TABLE VI

EQUATIONS $I_R = f(t_b)$ FOR DIFFERENT STATIONARY PHASES AT 100°C

r = Correlation coefficient, s = standard deviation, N = number of experimental points.

Stationary phase	*Equation*	r	s	N
Squalane	$I_R = 331.42 + 3.98\ t_b$	0.9985	12.0	14
OV-101	$I_R = 344.12 + 3.96\ t_b$	0.9986	11.9	15
SE-54	$I_R = 367.81 + 3.89\ t_b$	0.9973	17.6	15
OV-1701	$I_R = 371.76 + 3.99\ t_b$	0.9982	15.6	9
OV-215	$I_R = 375.27 + 4.09\ t_b$	0.9940	26.5	18

TABLE VII

EQUATIONS $I_R = f(t_b,\theta)$ WHERE θ IS M_R, V_M OR χ, FOR DIFFERENT STATIONARY PHASES AT 100°C

r = Correlation coefficient, s= standard deviation, N = number of experimental points.

Stationary phase	*Equation*	r	s	N
Squalane	$I_R = 52.56 + 4.68\ t_b + 6423.5/M_R$	0.9994	7.9	14
	$I_R = 220.70 + 4.23\ t_b + 9811.9/V_M$	0.9987	11.0	14
	$I_R = 98.94 + 4.57\ t_b + 517.1/\chi$	0.9992	8.5	14
OV-101	$I_R = -0.47 + 4.82\ t_b + 7890.1/M_R$	0.9998	4.3	15
	$I_R = 132.46 + 4.43\ t_b + 18717.1/V_M$	0.9994	7.6	14
	$I_R = 131.23 + 4.50\ t_b + 466.8/\chi$	0.9992	8.8	15
SE-54	$I_R = 15.96 + 4.85\ t_b + 7588.7/M_R$	0.9999	3.9	15
	$I_R = 75.40 + 4.60\ t_b + 24393.8/V_M$	0.9996	7.1	15
	$I_R = 184.39 + 4.42\ t_b + 363.9/\chi$	0.9989	11.2	15

expected, the statistical analysis (Table VI) showed that the solute–stationary phase interactions increase with increasing McReynolds' polarity of the stationary phase, which is reflected by the decrease in the correlation coefficient and the increase in the standard deviation. The existence of these interactions is also revealed by alterations in the elution order. The elution order on squalane is close to the increasing order of solute boiling points, whereas on stationary phases of higher polarity this relationship fails. Some examples of this are the change in the elution order of 1,3-cyclopentadiene and cyclopentene on OV-215 at 80 and 100°C, the gradual change of in the relative I_R values of methylenecyclohexane and 4-methylcyclohexene with polarity and similar changes for the pair formed by *cis*-bicyclo[3.3.0]oct-2-ene and ethylidenecyclohexane. The standard deviation of the equations $I_R = f(t_b)$ is too high to be used in the calculation of t_b.

When low-polarity solutes are chromatographed on stationary phases of low and medium polarity, the interactions are mainly dispersive and can be corrected by introducing dispersive descriptors, such as M_R, V_M or χ, in the correlation. The equations $I_R = f(t_b, \theta)$, where θ is M_R, V_M or χ, obtained with I_R on stationary phases of low and medium polarity at 100°C are shown in Table VII. It can be seen that the introduction of the new parameter improves the statistics, especially for OV-101 and SE-54 when M_R is used as the descriptor. The similar effect produced by these three parameters agrees with their high intercorrelation, which is shown in Table VIII.

TABLE VIII

CORRELATION COEFFICIENTS BETWEEN DIFFERENT SOLUTE PROPERTIES

Parameter	t_b	$1/M_R$	$1/V_M$	$1/\chi$
t_b	1.0000	0.9607	0.9419	0.9246
$1/M_R$		1.0000	0.9318	0.8573
$1/V_M$			1.0000	0.8591
$1/\chi$				1.0000

Unfortunately, the only parameter available for the calculation of t_b for methylcyclopentadiene dimers and iso-dimers is χ, which can be calculated from their structures[15,16]. Nevertheless, from equation $I_R = f(t_b,\chi)$ obtained from I_R values on squalane (Table VII), fairly good t_b values can be calculated, as shown in Table IX, where experimental and calculated t_b values are given. The standard deviation was less than $\pm 2.0°C$, which may be considered as acceptable taking into account the poor precision of some of the experimental t_b values. Sometimes, two different t_b values were found in the literature for the same compound. It is to be expected that the values calculated for MCPD dimers and iso-dimers bear a similar error.

The general expression of the equations in Table VII closely remembers the following equation deduced by Kaliszan[18] for the calculation of I_R on polar stationary phases from those on non-polar phases:

$$I_P = k_1 + k_2 I_{NP} - k_3 D \tag{3}$$

TABLE IX

EXPERIMENTAL AND CALCULATED t_b OF MCPD DIMERS AND OTHER CYCLOOLEFINS

Compound	t_b (°C)			
	Eqn. 1	*Eqn. 2*	*Exptl.*	*Ref*
1-Methylcyclopentene[a]	76.4	75.9	75.8	12
Cyclohexene[a]	84.5	84.2	83.0	10
2,5-Norbornadiene[a]	84.4	84.7	89.5	10
2-Norbornene	100.0	–	96.0	13
3-Methylcyclohexene[a,b]	103.0	104.6	104.0	10
Methylenecyclohexane	104.0	104.6	102–103[c]	10
4-Methylcyclohexene[a]	104.1	104.7	102.7	10
1-Methylcyclohexene[a]	110.1	110.4	110.1	10
Vinylcyclohexane[a,b]	127.4	128.8	128.0	11, 13
4-Vinylcyclohexene[a]	124.6	124.8	128.9	10
cis-Bicyclo[3.3.0]oct-2-ene	134.1	135.3	134.0	11
Ethylidenecyclohexane[a]	136.2	137.0	136.0	12
5-Vinyl-2-norbornene	137.9	139.1	141.0	11
cis-5-Ethylidene-2-norbornene[a,b]	144.0	144.9	147.3	14
trans-5-Ethylidene-2-norbornene	146.4	–	148.5	14
exo-Dicyclopentadiene	171.6	172.5	–	–
endo-Dicyclopentadiene[a]	171.9	172.8	170.0	10
Bicyclo[4.3.0]nona-3,6(1)-diene	175.5	175.1	178.0	13
1,4-(or 4,7-)Dimethyl-DCPD[d]	193.8	194.9	–	–
4,8-(or 4,9-)Dimethyl-DCPD[d]	198.6	199.6	–	–
cis-Cyclodecene[a]	199.4	199.5	198.0	12
1,3-(or 3,7-)Dimethyl-DCPD[d]	200.2	201.0	–	–
3,8-(or 3,9-)Dimethyl-DCPD[d]	205.4	206.7	–	–
4-Methyl-8-(or 9-)methylene-DCP[e]	206.1	206.6	–	–
3-Methyl-8-(or 9-)methylene-DCP[e]	212.2	212.4	–	–
trans,cis,cis-1,5,9-cyclododecatriene[a]	236.4	234.9	238.0	11
α-Cedrene[a,b,f]	263.8	262.2	262.5	12

[a] Compounds used as standards for eqn. I ($I_R = 98.94 + 4.57\, t_b + 517.1/\chi$).
[b] Compounds used as standards for eqn. 2 ($I_R = -0.99 + 4.84\, t_b + 725.7/\chi$).
[c] At 764 mmHg.
[d–f] See footnotes *b–d* in Table IV.

where I_P and I_{NP} are the retention indices on polar and non-polar stationary phases, respectively, and D is a dispersive descriptor.

The similarity between the equations in Table VII and eqn. 3 is not surprising, as for low-polarity solutes there is a clear correlation between their I_R values on non-polar stationary phases (I_{NP}) and the boiling points, as the equations in Table VI show.

In practice, the use of a large number of standards to obtain equations suitable for calculating t_b can be avoided. A small number of compounds selected at random with the only condition of covering the whole range of t_b may be sufficient. Thus, by choosing only the six cycloolefins marked with asterisks in Tables IV and V, the equation $I_R = -0.99 + 4.84t_b + 725.7/\chi$ was obtained. This equation provides t_b values very similar to those calculated from the former equation. They are also given in Table IX.

The method described offers the advantage of requiring only I_R and χ values of compounds for the calculation of their t_b. Although it is not as precise as those of Matukuma[19] and Sojak *et al.*[20], it provides better values than those obtained by the methods proposed by Kamlet *et al.* [21] and Lydesen[22].

CONCLUSIONS

Relationships between I_R values on several stationary phases of polarity ranging from 0 to 1550 (McReynolds' scale) and the physico-chemical properties of cycloolefins have been established that permit the calculation of approximate t_b values for dimethyldicyclopentadiene dimers and their iso-dimers with an exocyclic double bond, whose t_b data are not available in the literature.

ACKNOWLEDGEMENTS

The authors thank the Comisión Asesora de Investigación Científica y Técnica (CAICYT) and the Consejo Superior de Investigaciones Científicas (CSIC) for financial support.

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Journal of Chromatography, 508 (1990) 375–381
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 428

Note

Separation of porphyrins on cyclodextrin-bonded phases with a novel mobile phase

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(First received November 7th, 1989; revised manuscript received March 1st, 1990)

The isolation and purification of tetrapyrrole compounds, such as porphyrins, is one of the more interesting and important areas of separation in chemistry and the related disciplines. Interest in substituted porphyrins arises from their widespread occurrence in geological samples, such as petroleum, shales, coals and biological materials. Geoporphyrins which are thought to derive mainly from precursor chlorophyll[1] usually complexes with other metal ions, *e.g.* manganese and iron[2], vanadyl and nickel[3], copper and gallium[4]. The separation and analysis of geoporphyrins are important to the study of deposition of sediments and the geology of environment, whereas the determination of different porphyrins in biological tissues is useful in diagnosis of disease states and abnormal metabolism due to environmental intoxication or genetic disorders. The complexity of porphyrins has prompted the development of various chromatographic techniques for the separation and analysis of synthetic and naturally occurring porphyrins. Carboxylated porphyrins are one of the more common tetrapyrrole compounds found in biological materials (Table I). They are characterized by the number of carboxyl groups at the substituted positions. Numerous improved methods for the separation of porphyrins, which include a normal-phase chromatography using an aminopropyl-bonded silica stationary phase with a binary eluent[5], reversed-phase chromatography[6–12], and thin-layer chromatography[13], have been described earlier. More recently, reports dealing with the retention behavior of the polycarboxylic porphyrins in reversed phase chromatography[14] and the separation of individual carboxylated porphyrins on silicone polymer-coated silica gel modified with octadedyl groups[15] have appeared. Although there exists voluminous literature on the separation of porphyrins, isocratic separation of carboxylated porphyrins has not been successful. However, the previous methods have led to significant contributions to the understanding of, and improvement on the separation of porphyrins.

Cyclodextrin-bonded phases (CDs) show strong separation capability via high-performance liquid chromatography (HPLC). Separations of solutes on cyclodextrins result mainly from the formation of inclusion complexes which are formed when solute molecules enter the cavity of the CDs. The ability of the solute molecule to

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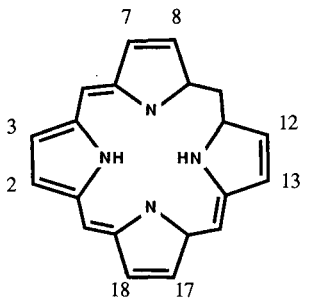
TABLE I

STRUCTURES OF PORPHYRINS



Polycarboxylic porphyrins	*Substituents[a] at position*							
	2	*3*	*7*	*8*	*12*	*13*	*17*	*18*
Uro-	A	P	A	P	A	P	A	P
Hepta-	M	P	A	P	A	P	P	A
Hexa-	M	P	M	P	A	P	P	A
Penta-	M	P	M	P	A	P	P	M
Copro-	M	P	M	P	M	P	M	P
Proto-	M	V	M	V	M	P	P	M
Meso-	M	E	M	E	M	P	P	M

[a] M = $-CH_3$; E = $-C_2H_5$; A = $-CH_2COOH$; P = $-CH_2CH_2COOH$; V = $-CH=CH_2$.

form an inclusion complex largely depends on the size, shape and chemical interactions between the solute molecule and the CDs. Other factors, such as Van der Waals forces and hydrogen bonding also affect the retention behavior of solutes. Different classes of compounds including carbohydrates and related molecules[16], optical, geometric and structural isomers[17–22], which are difficult to separate on reversed-phase columns, have been separated using CDs; thus, the separation of porphryins is attempted on the cyclodextrin bonded phases. However, the experiment is prone to some difficulty. The solubility and stability of polycarboxylic porphyrins are the major concerns in developing a chromatographic method.

