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Urbino, May 27–31, 1985

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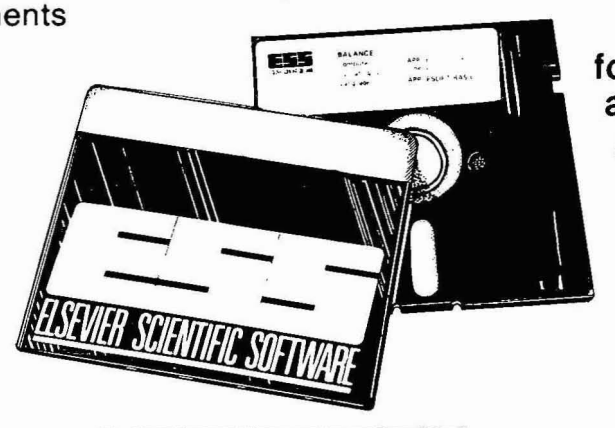


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## A. J. P. MARTIN HONORARY SYMPOSIUM, URBINO, MAY 27–31, 1985

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# FOREWORD

This volume contains most of the research papers presented as poster communications at the *A. J. P. Martin Honorary Symposium*, held in Urbino, Italy from May 27th till 30th, 1985. This symposium was organized to honour Archer John Porter Martin on the occasion of his 75th birthday. As is well known, the chromatographic community is highly indebted to Dr. Martin, whose discoveries made possible the enormous progress made in the last forty years in separation science. In fact, his research, carried out together with R. L. M. Synge, led to the invention of partition chromatography, with the first results published in 1941. The linearity of the partition isotherm, in contrast to the adsorption isotherm, the availability of a large variety of liquid phases and the possibility of achieving a large number of theoretical plates increased the analytical application of these chromatographic techniques so that they became the most widely used in all fields of chemistry.

The invention of paper chromatography, together with R. Consden and A. H. Gordon in 1944, made a great contribution to the development of spot analysis in inorganic and organic chemistry and in biochemistry, providing the scientific community with an effective and inexpensive analytical tool.

Martin and Synge were awarded the Nobel Prize in Chemistry in 1952 for the invention of partition chromatography. It is interesting to note that in the same year Dr. Martin, together with A. T. James, published the very first results on gas–liquid chromatography, opening up a new field of analytical applications of chromatographic techniques, which would have justified giving its inventor the Nobel Prize again.

I was gratified by the extremely positive reaction of the scientists involved in chromatographic research to the idea of organizing this symposium, and the presence at the meeting of most of the researchers who have made significant, and in many instances decisive contributions to the development of chromatographic science is the best demonstration of the esteem in which Dr. Martin is held all over the world.

The symposium was implemented with twenty-two plenary lectures that have been published in a book, entitled *The Science of Chromatography*, by Elsevier, as Vol. 32 in the *Journal of Chromatography Library*. Together with the plenary review lectures, about 75 research papers were presented in the form of posters by scientists from 20 countries. In addition to the scientific portion, a significant part of the symposium was the presentation of the "Martin Awards" by the Chromatographic Society. Professor Arnaldo Liberti from the University of Rome, and Dr. C. E. Roland Jones, Chairman of the Chromatographic Society (formerly the Chromatography Discussion Group), received the awards. Professor Liberti has made very important contributions to the development of gas chromatography since its earliest days. His activity mainly consisted of the study of new detectors, separation of natural organic mixtures, trace analysis of environmental interest and isotope separations by gas chromatography. A significant part of Dr. Liberti's activity was the development of new types of GC columns and stationary phases. Dr. C. E. R. Jones has made important contributions in the analytical applications of pyrolysis–gas chromatography. Lately, he has given a great impetus to the Chromatographic Society, making it a worldwide association.

The award of the Analytical Division of the Italian Chemical Society was

bestowed on Mr. Franco Poy of the DANI company for his contribution to the development of chromatographic instrumentation in the last thirty years.

At the conclusion of the symposium Dr. Martin was awarded the *Laurea Honoris Causa* in Biological Sciences by the University of Urbino.

It is worth noting that the symposium was held at a very old and glorious University, a significant symbol of the Italian Renaissance, to honour a man who, for his personality and activity, well deserves to be considered a Renaissance man.

I wish to thank all the scientists who made the symposium successful, covering in their outstanding lectures almost the entire field of chromatography. Special and warm thanks are given to Dr. M. Lederer, Dr. E. Heftmann, and to Elsevier Science Publishers who agreed to publish the papers presented at the symposium.