Although the formation of inclusion complexes is generally accepted to be the basic property of CDs to effect the separation of different compounds, such separation mechanism is not favorable for porphyrins. The cavities of β-CDs are relatively hydrophobic and have an internal diameter of 7.8 Å (Fig. 1). This small cavity does not allow porphyrins with bulky side-chain substituents to form the inclusion complexes; thus, the separation of porphryin acids cannot be via inclusion complex formation, but may be based on adsorption of the solutes on the outside of CDs and hydrogen-bonding interaction between solutes and solvents. Recent studies of the chemical reaction of porphyrins with cyclodextrins[23] and the separation of carbohydrates on CDs[16] have demonstrated that CDs can retain compounds via adsorption rather than inclusion complex formation.

In the present study, a method for the simultaneous separation of polycarboxylic

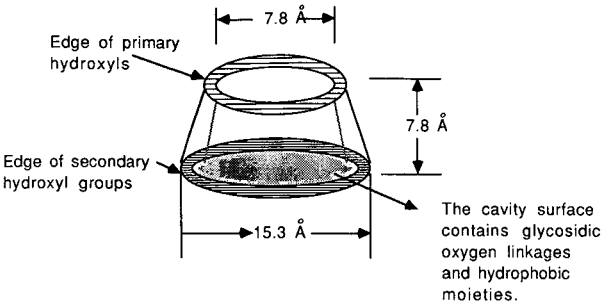


Fig. 1. The structure of β-cyclodextrin-bonded phase.

porphyrins on CDs with a novel mobile phase was described and the retention behavior of the seven porphyrins was investigated.

EXPERIMENTAL

Materials

All porphyrin acids were purchased from Porphyrin Products (Logan, UT, U.S.A.). Methanol, tetrahydrofuran, pyridine, acetonitrile and acetone (HPLC quality) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Potassium phosphate and 18-crown-6 were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

Instrumentation

Experiments were performed on a Varian 5000 liquid chromatograph equipped with a Rheodyne 7126 injector fitted with a 10-μl sample loop. A β-cyclodextrin Cyclobond I™ column (25 cm × 4.6 mm I.D.) was employed for all the experiments in this study. The column was a product from Advanced Separation Technologies (Whippany, NJ, U.S.A.). The detector was a variable-wavelength fluorescence spectrophotometer Model 650-15 (Perkin-Elmer, CT, U.S.A.) with a 12-μl flow cell attachment. All chromatograms were recorded with a Hewlett-Packard 3388A integrator,

Preparation of porphyrin acid solutions

Amounts of 20 nmol of each of the porphyrin acids were dissolved in 2 ml of 2 *M* hydrochloric acid. The dissolution was complete with sonication. The compounds were stable in solution at 4°C.

Chromatography

The mobile phase was prepared by dissolving 0.22 g of 18-crown-6 ether in 48 ml of acetone (58.5% v/v) and followed by the addition of 29 ml of pyridine (35.4%, v/v) to the solution. Subsequently, 5 ml of 0.06 *M* potassium phosphate solution were mixed with the solution. The pH of the mobile phase was adjusted to 6.37 on a digital ionalyzer with a Ross combination pH electrode from Orion Research (Cambridge, MA, U.S.A.). The separation of porphyrin acids was carried out using isocratic elution

at a flow-rate of 1.1 ml/min at ambient temperature. The injection volume was 1 μl. The excitation and emission wavelengths were set at 405 and 630 nm, respectively.

RESULTS AND DISCUSSION

The isocratic separation of the seven porphyrin acids by liquid chromatography has been studied by conventional chromatographic techniques but without success. Since CDs have shown remarkable separation capability and chiral resolution, β-CD-bonded phase is employed to study the separation of porphyrins in an attempt to resolve the seven compounds simultaneously. Although CDs have been widely used for separating different compounds in liquid chromatography, the mobile phase design for separating porphyrins on CD stationary phases is challenging and depends on the stability and the solubility of porphyrins. The porphyrin acids are soluble in strong acids. Their isoelectric points or pH values of minimal solubility are in the range 3.0–4.5. The results of the earlier studies indicated that a pH of 5.3 was necessary for the complete separation of porphyrin acids by liquid chromatography[6,14]. Common organic solvents, such as methanol, acetonitrile, ethanol, dimethyl sulfoxide and dimethylformamide in phosphate buffer, were used to develop a binary mobile phase for the isocratic separation of porphyrins on CDs. But the experiments met with a limited success. Solutes were either retained or co-eluted. As a result of the previous studies[14], it was also suggested that the addition of an organic modifier, tetrahydrofuran, was more effective to change the solvent selctivity; thus, different ternary mobile phases

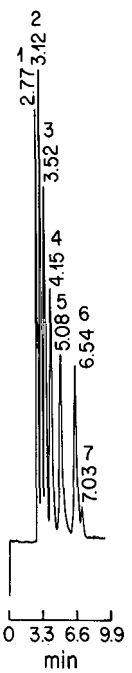


Fig. 2. Separation of porphyrin acids. Conditions were given in the Experimental section. Peaks: 1 = uroporphyrin; 2 = heptaporphyrin; 3 = hexaporphyrin; 4 = pentaporphyrin; 5 = coproporphyrin; 6 = mesoporphyrin; 7 = protoporphyrin.

were prepared from the previous methods with modifications[6,14]. Unfortunately, the ternary miobile phases produced poor retention and selectivity. Consequently, a mobile phase containing four components was developed for the isocratic separation of the porphyrin acids on the CD column. The chromatogram is shown in Fig. 2.

The influence of pyridine on retention was studied. The results showed that the capacity factors (k') decreased slightly with the increase of pyridine concentration as shown in Fig. 3. Overall retention changed only slightly for concentrations between 33 and 46% pyridine, accompanied by minor changes in selectivity. Pyridine was introduced into the mobile phase as the organic modifier to increase the solubility of porphyrin acids as well as to improve the mobile phase selectivity. It is understood that the carboxyl groups of porphyrins are strong proton donors, whereas both pyridine and the hydroxyl groups of the CDs are proton acceptors. But the hydroxyl group of CDs is a much weaker base than pyridine; thus, the hydroxyl group is not as good a proton acceptor as pyridine. Consequently, the acid–base interaction between pyridine and the porphyrin acids produced stronger hydrogen-bonding interaction, which resulted in increase in solubility, and also enhancing the preferential partition of porphyrins in the mobile phase. As a result, changes in pyridine concentration affected the elution strength of the mobile phase as observed.

The effect of crown ether on the retention of porphyrins was also studied. The results showed that the k' values decreased noticeably with the increase in crown ether concentration (Fig. 4). Although the elution strength was not significantly changed over the concentration range between 7 and 14 mM, the addition of crown ether in the mobile phase is essential to produce an acceptable solvent selectivity in this study. Crown ether is especially effective in that it contains both polar groups and the hydrophobic moieties. The presence of crown ether presumably facilitates a more efficient partition of porphyrins in the mobile phase due to its hydrophobic and polar interactions with the solutes. Furthermore, the addition of crown ether is necessary because earlier studies[6,14] have suggested that phosphate buffer is important in effecting the solubility and partitioning or porphyrins in the mobile phase. But phosphate buffer is only slightly soluble in the organic phase developed in this study. Nevertheless, crown ether can solubilize potassium phosphate in the non-polar organic phase by forming specific complexes with potassium cations.

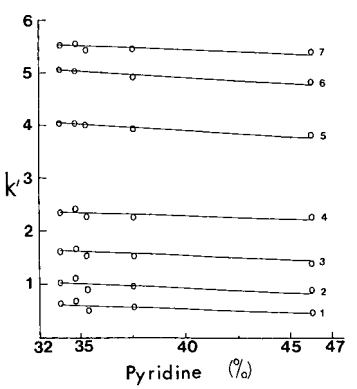


Fig. 3. Effects of pyridine content in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

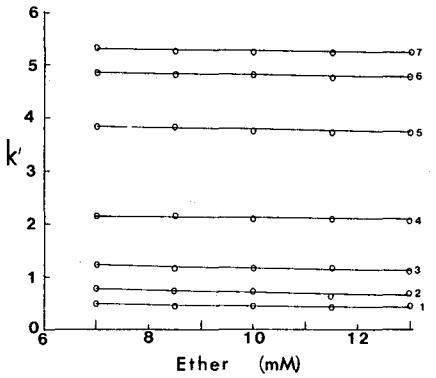


Fig. 4. Effects of crown ether (18-crown-6) in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

As related to the formation of potassium cation–crown ether complexes, the influence of phosphate content on the retention and selectivity was also studied. The results showed that 0.06 *M* of phosphate solution was the optimal concentration to produce a good retention and selectivity as evidenced by the separation of porphyrins shown in Fig. 2. Higher phosphate concentration (> 0.06 *M*) would, however, produce precipitate in the mobile phase, whereas lower phosphate concentration would result in significant decrease in resolution among porphyrins.

In addition, the effect of acetone concentration on retention was studied (Fig. 5). The results indicated that the k' values showed marked but similar changes as the proportion of either acetone or pyridine was changed. However, significant changes in retention and selectivity could be obtained by altering the volume composition of acetone of the mobile phase. The results suggested that acetone has a greater affinity than porphyrins for the adsorption site. Consequently, an increase in acetone concentration reduces the interaction between solutes and CDs; thus, the k' values decrease as the volume composition of acetone increases. In addition, the present study showed that a relative smaller change in acetone concentration in the mobile phase resulted in greater changes in the k' values, suggesting that acetone was more effective in changing the elution strength of the mobile phase. The different porphyrin retentions appear to be based on the adsorption process through the acetone interactions with the carboxyl groups of porphyrins and CDs.

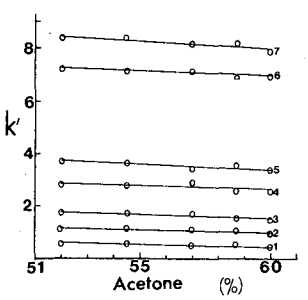


Fig. 5. Effects of acetone content in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

CONCLUSIONS

The isocratic separation of porphyrin acids on β-CD-bonded phases with a novel mobile phase has been demonstrated. As the solvent selectivity for solutes becomes better characterized, it is possible to develop novel mobile phases for separating different compounds especially macromolecules. The results of this study have also shown that compounds with similar structures, such as mesoporphyrin and protoporphyrin bearing identical substituents except an additional double bond at the position three (Table I), can be easily separated. The retention of porphyrins is reasonably believed to be governed by an adsorption process on the outside of CDs. More polar porphyrins eluted before the less polar ones. It appears that the separation of porphyrins is closely related to the number of carboxyl groups on porphyrins. Although the separation of porphyrins on CDs is intrigued by various interactions between solutes and solvents, the results of the present study have demonstrated one other possible technique to resolve some macrocycles using CDs via adsorption process.

ACKNOWLEDGEMENTS

This work was supported by Grant Nos. MCJ360476020 and 435 from the Bureau of Community Health Services, Department of Health and Human Services.

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Journal of Chromatography, 508 (1990) 382–385
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Note

Thin-layer and high-performance liquid chromatographic separation of glycerolipid subclasses as benzoates

Derivatives of ether and ester analogues of phosphatidylcholine, phosphatidylethanolamine and platelet activating factor

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Methods for separation and quantitation of glycerolipid subclasses containing alk-1-enyl, alkyl and acyl groups are important tools in lipid research. Many of these methods are based on the chromatographic separation of derivatives that are prepared from the original lipid class. Although extensive use has been made of acetate derivatives[1], groups that have a reasonably high molar absorptivity such as nitrobenzoates[2], dinitrobenzoates[3], dinitrophenylurethanes[4] and benzoates[5] are better suited for direct on-line quantitation by high-performance liquid chromatography (HPLC). Three of these derivatives have been used for the HPLC separation of the subclasses of long-chain diradylglycerols[1,5,6], derived from phospholipids via phospholipase C hydrolysis. In this paper the HPLC methodology for the separation of diradylglycerol benzoates[5] is extended to include several other lipids. Data on the relative migration of these benzoate derivatives during thin-layer chromatography (TLC) are also included. This information should be particularly valuable for investigators in the lipid mediator field who are interested in the analytical identification of radiolabeled lipids that migrate in the same general area as 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) during thin-layer chromatography (TLC).

MATERIALS AND METHODS

1-Alk-1′-enyl-*sn*-glycerol(1-alk-1′-enyl-Gro) and 1-alkyl-Gro were obtained as previously described[7]. 1-Acyl-Gro (monoolein) was purchased from Sigma (St. Louis, MO, U.S.A.). 1-Alk-1′-enyl-2-acetyl-Gro and 1-alkyl-2-acetyl-Gro were prepared by acetylation of the corresponding 1-radyl-Gro followed by treatment of the diacetates with pancreatic lipase and isolation of both the 1-radyl-2-acetyl-Gro and 1-radyl-3-acetyl-Gro by TLC as described[8], except that the radylacetyl-Gro were

extracted from the TLC plates with diethyl ether instead of hexane. 1-Acyl(palmitoyl)-2-acetyl-Gro was prepared by acetylation of palmitoyllysoglycerophosphocholine (Sigma) and subsequent hydrolysis of this product by phospholipase C (ref. 9). Sources of the long-chain diradyl-Gro subclasses were described previously[5]. A preparation of ceramides from the sphingomyelin of bovine brain was purchased from Sigma.

Benzoylation of the compounds was accomplished as previously described[5,7] except that 1 ml of concentrated ammonium hydroxide at room temperature, instead of 0.1 *M* sodium hydroxide at 0°C, was used to remove excess benzoic anhydride and benzoic acid at the termination of the reactions. Benzoate derivatives were separated on silica G (EM Science, Gibbstown, NJ, U.S.A.)-coated (250 μm) TLC plates in chromatographic tanks lined with filter paper using a solvent system of benzene–hexane–diethyl ether (50:45:5, v/v/v). TLC R_F values were measured after charring the developed plates with sulfuric acid spray and heat (180°C, 30 min). All benzoate derivatives were purified by TLC[10] and redissolved in cyclohexane before analysis by normal-phase HPLC. Except for the hardware (an isocratic, single-pump HPLC system purchased from Rainin, Woburn, MA, U.S.A.) normal-phase HPLC was carried out exactly as described previously[5]. The mobile phase consisted of cyclohexane–diethyl ether–glacial acetic acid (97:3:0.07, v/v/v) at a flow-rate of 1 ml/min. Separations were made on an Altex 250 × 4.6 mm I.D. column packed with Ultrasphere-Si (5 μm; Beckman, Norcross, GA, U.S.A.) and the benzoate derivatives were detected by measuring the absorbance at 230 nm.

RESULTS AND DISCUSSION

Normally a difference in TLC R_F values of about 0.05 is needed to achieve a separation of two compounds when the distance from the origin to the solvent front is 14–15 cm. The TLC solvent system described separated the benzoate derivatives of the alk-1-enyl, alkyl and acyl subclasses within each class of glycerolipid (Table I). If 1-radylglycerodibenzoates are excluded, the TLC system is capable of separating the

TABLE I

TLC MOBILITIES OF BENZOATE DERIVATIVES OF VARIOUS LIPID CLASSES

Benzoylated compounds	*TLC R_F values*[a]
(A) 1-Alk-1'-enyl-2-acyl-Gro	0.54 ± 0.02
(B) 1-Alkyl-2-acyl-Gro	0.46 ± 0.02
(C) 1,2-Diacyl-Gro	0.35 ± 0.01
(D) 1-Alk-1'-enyl-2-acetyl-Gro	0.30 ± 0.02
(E) 1-Alkyl-2-acetyl-Gro	0.22 ± 0.01
(F) 1-Acyl-2-acetyl-Gro	0.16 ± 0.01
(G) 1-Alk-1'-enyl-Gro	0.42 ± 0.02
(H) 1-Alkyl-Gro	0.36 ± 0.02
(I) 1-Acyl-Gro	0.26 ± 0.01
(J) Ceramide	0.07 ± 0.00

[a] Average TLC R_F values ± S.D. were calculated from plates ($n = 3$ different days) that were chromatographed as described in Materials and Methods.

remaining six subclasses of glycerolipid benzoate derivatives and the benzoate derivatives of ceramides. We have found the benzoate derivatives to be particularly important in discerning the amount of radiolabeled acetate incorporated into sphingomyelin (which has a TLC R_F similar to PAF) relative to the amount actually incorporated into PAF by intact cells.

Separation of several benzoate derivatives by normal-phase HPLC is shown in Fig. 1. As expected, the HPLC resolution of these derivatives is better than with TLC. In fact, as indicated by the multiple peaks within some subclasses, there is even a partial resolution of molecular species. The only major overlap of peaks occurred with the benzoates of 1-alk-1′-enyl-Gro and 1-alkyl-2-acyl-Gro (peaks B and G in Fig. 1). If there is doubt about the presence of these two components in a sample, the peak can be collected from HPLC, treated with acid to hydrolyze the labile vinyl ether linkage, and then rerun the sample on HPLC. Any peak found at this elution time, after the acid treatment, should be representative of the 1-alkyl-2-acyl-Gro subclasses. The benzoate derivative of cholesterol, which could be a contaminant in the TLC isolation of diglycerides from a sample of total lipids, elutes from this normal-phase HPLC system 4 to 4.5 min after injection (not shown in Fig. 1) and therefore, would not interfere.

1-Radyl-2-acetyl-*sn*-glycero-3-phosphocholine and 1-radyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine can contain 1-alk-1′-enyl, 1-alkyl, and/or 1-acyl groups at the *sn*-1 position of glycerol[11,12]. Therefore, normal-phase HPLC separation[5,13] of these subclasses, after phospholipase C hydrolysis and benzoylation, provides another method for analysis of these subclasses of PAF analogues (see peaks D, E, and F in Fig. 1); this technique is particularly helpful in experiments that have utilized radiolabeled acetate as a measure of PAF production. Samples suspected of containing the PAF analogues should be benzoylated immediately after phospholipase C hydrolysis to minimize isomerization of acetate from the *sn*-2 position to the *sn*-3 position. If isomerization of the acetate has occurred, the 1-radyl-2-benzoyl-3-acetyl-Gro derivatives would elute at about 19, 25 and 55 min for the alk-1-enyl, alkyl, and acyl subclasses, respectively, in the HPLC system described (peaks not shown in Fig. 1). As an example of sensitivity using our instrumentation, 0.2 nmol of 1-alkyl-2-

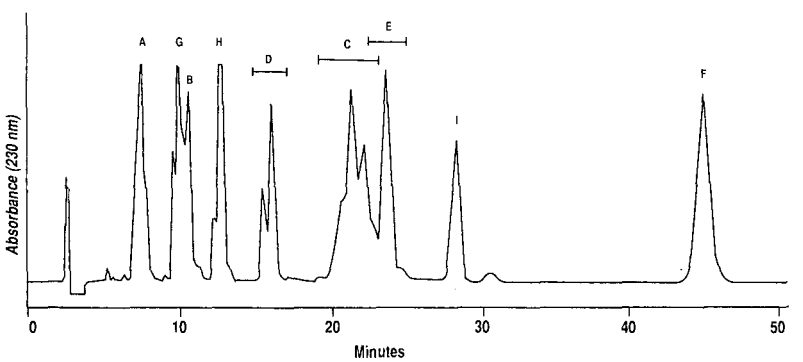

Fig. 1. Normal-phase HPLC separation of various glycerolipid subclasses as their benzoate derivatives was carried out as described in Materials and Methods. Letters for peak identification refer to Table I. Detection at 0.020 a.u.f.s.; peak A represents 1.2 nmol.

acetyl-3-benzoyl-Gro yields an HPLC peak with twenty-fold greater height than any baseline fluctuations.

The chromatographic techniques described here (TLC and HPLC of benzoate derivatives) have proven extremely useful in our investigations of the alkyl-, alk-1-enyl- and acyl subclasses of choline and ethanolamine glycerophosphatides and diradylglycerols. The information in this report should assist others in interpreting data obtained with the phospholipase C/benzoylation methodology[5].

ACKNOWLEDGEMENTS

This work was supported by DOE (DE-ACO5-760R00033), ACS (BE-26U) and NHLBI (27109-09).

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Journal of Chromatography, 508 (1990) 386–390
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 432

Note

Separation and detection of monensin, lasalocid and salinomycin by thin-layer chromatography/bioautography

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Monensin, lasalocid and salinomycin are three compounds (Fig. 1) known as carboxylic-polyether antibiotics[1]. These structures are ionophores, which cause ionic fluxes and altered membrane potential[2]. The high molecular weights and multiple functionalities of these ionophores hinder analysis[3]. These compounds are presently used primarily as anticoccidial agents for poultry[4]. They are also being used in other species (such as beef cattle) as growth stimulants and to improve the efficiency of feed utilization[3].

Several reports have appeared recently on the toxic effects of each of these compounds[5,6]. Toxicities arise primarily from three avenues[7]: first, from feeding a dose higher than the recommended level of the ionophore; second, from the inadvertent inclusion of the ionophore in a feed presented to a species for which it was not intended; and third, from an adverse interaction between the ionophore and a simultaneously administered alternate ionophore or drug.

It is believed that the increasing use of these compounds in animal husbandry may induce bacterial resistance, leave residues in tissues and lead to environmental pollution[8–12]. In addition, owing to the potential for toxicity from low level mixtures of these ionophores, alone and in combination with other drugs, a precise assay that will separate and quantify each individual ionophore is essential.

Multiresidue methods for the separation of these three ionophores have been developed, using a variety of chromatographic technology. Asukabe *et al.*[1] have achieved this separation using high-performance thin-layer chromatography (HPTLC), Blanchflower *et al.*[7] by high-performance liquid chromatography (HPLC) and Martinez and Shimoda[13] by LC methods. The method described here is believed to be the first to attempt such a separation using TLC/bioautography. This method is based on one currently available for the quantitative analysis of monensin in poultry tissues[14].

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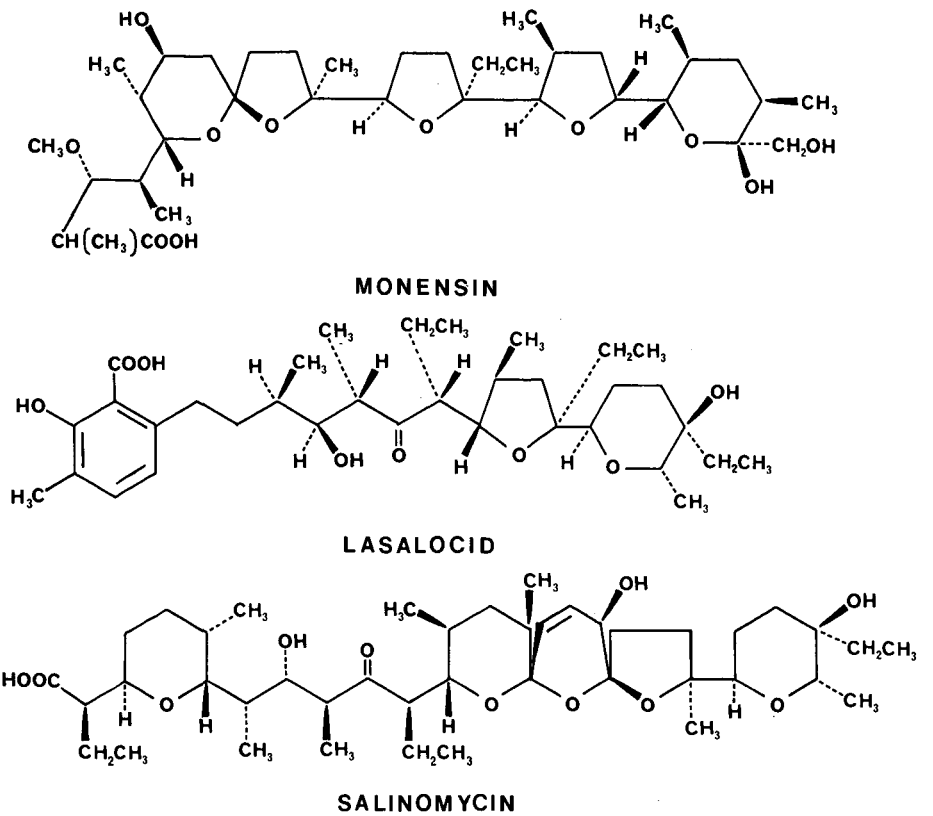

Fig. 1. The chemical structures of the monocarboxylic polyether inophores monensin, lasalocid and salinomycin.

EXPERIMENTAL

Apparatus and reagents

For a complete listing of the apparatus and reagents involved see VanderKop and MacNeil[14].

Preparation of tissue extracts

All experimental glassware must be silanized before use in order to increase ionophore recovery. The preparation of tissue extracts is as described by VanderKop and MacNeil[14]. Briefly, the method involves obtaining 10 g of frozen tissue, dicing, homogenizing for 20 s, adding methanol, mechanically shaking for 15 min, centrifuging for 10 min at 1700 *g* then collecting the supernatant. This supernatant is transferred to a round-bottomed flask, extracted with 90 ml carbon tetrachloride, rotary evaporated, collected with 8 ml hexane, reevaporated by nitrogen flow, and then the final residue is dissolved in 1 ml methanol.

Thin-layer chromatography

The adsorbent zone of a 20 cm × 20 cm pre-scored 19-channel silica gel TLC

plate with pre-adsorbent spotting zone (Whatman LK6D) is preheated by placing it over a heating strip attached to a variable autotransformer, set to provide a temperature of 20°C. Using a micro-syringe, 30 μl of the extract are spotted onto the preheated TLC plate and allowed to dry for half an hour. The plate is then developed for 35 min in a sealed glass tank containing 200 ml of ethyl acetate–acetonitrile (50:50, v/v), that had been allowed to equilibrate for at least 2 h. The TLC plate is removed from the chamber and the solvent front marked immediately with a pencil. The TLC plate is air-dried for 2 h in a fumehood.

Bioautography

The bioautography is as reported by Salisbury *et al.*[15], with the exception of adjusting the SAM-3 medium pH to 5.5 by adding either 0.1 *M* citric acid or 0.1 *M* sodium hydroxide, as required, and using 0.6 ml of *Bacillus subtilis* spore suspension to seed the media.

RESULTS AND DISCUSSION

This method is based on one developed specifically for the detection and determination of monensin in poultry tissues[14]. In the original method, using a developing system of chloroform–methanol–acetone–glycerol, the three ionophores could not be chromatographically resolved. Lasalocid could be identified by the characteristic "teardrop" shape of its zone of inhibition. Monensin and salinomycin, however, both produced circular zones of inhibition and could not be distinguished by R_F.

To permit ionophore identification, a variety of solvent developing systems were tested (Table I). A minimum of ten replications per system was done. The data for the R_F values (within each solvent system) were analyzed statistically using an analysis of variance with the level of significance set at 0.05 (ref. 16). The relationship between tissue concentration of ionophore and the size of the zone of inhibition

TABLE I

SEPARATIONS SEEN ON BIOAUTOGRAPHIC MEDIA BETWEEN MONENSIN, LASALOCID AND SALINOMYCIN WHEN USING A VARIETY OF SOLVENT SYSTEMS

R_F values are means of ten replications.

Solvent system, 200 ml	R_F *value*		
	Monensin	*Lasalocid*	*Salinomycin*
Chloroform–methanol–acetone–glycerol (98:60:40:2)	0.80^a	0.80^a	0.80^a
Ethyl acetate	0.15^a	0.57^b	0.21^a
Cyclohexane–2-propanol (150:50)	0.48^a	0.50^a	0.58^b
Ethyl acetate–acetonitrile (100:100)	0.12^a	0.34^b	0.21^c

$^{a-c}$ Letters represent significant differences in R_F values between the three ionophores within a solvent system ($p > 0.05$). For each solvent system tested, R_F values with different superscripts are significantly different, while those with the same superscript do not differ significantly.

produced on the bioautographic media was measured by simple correlation[16]. Monensin and salinomycin were separated from lasalocid using 100% ethyl acetate in the developing system. However, monensin and salinomycin were not well separated from each other. Since lasalocid can be identified as distinct by its zonal shape, it is only critical that it have a different R_F value when it is suspected to occur in combination with either monensin or salinomycin. Through further experimentation it was observed that lasalocid could be well separated from salinomycin, but not from monensin, by using a developing system with cyclohexane–2-propanol (150:50, v/v). Similarly, the solvent system originally developed by Martinez and Shimoda[17] for monensin residues in feeds and subsequently adapted to tissue was reported to give similar R_F values for monensin and lascalocid[13]. The best solvent developing system for the complete chromatographic separation of monensin, lasalocid and salinomycin tested in this research was one combining ethyl acetate and acetonitrile in a 50:50 ratio (see Fig. 2).

Problems with non-specific inhibitory substances (*i.e.* enzymes) also present in the tissue matrices were not observed. These substances migrate at different rates than do the ionophoric compounds, and leave a characteristic inhibitory pattern on the bioautographic medium. This mark is typically seen as a faint line running in the middle of the channel on the TLC plate.

Thus far, this method has only been tested in chicken liver and kidney tissues. Using the method as listed, the following detection limits were achieved: monensin

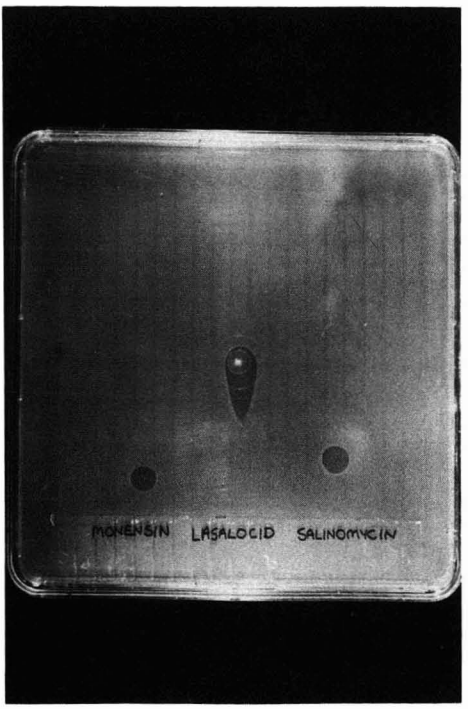

Fig. 2. Bioautogram showing TLC separation of monensin (R_F = 0.12), salinomycin (R_F = 0.21) and lasalocid (R_F = 0.34) on pre-scored 19-channel silica gel plate.

0.45 μg/g, lasalocid 1 μg/g and salinomycin 1 μg/g. A linear relationship between inhibition zone size and concentration was observed for monensin (r = 0.98), but poor linearity (r = 0.60) was found for both lasalocid and salinomycin, using linear regression analysis.

The methodology provides a simple screening test to distinguish qualitatively between residues of the three ionophores, monensin, lascalocid and salinomycin in tissue, and permits an estimation of the concentration range of the residue. For laboratories with access to more sophisticated equipment, quantitation by HPLC, and mass spectral confirmation, as described by Martinez and Shimoda[13] may be attempted. However, the latter approach does involve extensive derivatization with a reagent of limited availability and stability and thus may not be practical for laboratories performing occasional analyses in support of toxicological investigations. The HPLC separation of the ionophores described by Blanchflower *et al.*[7] is specifically for the analysis of residues in feeds and has not been tested for residues in animal tissue.

CONCLUSIONS

A method was developed for the separation of monensin, lasalocid and salinomycin, three ionophore antibiotics. A linear response (inhibition zone size *versus* concentration) was observed for monensin, but linearity was poor for lasalocid and salinomycin. The TLC/bioautography approach is useful as it is a simple method of relatively low cost and skill-level. Screening methods used in slaughter plants for antibiotics in animal tissues are based on bioassay, so this technique enables separation between those ionophores while confirming the initial bioassay response.

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Journal of Chromatography, 508 (1990) 391–398
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 316

Note

Application of gas chromatography–mass spectrometry to the identification of isoflavonoids in lupine root extracts

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Isoflavonoids present in the genus *Lupinus* have been isolated and many components, derivatives of genistein and 2′-hydroxygenistein, have been identified[1–4]. Because of intensive research on the biological activities of this class of compounds[5,6], it became necessary to find a method for the identification of flavonoids or isoflavonoids in mixtures after isolation from plant material. The separation and identification of this class of compounds by high-performance liquid chromatography (HPLC) have been described by several workers and gas chromatography has also been used[7–10]. This paper describes an attempt to use gas chromatography–mass spectrometry (GC–MS) for the identification of isoflavonoids, isolated from lupine root extracts, as methyl and trimethylsilyl derivatives.

EXPERIMENTAL

Chemicals

Solvents of analytical-reagent grade were obtained from POCh (Gliwice, Poland). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMS) and methyl iodide, used as derivatizing reagents, were obtained from Fluka (Buchs, Switzerland). Silica gel H60 used for column chromatography and plates for thin-layer chromatography (TLC) with silica gel 60 F_{254} were purchased from E. Merck (Darmstadt, F.R.G.).

Gas chromatography–mass spectrometry

GC–MS analyses were carried out with a Hewlett-Packard Model 5890 instrument equipped with an HP-1 fused-silica capillary column (12 m × 0.25 mm I.D.). The carrier gas was helium at a flow-rate of 1.5 ml/min. The column temperature was programmed from 200°C (held for 2 min) at 5°C/min to 280°C, which was held for 10 min. The injector temperature was 250°C. Injections were made in the splitless mode.

Biological samples

Methanolic root extract obtained from 100 g of fresh roots of 3-week-old seedlings of bitter lupine plants (*Lupinus albus* L. *cv.* BAC) was evaporated to dryness,

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giving a brown syrup which was dissolved in 300 ml of water. In order to isolate isoflavonoids, the aqueous solution was extracted five times with the same volume of ethyl acetate. The isoflavonoid fraction, concentrated on a Rotavapor, was adsorbed on silica gel H60 (20 g) and dried. The gel coated with the extract was transferred to the column filled with the same silica gel (50 g). The column was eluted in a stepwise mode with benzene and increasing amounts of ethyl acetate (0, 5, 15, 30, 40, 55, 70 and 100%) in benzene, 75 ml per fraction. Elution of the isoflavonoid fractions was monitored by TLC with chloroform–methanol (50:3) as the developing solvent. Compounds of interest were detected by inspection of the plates under UV radiation of 254 and 360

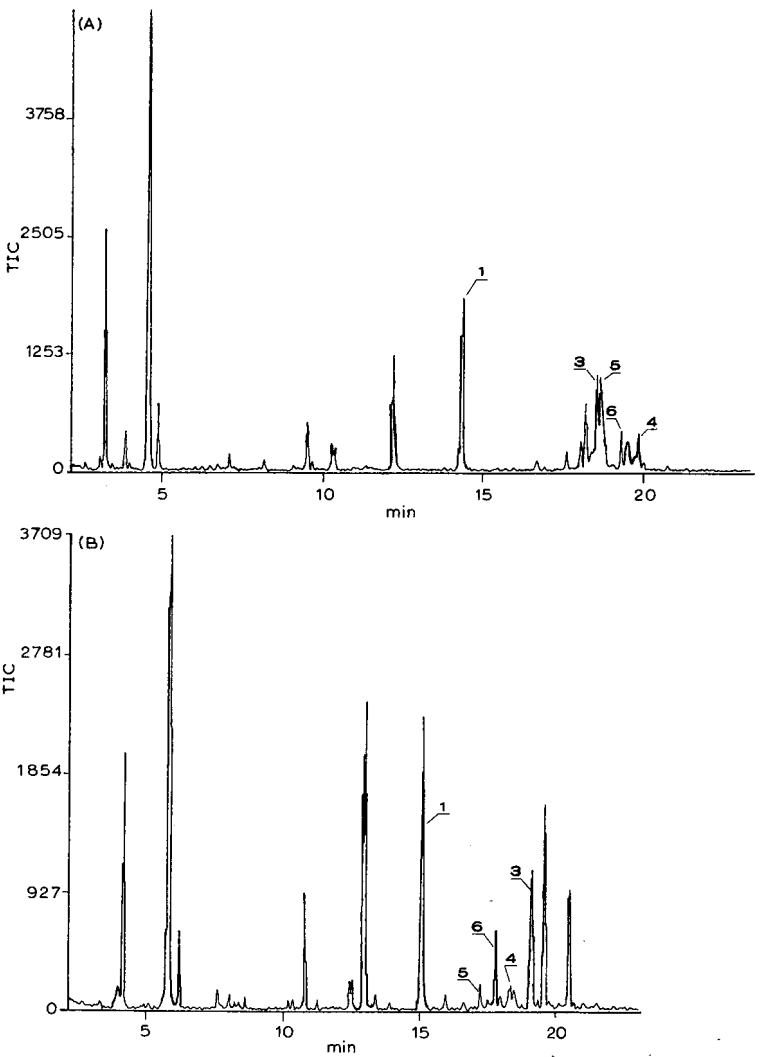

Fig. 1. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction 1. For conditions, see Experimental. Peaks: 1 = genistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

nm and by the characteristic colours (blue and violet) formed with Gibbs reagent[11].

Isoflavonoids were eluted from the column with solvent mixtures containing from 0 to 40% of ethyl acetate in benzene. Fractions with similar spot patterns of isoflavonoids were pooled into three solutions and, after derivatization, analysed by GC–MS.

Derivatization of samples for GC–MS analysis

Isoflavonoids were methylated with methyl iodide in dimethyl sulphoxide following the procedure of Ciucianu and Kerek[12]. Samples for methylation (2–4 mg)

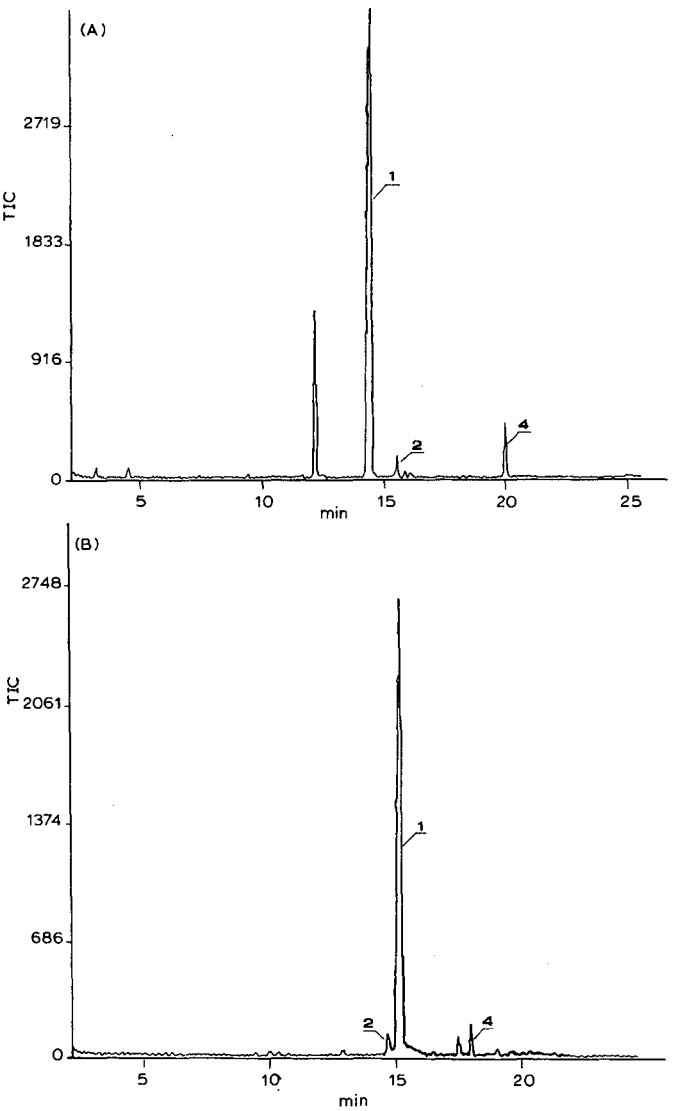

Fig. 2. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction II. For conditions, see Experimental. Peaks: 1 = genistein; 2 = 2'-hydroxygenistein; 4 = luteone.

were transferred into test vials, evaporated to dryness in a stream of nitrogen and dried over P_2O_5 overnight. The dried samples were dissolved in 1 ml of dimethyl sulphoxide and 70 mg of powdered potassium hydroxide and 200 μl of methyliodide were added. The vials were vortex mixed for 5 min, then the reaction was stopped with 2 ml of water. The reaction mixtures were extracted three times with 2 ml of chloroform. The combined organic layers were extracted twice with 3 ml of water. The chloroform solutions were evaporated to dryness and the residue was dissolved in 200 μl of methylene chloride. The solution obtained was used for GC–MS analysis.

The preparation of the samples for trimethylsilylation was identical with that for methylation. The dried samples were dissolved in 150 μl of pyridine and mixed with 150

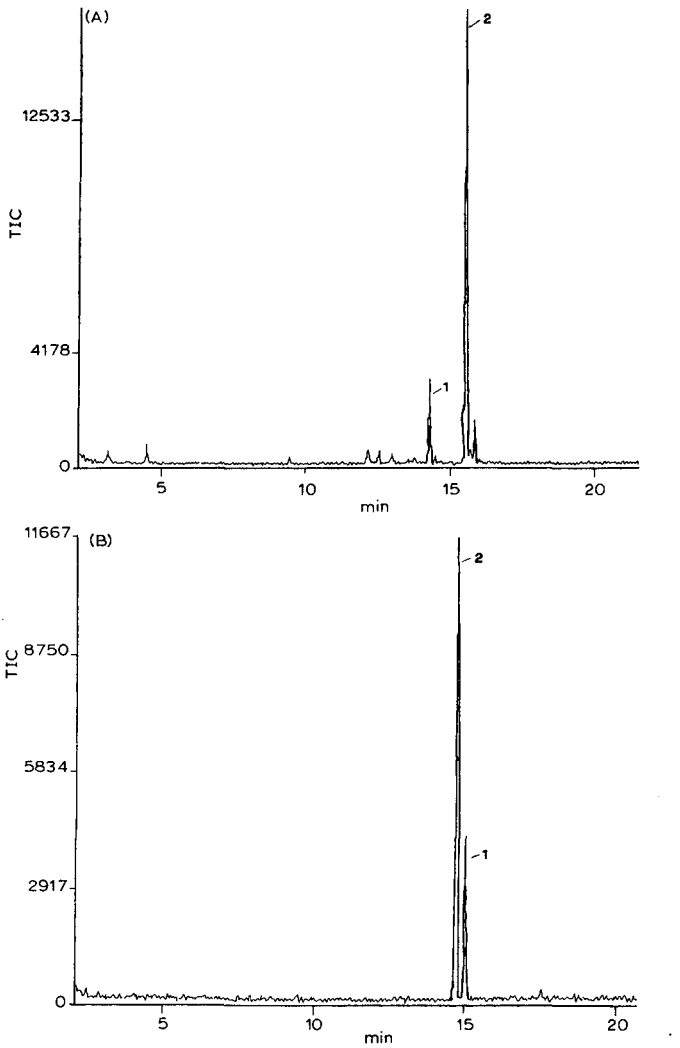

Fig. 3. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction III. For conditions, see Experimental. Peaks: 1 = genistein; 2 = 2′-hydroxygenistein.

TABLE I

GC RETENTION TIMES AND FRAGMENT IONS IN MASS SPECTRA OF METHYL DERIVATIVES OF ISOFLAVONOIDS IDENTIFIED IN FRACTIONS ISOLATED FROM LUPINE ROOT EXTRACTS

Ion	Isoflavonoid[a]											
	1 (14.38)[b]		*2 (15.55)*[b]		*3 (18.52)*[b]		*4 (19.85)*[b]		*5 (18.55)*[b]		*6 (19.33)*[b]	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
M^+	312	100	342	100	380	72	410	75	394	25	394	100
$M-CH_3$	297	5	327	8	365	66	395	21	379	100	379	8
$M-OCH_3$	281	20	311	58	349	22	379	100	363	23	363	45
$M-(OCH_3+CH_3)$	266	25	296	13	–	–	–	–	–	–	–	–
M−41	–	–	–	–	–	–	–	–	353	18	–	–
M−68	–	–	–	–	–	–	–	–	–	–	326	10
M−69	–	–	–	–	311	100	341	38	–	–	–	–
$M-(69+OCH_3)$	–	–	–	–	281	11	311	6	–	–	–	–
M−83	–	–	–	–	–	–	–	–	311	40	–	–
M−108	204	12	–	–	–	–	–	–	–	–	–	–
M−138	–	–	204	6	–	–	–	–	–	–	–	–
M−161	–	–	–	–	–	–	–	–	–	–	233	7
$C_9H_8O_4$	180	6	180	17	–	–	–	–	–	–	–	–
$C_{10}H_{10}O_2$	–	–	162	19	162	16	–	–	162	11	162	16
$C_8H_9O_2$	137	17	137	10	–	–	137	6	137	7	137	4
C_9H_8O	132	33	–	–	132	12	–	–	–	–	–	–
C_7H_7O	107	5	–	–	107	8	–	–	–	–	–	–

[a] 1 = Genistein; 2 = 2′-hydroxygenistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

[b] Retention times (min) in parentheses.

TABLE II

GC RETENTION TIMES AND FRAGMENT IONS IN MASS SPECTRA OF TRIMETHYLSILYL DERIVATIVES OF ISOFLAVONOIDS IDENTIFIED IN FRACTIONS ISOLATED FROM LUPINE ROOT EXTRACTS

Ion	Isoflavonoid[a]											
	1 (15.12)[b]		*2 (14.70)*[b]		*3 (19.15)*[b]		*4 (18.30)*[b]		*5 (17.22)*[b]		*6 (17.80)*[b]	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
M^+	486	4	574	3	–	–	642	4	568	9	–	–
$M-CH_3$	471	100	559	45	539	15	627	25	553	72	553	74
$M-(CH_3+TMS)$	399	10	487	5	467	15	–	–	481	13	481	3
$M-(CH_3+2TMS)$	327	3	415	3	–	–	483	10	–	–	–	–
$C_6H_{15}OSi_2$	147	3	147	8	147	26	147	8	147	7	147	15
TMS	73	23	73	100	73	100	73	100	73	100	73	100

[a] 1 = Genistein; 2 = 2′-hydroxygenistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

[b] Retention times (min) in parentheses.

μl of BSTFA containing 1% TMS. Derivatization was conducted for 1 h at 80°C to give samples for GC–MS analysis.

RESULTS AND DISCUSSION

All samples were subjected to GC–MS analysis. Total ion current (TIC) chromatograms of the methyl and trimethylsilyl derivatives are shown in Fig. 1–3. Peaks of isoflavonoids and of other substances, probably phenolics related to the analysed class of compounds, were observed. Mass spectral data for particular isoflavonoid peaks in the TIC chromatograms of the three fractions studied are given in Tables I and II. In the TIC chromatograms of the trimethylsilyl and methyl derivatives, the peaks related to isoflavonoids were clearly resolved. The only exception was the TIC chromatogram of the methyl derivatives of fraction I, where the peaks of two isoflavonoids (wighteone and lupinisoflavone A) were very close. In the studied samples, six isoflavonoids were recognized and were identified on the basis of their molecular weights (MW) and mass fragmentation pathways.

In the mass spectra of methylated compounds, very abundant molecular ions

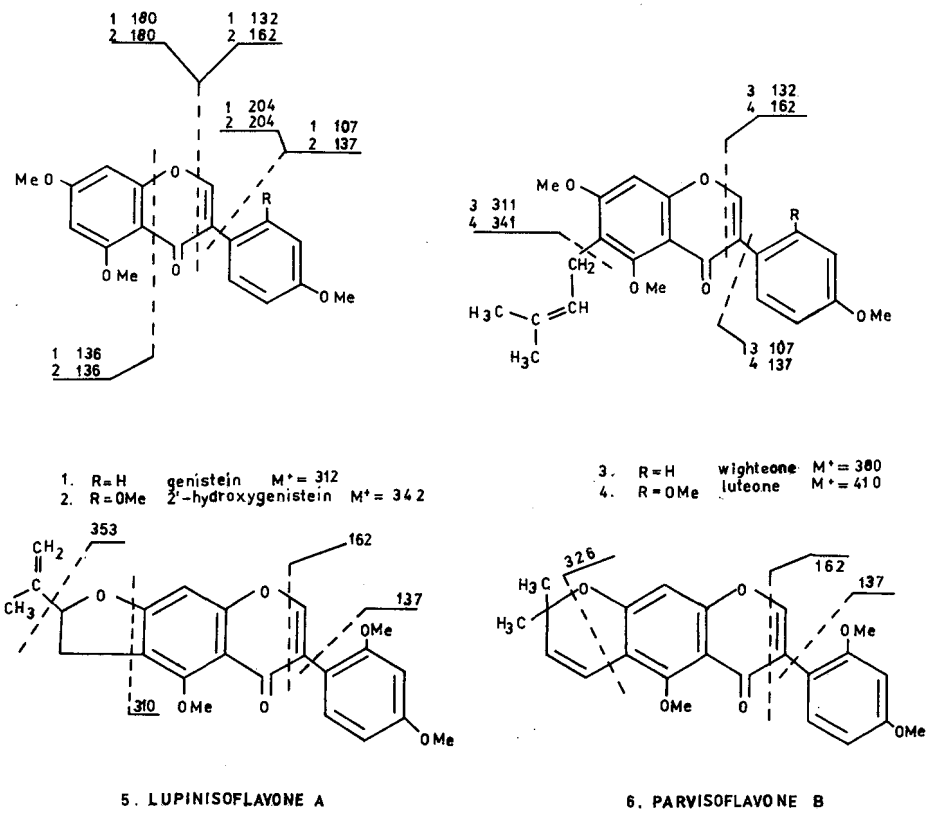

Fig. 4. Characteristic fragmentation pathways of methyl derivatives of isoflavonoids identified in lupine root extracts. Me = Methyl.

(M^+) and fragment ions were observed, giving structural information about the various isoflavonoids present in the studied extracts. On the other hand, in the mass spectra of TMS derivatives small molecular ions were observed for genistein, 2′-hydroxygenistein, luteone and lupinisoflavone A. The main ions observed were those created during elimination of the methyl radical from molecular ions. These mass spectra were dominated by fragment ions created during fragmentation of trimethylsilyl substituents at *m/z* 73 and 147, so apart from information about the molecular weights of compounds from molecular ions or [M − 15] ions there was no structural information.

Genistein, wighteone, lupinisoflavone A, parvisoflavone B and luteone were identified in fraction I, genistein, 2′-hydroxygenistein and luteone in fraction II and genistein and 2′-hydroxygenistein in fraction III. Identification of the genistein and 2′-hydroxygenistein peaks in the TIC chromatogram of methylated samples was based on the characteristic fragment ions present in the mass spectra created during cleavage of the C ring of the isoflavonoid core (see Fig. 4)[13]. In the mass spectrum of genistein these were ions at *m/z* 137 and 132, whereas for 2′-hydroxygenistein these were ions at *m/z* 137 and 161.

In the mass spectra of wighteone and luteone, fragment ions (M − 69) were observed at *m/z* 311 and 341, respectively, due to cleavage of an isoprene substituent from the molecular ions. Additionally, the presence of fragment ions at *m/z* 132 (wighteone) and *m/z* 161 (luteone) in the mass spectra of these compounds and a lack of ions at *m/z* 137 indicated that the isoprene group is attached at C-6 of the isoflavonoid core (Fig. 4).

The last two compounds, lupinisoflavone A and parvisoflavone B, have an additional furan or pyran ring owing to cyclization of the isoprene group at C-6 with the hydroxyl group at C-7 in the isoflavonoid moieties. The establishment of the substitution sites of the dihydrofuran or pyran ring in the molecules of these two compounds was possible because of the presence of the fragment ions at *m/z* 161 and a lack of fragments at *m/z* 137 and 180 (Fig. 4). Differentiation between lupinisoflavone A and parvisoflavone B was based on the observation of the fragment ion at *m/z* 353 in the mass spectrum of lupinisoflavone A. This ion is created by the cleavage of the isoprene radical from the dihydrofuran ring of the molecular ion. The ion mentioned above was absent from the mass spectrum of parvisoflavone B.

Genistein and 2′-hydroxygenistein were two main components among all the isoflavonoids present in the lupine root extract. Genistein was present in all three fractions studied, but in fraction III it occurred in only a relatively small amount. 2′-Hydroxygenistein was identified in fractions II and III. In the latter, 2′-hydroxygenistein was the basic component. The remaining four isoflavonoids were identified only in fraction I, except for luteone, which was found also in fraction II.

CONCLUSIONS

GC–MS is useful for the study of isoflavonoid mixtures, where it is recommended that both methyl and trimethylsilyl derivatives are analysed. Application of methyl derivatives makes it possible to obtain better structural information from mass spectral data than with TMS derivatives. However, the latter derivatives give a better resolution of the TIC chromatograms. In studies of methoxylated isoflavonoids

present in plant materials, utilization of both types of derivative for structural determination would give complementary information.

ACKNOWLEDGEMENTS

Thanks are due to Dr. E. Kujawa and Miss M. Krzyżaniak (Institute of Medicinal Plants, Poznań, Poland) for help with the GC–MS analyses. This work was supported by grant RPBR 01.15.

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Erratum

J. Chromatogr., 504 (1990) 464–468

Page 466, Table I, last line of first column should read "$K_3[Co(Ox)_3]$" (where Ox = oxalate).

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journal of chromatography news section

NEW BOOKS

Chromatography and Modification of Nucleosides, Part A; Analytical Methods for Major and Modified Nucleosides — HPLC, GC, MS, NMR, UV and FT-IR (*Journal of Chromatography Library,* Vol. 45A), edited by C.W. Gehrke and K.C.T. Kuo, Elsevier, Amsterdam, 1990, 400 pp., price Dfl. 275.00, US$ 141.00, ISBN 0-444-88540-4.

Chromatography and Modification of Nucleosides, Part B: Biological Roles and Function of Modification (*Journal of Chromatography Library,* Vol. 45B), edited by C.W. Gehrke and K.C.T. Kuo, Elsevier, Amsterdam, 1990, 370 pp., price Dfl. 300.00, US$ 153.75, ISBN 0-444-88505-6.

Selectivity and Detectability Optimizations in HPLC, by S. Ahuja, Wiley, Chichester, 1989, *ca.* 742 pp., price *ca.* US$ 97.75, ISBN 0-471-62645-7.

Polymer Thermodynamics by Gas Chromatography (*Studies in Polymer Science,* Vol. 4), by R. Vilcu and M. Leca, Elsevier, Amsterdam, 1989, VIII+204 pp., price Dfl. 190.00, US$ 92.75, ISBN 0-444-98857-2.

Thin-Layer Chromatography, Reagents and Detection Methods, Vol. 1a, Physical and Chemical Detection Methods: Fundamentals, Reagents I, by H. York, W. Funk, W. Fisher and H. Wimmer, VCH Verlagsgesellschaft, Weinheim, 1989, 368 pp., price DM 108.00, ISBN 3-527-26527-9.

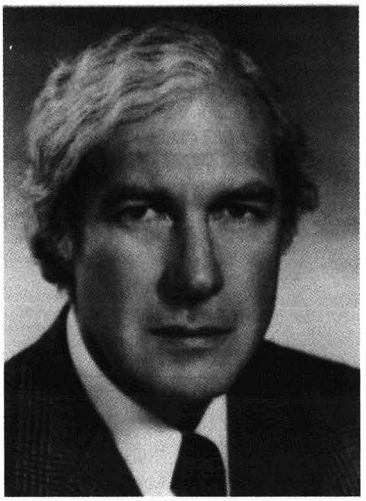
Advances in Electrophoresis, Vol. 3, edited by A. Chrambach, M. J. Dunn and B.J. Radola, VCH Verlagsgesellschaft, Weinheim, 1989, 456 pp., price DM 178.00, ISBN 3-527-27918-0.

Immobilized pH Gradients: Theory and Methodology (*Laboratory Techniques in Biochemistry and Molecular Biology,* Vol. 20), by P.G. Righetti, Elsevier, Amsterdam, 1989, 408 pp., price: hardback, Dfl. 298.00 US$ 152.75, ISBN 0-444-81301-2; paperback, Dfl. 91.00, US$ 46.75, ISBN 0-444-81315-2.

Dictionary of Chemistry and Chemical Technology, in Japanese, English and Chinese, by C. Huimin and B. Wenchu, Elsevier Science Publishers, Amsterdam, Oxford, New York, Tokyo, 1989, 1720 pp., US$ 210.50, Dfl. 400.00, ISBN 0-444-87371-6.

OBITUARY

MEMORIAL TO VICTOR PRETORIUS



My old friend and distinguished colleague Victor Pretorius died at the age of sixty one, on December 28th, 1989 at his home in Pretoria, South Africa after a short but serious illness.

It seems only yesterday that we had a long discussion over innumerable cups of coffee in London's Waterloo Station during his last visit to the U.K. in May 1989, when he told me of all his plans for his retirement from his last post as Head of the Research Institute for Chromatography at the University of Pretoria. It is sad to think that the splendid laboratory and workshop he was building in his garden with so much enterprise and effort, will not now be used for all his imaginative new ideas in molecular separation.

Victor had a fascinating career. As an energetic young man he enjoyed to the full the delights of the then hardly disturbed Southern African environment until he was awarded a Rhodes Scholarship in 1952. After much heart searching he eventually decided to come to England and continue his scientific training at Oxford. In his characteristic way he fully exploited all that this wonderful university offered in those days, both social and scientific. He soon acquired a magnificent veteran car in which he explored the beautiful English countryside in between obtaining an excellent Ph.D.

Returning to the University of Pretoria he soon became Professor of Physical Chemistry. He set up the Research Institute of Chromatography there and became its first Director. After a disastrous fire he had to start all over again. He was the only pioneer in the field that had the imagination and interest to collect specimens of original equipment from busy research men, and form a museum of chromatography. This also comprised a collection of video tapes made in the University Photographic Department by all the pioneers who visited him.

In spite of his isolation in the Transvaal 7000 miles away from the mainstream of hectic chromatographic development in the UK in the fifties, he started work with this fascinating new tool as early as 1953 with occasional visits to Archer Martin, Tony James, Jim Lovelock, Sandy Lipsky and Al Zlatkis amongst the small core of less than forty working in the UK and the USA. Victor always went back home vastly stimulated by such contacts and endeavoured to put into practice all the new ideas generated in his mind by actual participation in the experimental work of such men.

As early as 1955 he nearly stumbled on the electron-capture detector, in a few years to be so beautifully defined by Jim Lovelock. It was, however, his origination of the elegant simple flame ionization detector with which he is most identified. He has told the story of his thinking at the time that led him to the first primitive unit and demonstrated its amazing omnivorous sensitivity and linearity range.

Once again in 1956 he came very close to a demonstration of capillary columns but this fundamental advance eluded him and it was left to Marcel Golay in 1958 to describe this magnificent concept.

Over the two decades of the fifties and sixties I had said much about the potential of producing villi structure on the inside wall of capillary columns but had not at that time been able to think of a practical approach. I remember so well Victor proudly showing me, at one of Al Zlatkis' Symposia in the USA, his first electron micrographs of micro inorganic whiskers grown on the inside wall of a tiny glass capillary only a quarter of a millimetre in diameter. The photographs alone were a significant achievement but the whiskers themselves were a fabulous manifestation of my concept in reality. Even today I do not understand why Victor failed to mature this amazing creative effort into practical columns. I must confess, however, that it took me over ten years to conceive a way of coating such capillaries with porous alumina covered by a uniform layer of molecular grass.

In the seventies and eighties Victor became more occupied with organisation, administration and theoretical mathematical treatments of the chromatography system in various forms but he still found

time to actively contribute experimental work on preparative chromatography and electro-osmotic pumping.

In the best sense Victor was the South African gentleman of chromatography. He obviously enjoyed life and brought much to the lives of others with his controlled enthusiasm, humility and courage in dealing with difficult problems. He liked his wine, and particularly chain smoking: I recal seeing him puffing away on a cigarette, while playing a vigorous game of tennis on a covered court in Hinderlang.

We shall all miss him with a real sense of loss. I still expect any moment to hear the phone ring and hear his infectious voice announcing a visit to me in the near future.

His wife Yvonne and his two children Tania and Marcus have all our sympathy in their great grief at his passing.

D.H. DESTY

COURSE

ANALYTICAL CHEMISTRY SHORT COURSES, LOUGHBOROUGH, U.K.

The following short courses will be held this Summer at the Department of Chemistry, University of Technology, Loughborough, U.K.

— High-Performance Liquid Chromatography; July 9–13, 1990; fee £550 including residence and all meals, non-residents £430.

— Statistics for Analytical Chemistry; July 10–13, 1990; fee £440 including residence and all meals, non-residents £345.

— Radioisotope Techniques; July 2–6, 1990; fee £550 including residence and all meals, non-residents £430.

— Microcomputers in the Laboratory; September 17–21, 1990; fee £550 including residence and all meals, non residents £430.

For further details contact: Mrs. B.E. Cattell, Department of Chemistry, Loughborough University of Technology, Loughborough, Leics. LE11 3TU, U.K. Tel.: (0509) 222575.

ANNOUNCEMENTS OF MEETINGS

GORDON RESEARCH CONFERENCES, "FRONTIERS OF SCIENCE"

The Gordon Research Conferences for the summer of 1990 will be held in New Hampshire and Rhode Island. The object and exclusive purpose of the Gordon Research Conferences is to foster and promote education and science by organizing and operating meetings of research scientists with common interests in the fields of chemistry or related sciences for the purpose of discussions and the free 3exchange of ideas, thereby stimulating advanced thinking in research at universities, research foundations, and industrial laboratories. This type of meeting is a valuable means of disseminating information and ideas to an extent that could not be achieved through the usual channels of publication and presentation at scientific meetings. It is hoped that each conference will extend the Frontiers of Science by fostering a free and informal exchange of ideas among persons actively interested in the subjects under discussion.

Some meetings in related areas to this journal are: Analytical Chemistry (New Hampton, NH, U.S.A., August 6–10, 1990) and Separation and Purification (New London, NH, U.S.A., August 13–17, 1990).

The complete program for the 1990 Gordon Research Conferences in published in *Science* (*Washington, D.C.*), March 2, 1990. Reprints are available on request.

Requests for applications to the conferences, or for additional information, should be addressed to: Dr. Alexander M. Cruickshank, Gordon Research Conferences, Gordon Research Center, University of Rhode Island, Kingston, RI 02881-0801, U.S.A. Tel.: (401) 783-4011 or (401) 783-3372; Fax: (401) 783-7644; Bitnet: BCP101@URIACC.

MEETING ON CHEMOMETRICS IN PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, STOCKHOLM, SWEDEN, NOVEMBER 27–28, 1990

Chemometrics has as a discipline successfully grown and is now a very useful tool in several branches of chemistry. The amount of data that is produced by modern analytical instrumentation is often so overwhelming that some kind of statistical treatment, such as chemometrics, is necessary in order to get the relevant information in an understandable form. Numerous applications in analytical chemistry demonstrate that new possibilities are opened in that way.

In the broad field of pharmaceutical and biomedical analysis chemometrics has been applied for more than a decade. In this meeting scientists with experience from different areas within the chemical, pharmaceutical and biomedical fields will come together with the aim to review, update and discuss the role of chemometrics in pharmaceutical and biomedical analysis and related areas.

Poster abstracts may be submitted up to August 15, 1990. Information as to whether a poster has been accepted or not will be given before September 15, 1990.

The proceedings of this meeting will be published in a separate issue of the *Journal of Pharmaceutical and Biomedical Analysis* in August, 1991. All registered participants will receive a copy of the proceedings free of charge.

The official language of the meeting will be English.

The participation fees are: for members of the Section for Pharmaceutical and Biomedical Analysis: SEK 2800, for non-members: SEK 3200, if received before October 17, 1990, and SEK 3300 and SEK 3700 respectively after this date.

For further details, contact: "Meeting on Chemometrics in Pharmaceutical and Biomedical Analysis". The Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-111 81 Stockholm, Sweden. Telephone: +46 8 24 50 85, Telefax: +46 8 20 55 11.

2nd INTERNATIONAL SYMPOSIUM ON HYPHENATED TECHNIQUES IN CHROMATOGRAPHY, ANTWERP, BELGIUM, FEBRUARY 18–21, 1992

The 2nd International Symposium on Hyphenated Techniques will be held February 18–21, 1992 at the University of Antwerp (U.I.A.), Antwerp, Belgium. It will cover all fundamental aspects, instrumental developments and applications of the various hyphenated chromatographic techniques (*e.g.* GC–MS, GC–MS–MS, GC–FT-IR, GC–AED, HPLC–MS, HPLC–GC–MS, SFC–MS, SFE–HPLC, TGA–GC–MS, HPLC–UV, GPC–FT-IR, CZE–MS, CCC/MS, ...). It is indisputable that these techniques will continue to grow in importance and sophistication. The purpose of this symposium will therefore be to highlight all advances and recent developments in the field of hyphenations.

The scientific programme will comprise oral presentations in plenary and parallel sessions and poster presentations. The official symposium language will be English. No simultaneous translation facilities will be provided. Papers are invited in the above, and related, areas. Papers presented at the symposium will be reviewed for publication in a special volume of the *Journal of Chromatography*.

An exhibition of chromatographic equipment and accessories will ensure strong interaction between instrumentation and scientific communication.

The deadline for submitting *abstracts* is June 30, 1991. Abstracts should be submitted to KVCV, Royal Flemish Chemical Society, co/ Dr. R. Smits, BASF Antwerpen N.V., Scheldelaan, B-2040 Antwerp, Belgium. Abstracts will be assessed by a panel of referees. Authors will be informed by September, 1991 whether or not their abstract has been accepted and in which form the contributed paper is to be presented (oral or poster). For *manuscripts* the deadline will be the last day of the symposium (February 21, 1992). The papers will be published in *Journal of Chromatography* after the usual reviewing.

Programme, registration and poster information may be obtained from the Symposium Chairman: Dr. R. Smits, p.a. BASF Antwerpen N.V., Scheldelaan, B-2040 Antwerp, Belgium. Tel.: (323) 5682831; Telex 31047 basant b: Fax: (323) 5683355.

4th INTERNATIONAL CONFERENCE ON FUNDAMENTALS OF ADSORPTION, TOKYO, JAPAN, MAY 17–22, 1992

The Japan Society on Adsorption, Kawasaki, will be sponsoring the Fourth International Conference on Fundamentals of Adsorption, to be held May 17–22, 1992, at the Holiday Inn in Kyoto, Japan.

The tentative program outline comprises the following topics: characteristics of adsorbents, novel adsorbents, adsorption equilibrium and kinetics, industrial processes, biological adsorption systems, and molecular simulations. In addition, a poster session will be scheduled. A booklet with abstracts will be distributed at the conference, a proceedings volume will be published later. Submission deadlines are February 28, 1991, for abstracts; March 31, 1992, for complete manuscripts. Authors will be informed of acceptance by end of August, 1991.

For further details, contact Professor M. Suzuki, Conference Chairman, Institute of Industrial Science, University of Tokyo, 7-22-1 Roppongi, Minato-ku, Tokyo 106, Japan.

Announcements are included free of charge. Information on planned events should be sent well in advance (preferably 6 months or more) to: Journal of Chromatography, News Section, P.O. Box 330, 1000 AH Amsterdam, The Netherlands, Fax: (31) 20-5862845.

CALENDAR OF FORTHCOMING EVENTS

June 19–20, 1990
Innsbruck, Austria

*__Chromatography — Past, Present and Future; Honorary Symposium on the Occasion of the 90th Birthday of Professor Erika Cremer__
Contact: Dr. G.K. Bonn, Institute of Radiochemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria. Tel.: (512) 507-3213; Fax: (512) 580-519. (Further details published in Vol. 504, No. 2.)

June 25–29, 1990
Waterford, Ireland

*__Euro HPLC Training Course__
Contact: Mr. J. Griffith, Department of Chemical and Life Sciences, Waterford Regional College, Waterford, Ireland. Tel.: (05) 175934; Fax: (05) 178292. (Further details published in Vol. 504, No. 2.)

July 9–11, 1990
Wrexham, U.K.

__Ion-Ex 90, International Conference and Industrial Exhibition on Industrial, Analytical and Preparative Applications of Ion-Exchange Processes__
Contact: Ion-Ex 90, Conference Secretariat, Faculty of Research and Innovation, The North East Wales Institute, Connah's Quay, Deeside, Clwyd CH5 4BR, U.K. Tel.: (0244) 817531, ext. 276 or 234, telex: 61629 NEWI G, fax: (0244) 822002. (Further details published in Vol. 464, No. 2.)

July 23–25, 1990 Brno, Czechoslovakia	**International Symposium on Polymer Analysis and Characterization** Contact: Dr. Howard G. Barth, ISPAC Chairman, DuPont Company, Experimentalo Station E228/238, P.O. Box 80228, Wilmington, DE 19880-0228, U.S.A. Tel.: (302) 695-4354. (Further details published in Vol. 483.)
Aug. 7–11, 1990 Changchun, China	**1st Changchun International Symposium on Analytical Chemistry** Contact: Professor Qinhan Jin, Department of Chemistry, Jilin University, Changchun, Jilin 130021, China. (Further details published in Vol. 477, No. 2.)
Aug. 14–17, 1990 Budapest, Hungary	**Budapest Chromatography Conference** Contact: Intercongress IPV, Dózsa Gy. út 84/a, Budapest, H-1068 Hungary. (Further details published in Vol. 477, No. 2.)
Aug. 15–17, 1990 Amsterdam, The Netherlands	* **Amsterdam Summercourse on Capillary Zone Electrophoresis** Contact: The Amsterdam Summerschool, University of Amsterdam, Laboratory for Analytical Chemistry, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (31 20) 525-6515/6539/-6546/6567; Fax: (31 20) 525-5802 CCITT3. (Further details published in Vol. 504, No. 2.)
Aug. 19–22, 1990 Aronsborg, Sweden	**2nd International Symposium on Microcolumn Separation Methods** Contact: The Swedish Chemical Society, The Analytical Division, Wallingatan 26B, S-111 24 Stockholm, Sweden. Tel.: 08-115260. (Further details published in Vol. 502, No. 2.)
Aug. 19–23, 1990 Ghent, Belgium	**8th International Symposium on Mass Spectrometry in Life Sciences** Contact: Professor Dr. A. De Leenheer, Laboratoria voor Medische Biochenie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 218951, ext. 324; Fax: (091) 217902. (Further details published in Vol. 502, No. 2.)
Aug. 20–22, 1990 Stockholm, Sweden	**2nd International Symposium on Microcolumn Separation Methods** Contact: The Swedish Chemical Society, The Analytical Division, Wallingatan 26B, S-111 24 Stockholm, Sweden. (Further details published in Vol. 477, No. 2.)
Aug. 22–24, 1990 York, U.K.	* **International Symposium on Capillary Electrophoresis** Contact: Dr. Carys Calvert, Department of Chemistry, University of York, Heslington, York, Y01 5DD, U.K. Tel.: 0904 432576 or 432511; Telex: 57933 YORKUL; Fax: 0904 433433. E-mail: CGGC1@VAXA.YORK.AC.UK. (Further details published in Vol. 504, No. 2.)
Aug. 26–31, 1990 Vienna, Austria	**Euroanalysis VII, 7th European Conference on Analytical Chemistry** Contact: Professor Dr. M. Grasserbauer, c/o Interconvention, Austria Center Vienna, A-1450 Vienna, Austria. Tel.: (43) 222-2369/647; telex: 111803 icos a, Fax: (43) 222-2369/648. (Further details published in Vol. 445, No. 1.)

Aug. 26–31, 1990 Prague, Czechoslovakia	**10th International Congress on Chemical Engineering, Chemical Equipment Design and Automation** Contact: Congress CHISA '90, P.O. Box 857, 111 21 Prague 1, Czechoslovakia. Telex: 121114 chp c.
Sept. 3–7, 1990 Balatonfüred, Hungary	**6th International Symposium on Ion Exchange** Contact: Professor J. Inczédy, Department of Analytical Chemistry, University of Veszprém, P.O. Box 158, H-8201 Veszprém, Hungary. Tel.: (3680) 22022; fax: (3680) 26016; telex: 32297. (Further details published in Vol. 477, No. 2.)
Sept. 4–7, 1990 Prague, Czechoslovakia	**Symposium on Bioanalytical Methods** Contact: Symposium on Bioanalytical Methods, House of Technology, Ing. Jirina Kostalova, Gorkeho nam 23, 111 28 Prague 1, Czechoslovakia. Tel.: (422) 236485; Telex: 121024 roh. (Further details published in Vol 483.)
Sept. 10–12, 1990 Hatfield, U.K.	*__Gas Chromatography Training Course for Technicians and Young Gradients__ Contact: Short Course Administrator, Division of Chemical Sciences, Hatfield Polytechnic, College Lane, Hatfield, Herts AL10 9AB, U.K.
Sept. 10–13, 1990 New Orleans, LA, U.S.A.	*__104th AOAC Annual Meeting and Exposition__ Contact: Marketing Department AOAC, 2200 Wilson Boulevard, Suite 400-CS, Arlington, VA 22201-9907, U.S.A.
Sept. 10–13, 1990 Cambridge, U.K.,	**6th International Symposium on Bioluminescence and Chemiluminescence** Contact: Professor L.J. Kricka, Department of Pathology and Laboratory Medicine, 3400 Spruce Street, 784 Founders Pavilion, Philadelphia, PA 19104-4283, U.S.A.
Sept. 11–14, 1990 Kobe, Japan	**12th International Symposium on Capillary Chromatography** Contact: Dr. K. Jinno, School of Materials Science, Toyohashi University of Technology, Toyohashi 440, Japan. (Further details published in Vol 483.)
Sept. 18–21, 1990 Verona, Italy	**1st International Symposium on Applications of HPLC in Enzyme Chemistry** Contact: Dr. F. Tagliaro, Istituto di Medicina Legale, Università di Verona, Policlinico di Borgo Roma, 37134 Verona, Italy. Tel.: +39 45 504073; Fax: +39 45 58212. (Further details published in Vol. 479, No. 2.)
Sept. 19–21, 1990 Manchester, U.K.	*__6th International Symposium on Environmental Radiochemical Chemistry__ Contact: Mrs F.J. Johnson, Concilia, P.O. Box 18, Ikley LS29 6RA, U.K. (Further details published in Vol. 504, No. 2.)

Sept. 23–28, 1990 Amsterdam, The Netherlands	**18th International Symposium on Chromatography** Contact: 18th International Symposium on Chromatography, RAI Organisatie Bureau Amsterdam bv, Europaplein 12, 1078 GZ Amsterdam, The Netherlands. Tel.: (31-20) 549 1212; telex: 13499 raico nl; Fax: (31-20) 464469. (Further details published in Vol. 464, No. 2 and Vol. 483.)
Oct. 2–4, 1990 High Tatras, Czechoslovakia	**7th International Symposium on Capillary Electrophoresis and Isotachophoresis** Contact: 7th International Symposium on CE and ITP, Dr. D. Kaniansky, Institute of Chemistry, Comenius University, Mlynská, Dolina CH-2, CS-84215 Bratislava, Czechoslovakia. Tel.: (70) 320003. (Further details published in Vol. 483.)
Oct. 7–12, 1990 Cleveland, OH, U.S.A.	* **FACSS, 7th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: C.J. Belle, Lucas Aerospace, PEC, 4259 W. 192 Street, Fairview Park, OH 44126, U.S.A.; or C. Paxton, BP Research, 4440 Warrensville Ctr. Rd., Warrensville Hts., OH 44128, U.S.A.; or J. Grasselli, 151 Greentree Rd., Chagrin Falls, OH 44022, U.S.A.
Oct. 8–12, 1990 Montpellier, France	* **Euro HPLC Training Course** Contact: Professor H. Fabre, Laboratoire de Chimie Analytique, Faculté de Pharmacie 34060 Montpellier Cedex, France. Tel.: (67) 635432; Fax: (67) 611622. (Further details published in Vol. 504, No. 2.)
Oct. 19–23, 1990 Adelaide, Australia	**27th Meeting of the International Association of Forensic Toxicologists** Contact: V.J. McLinden, Chemistry Center (WA), 125 Hay Street, Perth, Western Australia 6000, Australia. (Further details published in Vol. 467, No. 2.)
Oct. 22–24, 1990 San Francisco, CA, U.S.A.	**ANABIOTEC '90, 3rd International Symposium on Analytical Methods in Biotechnology** Contact: Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 448, No. 3.)
Oct. 29–31, 1990 Wiesbaden, F.R.G.	**10th International Symposium on HPLC of Proteins, Peptides and Polynucleotides** Contact: Secretariat 10th ISPPP, P.O. Box 28, S-751 03 Uppsala, Sweden. (Further details published in Vol. 502, No. 2.)
Oct. 31–Nov. 2, 1990 Montreux, Switzerland	**7th Symposium on Liquid Chromatography–Mass Spectroscopy (LC–MS, MS–MS, SFC–MS)** Contact: M. Frei-Häusler, Strengigässli 20, CH-4123 Allschwil, Switzerland. (Further details published in Vol. 475.)

Nov. 27–28, 1990 Stockholm, Sweden	* **Chemometrics in Pharmaceutical and Biomedical Analysis** Contact: Swedish Academy of Pharmaceutical Sciences, Chemometrics in Pharmaceutical and Biomedical Analysis, P.O. Box 1136, S-111 81 Stockholm, Sweden.
Dec. 3–5, 1990 Baltimore, MD, U.S.A.	* **Seminar on Analytical Molecular Biology** Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772; Fax: (301) 898-5596.
Jan. 15–17, 1991 Park City, UT, U.S.A.	* **1991 Symposium on Supercritical Fluid Chromatography** Contact: Dr. M.L. Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602, U.S.A. Tel.: (801) 378-2135; Fax: (801) 378–5474. (Further details published in Vol. 504, No. 2.)
Jan. 22–24, 1991 Paris, France	* **Congrès de Chromatographies en Phases Liquide et Supercritique** Contact: Société Française de Chimie, Division "Chimie Analytique", Congrès CPL/CPS, 250 rue Saint-Jacques, 75005 Paris, France. Tel.: (1) 43252078; Fax: (1) 40468380.
Feb. 3–6, 1991 San Diego, CA U.S.A.	* **HPCE '91, 3rd International Symposium on High Performance Capillary Electrophoresis** Contact: HPCE '91, Ms. Shirley E. Schlessinger, Symposium Manager, 400 East Randolph Drive, Suite 1015, Chicago, IL 60601, U.S.A. (Further details published in Vol. 504, No. 2.)
Feb. 10–15, 1991 Melbourne, Australia	**POLYMER '91, Polymer Materials: Preparation, Characterization and Properties** Contact: POLYMER '91 Secretary, P.O. Box 224, Belmont, Vic. 3216, Australia.
March 4–7, 1991 Les Diablerets, Switzerland	**4th Hans Wolfgang Nürnberg Memorial Workshop on Toxic Metal Compounds (Interrelation Between Chemistry and Biology)** Contact: Dr. Ernest Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland.
March 11–13, 1991 Lausanne, Switzerland	**2nd Soil Residue Analysis Workshop** Contact: Professor J. Tarradellas, IGE-EPFL, 1015 Lausanne, Switzerland.
May 27–31, 1991 Rome, Italy	**2nd International Symposium on Chiral Discrimination** Contact: Professor D. Misiti or Professor F. Gasparrini, Laboratori di Chimica Organica, Facoltà di Farmicia, Università "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy. Tel.: (06) 4452900; fax: (06) 49912780. (Further details published in Vol. 477, No. 2.)

May, 27–31, 1991 Ghent, Belgium	**IVth International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. Willy R.G. Baeyens, Symposium chairman, State University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 483.)
June 3–7, 1991 Basel, Switzerland	**HPLC '91, 15th International Symposium on Column Liquid Chromatography** Contact: Secretariat HPLC '91, Convention Center Basel, Congress Department, P.O. Box, CH-4021 Basel, Switzerland. (Further details published in Vol. 477, No. 2.)
June 9–14, 1991 Bergen, Norway	**XXVII Colloquium Spectroscopicum Internationale** Contact: Secretariat XXVII CSI, HSD Congress-Conference, P.O. Box 1721 Nordnes, N-5024 Bergen, Norway. Tel.: (475) 318414; Telex: 42607 hsd n, Fax: (475) 324555.
July 16–18, 1991 London, U.K.	***Two-Dimensional Polyacrylamide Gel Electrophoresis** Contact: Conference Secretariat 2-D PAGE 1991, Department of Cardiothoracic Surgery, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY, U.K.
Aug. 17–22, 1991 Budapest, Hungary	***33rd IUPAC Congress** Contact: 33rd IUPAC Congress, E. Pungor, c/o Hungarian Academy of Sciences, Gellèrt ter 4, H-1111 Budapest, Hungary.
Aug. 21–24, 1991 Kumamoto, Japan	**5th International Conference on Flow Analysis** Contact: Professor Ishibashi, Department of Applied Analytical Chemistry, Faculty of Engineering 36, Kyushu University, Hokazaki, Higashiku, Fukuoka 812, Japan. (Further details published in Vol. 475.)
Aug. 25–31, 1991 Makuhari, Japan	**ICAS '91, IUPAC International Congress on Analytical Sciences** Contact: ICAS '91 Secretariat, The Japan Society for Analytical Chemistry, 1-26-2 Nishigotande, Shinagawa, Tokyo 141, Japan. Tel.: (813) 490-3351; fax: (813) 490-3572. (Further details published in Vol. 483.)
Sept. 1–6, 1991 Lubeck-Travemunde, F.R.G.	**8th International Conference on Fourier Transform Spectroscopy** Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt 90, F.R.G. Tel.: 17-366/360; Fax: (79) 17475; Telex: 4170497 gdch d.
Sept. 2–6, 1991 Warsaw, Poland	**8th Danube Symposium on Chromatography** Contact: 8th Danube Symposium on Chromatography, Janusz Lipkowski, Institute of Physical Chemistry of the Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland. (Further details published in Vol. 502, No. 2.)

Sept. 4–6, 1991 Bilthoven, The Netherlands	**3rd Workshop on Chemistry and Fate of Modern Pesticides** Contact: Pesticides Workshop Office, Dr. P. van Zoonen, RIVM, P.O. Box 1, 3720 Bilthoven, The Netherlands. (Further details published in Vol. 472, No. 2.)
Sept. 24–28, 1991 Yokohama, Japan	*__9th International Symposium on Affinity Chromatography and Biological Recognition__ Contact: Professor Ken-ichi Kasai, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukui, Kanagawa 199-01, Japan.
Oct. 14–18, 1991 Budapest, Hungary	*__ECASIA 91, 4th European Conference on Applications of Surface and Interface Analysis__ Contact: ECASIA 91, MTA ATOMKI, Pf. 51, H-4001 Debrecen, Hungary. Tel.: (36) 52-16181; Telex: 72210 (atom h); Fax: (36) 52-16181.
Feb. 18–21, 1992 Antwerp, Belgium	*__2nd International Symposium on Hyphenated Techniques in Chromatography__ Contact: Dr. R. Smits, p.a. BASF Antwerpen N.V., Scheldelaan B-2040 Antwerp, Belgium. Tel.: (32) 5682831; Fax: (323) 5683355; Telex: 31047 basant b.
May 17–22, 1992 Kyoto, Japan	*__4th International Conference on Fundamentals of Adsorption__ Contact: Prof. M. Suzuki, Conference Chairman, Institute of Industrial Science, University of Tokyo, 7-22-1 Roppongi, Minatoku, Tokyo 106, Japan.

* Indicates new or amended entry

PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	
Journal of Chromatography	498/1 498/2 499	500 502/1	502/2 503/1 503/2 504/1	504/2 505/1	505/2 506 507 508/1	508/2 509/1 509/2 510	The publication schedule for further issues will be published later
Cumulative Indexes, Vols. 451–500		501					
Bibliography Section		524/1		524/2		524/3	
Biomedical Applications	525/1	525/2	526/1	526/2 527/1	527/2	528/1 528/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 478, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

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R: –C(=O)–N(H)–C*(H)(CH3)–

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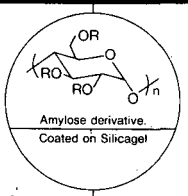
for β-Lactam antibiotics



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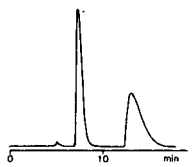
●CHIRALPAK AD

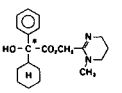
R: –C(=O)–N(H)–

4-Acetoxy-2-azetidine

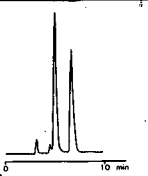
Eluent : Hexane/Ethanol = 8/2
Flow rate : 1.0ml/min
Temperature: r.t.
Detection : UV254nm



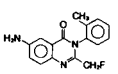
Oxyphencyclimine



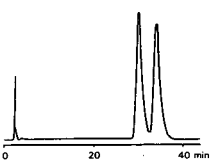
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature: r.t.
Detection : UV254nm



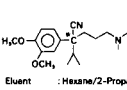
Afloqualone



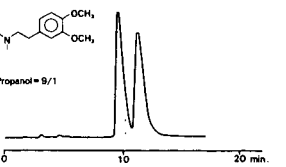
Eluent : Hexane/EtOH = 95/5
Flow rate : 1.3ml/min
Temperature: 50°C
Detection : UV254nm



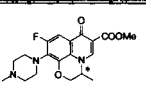
Verapamil



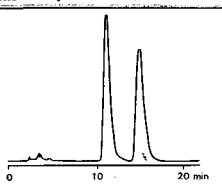
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature: r.t.
Detection : UV254nm



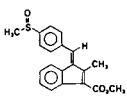
Ofloxacine methyl ester



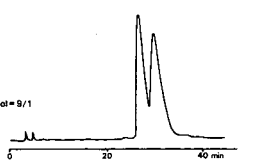
Eluent : Hexane/EtOH = 8/2
Flow rate : 1.2ml/min
Temperature: 40°C
Detection : UV254nm



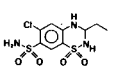
Sulindac methyl ester



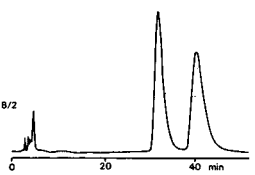
Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature: r.t.
Detection : UV254nm



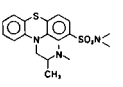
Ethiazide



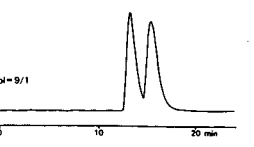
Eluent : Hexane/Ethanol = 8/2
Flow rate : 1.0ml/min
Temperature: 40°C
Detection : UV254nm



Dimethotiazine



Eluent : Hexane/2-Propanol = 9/1
Flow rate : 1.0ml/min
Temperature: r.t.
Detection : UV254nm



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