*Urbino (Italy)*
*September 4, 1985*

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CHROMSYMP. 695

# RESOLUTION OF *cis*- AND *trans*-DIMETHYLCYCLOHEXANES BY PARTITION GAS CHROMATOGRAPHY THROUGH CYCLODEXTRIN COMPLEXES

TOMASZ KOŚCIELSKI and DANUTA SYBILSKA*
*Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw (Poland)*

## SUMMARY

α- and β-Cyclodextrins, dissolved in formamide or ethylene glycol, were applied as stationary phases in gas chromatography for the resolution of isomeric *cis*- and *trans*-1,2-, 1,3-, and 1,4-dimethylcyclohexanes. It was found that stereoselectivity arising from inclusion of dimethylcyclohexanes in cyclodextrin cavities is more distinct for β-cyclodextrin. Of the two matrix solvents, which influence not only the capacity factors but also the separation factors, formamide is the more efficient medium for stereoselective cyclodextrin inclusion processes. An evaluation of stability constants of (β-cyclodextrin · dimethylcyclohexane) complexes was attempted. Almost complete separation of a mixture containing the six isomers investigated was achieved by using concentrated β-cyclodextrin (1.48 mol. %) in formamide solution.

## INTRODUCTION

The selective properties of cyclodextrins (CD), based on their ability to form inclusion compounds with various molecules and ions[1], have been widely used in many separation techniques, including liquid chromatography (LC)[2,3].

Our recent studies have shown that α- or β-CD can be successfully applied for analytical purposes, not only in LC but also in gas chromatography (GC), for imparting to a liquid stationary phase the stereoselectivity needed for the efficient separation of *o*-, *m*- and *p*-xylenes[4] and diethylbenzenes[5], as well as for the resolution of α- and β-pinenes into enantiomers[6].

These results encouraged us to undertake further systematic studies on the application of the same procedure for the separations of other kinds of isomer. Our aim is to establish some general principles that relate the shape and size of molecules to their chromatographic behaviour in GC systems containing α- or β-CD in the stationary phase solution.

In selecting compounds for our studies we have also taken into account the actual analytical needs and difficulties in their separation. This paper reports the results of the resolution of six isomeric *cis*- and *trans*-1,2-, 1,3-, and 1,4-dimethylcyclohexanes (DMCH), abbreviated respectively as: c-1,2; t-1,2; c-1,3; t-1,3; c-1,4; t-1,4.

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Analysis of DMCH mixtures is of crucial value in monitoring the course of xylene hydrogenation. The partial separation of DMCH by GC methods has already been achieved with a molecular sieve 13X porous-layer open-tubular (PLOT) column[7] or columns filled with graphitized thermal carbon black[8]. Numerous retention data relative to DMCH behaviour on various stationary phases have also been reported[9–13]. All these investigations concluded that the complete separation of DMCH mixtures poses difficulties.

## EXPERIMENTAL

### *Reagents*

α- and β-CD were supplied by Chinoin (Budapest, Hungary). Celite, 80–100 mesh, for GC was from BDH (Poole, U.K.). DMCH puriss. samples were obtained from Fluka (Buchs, Switzerland). All other materials were of analytical or reagent grade and were used without further purification.

### *Apparatus and procedures*

Chromatographic studies were performed using a Hewlett-Packard 7620 A gas chromatograph, equipped with dual flame ionization detectors. Glass columns (2 m × 4 mm I.D. and 5 m × 4 mm I.D.) were used. The compounds were injected separately (0.2 μl) or as mixtures, with Hamilton microsyringes.

The stationary phases were prepared as follows. Celite, 80–100 mesh, was coated with solutions of α- or β-CD in formamide or ethylene glycol, using 4.54 g of the solvent and 20.0 g of Celite. The amounts of α- or β-CD varied from 0.0 to 1.5 g in different coated supports. The detailed preparation procedure was described earlier[4]. The mass of the coated support in the column was determined by weighing the columns before and after packing: the mean value for all the columns of 2-m length was 10.2 ± 0.5 g, and for the 5-m columns it was 24.5 g.

In all experiments two columns were used: the first with formamide or ethylene glycol solutions of α- or β-CD and the second (reference), containing only formamide or ethylene glycol, respectively. This two-column system enabled us to perform comparative measurements and excluded many sources of error.

The studies were carried out in the temperature range 45–70°C. Stability constants of the β-CD complexes were evaluated on the assumption that only complexes of 1:1 stoichiometry are formed, using the following equation

$$t'_{\beta\text{-CD}} = t'_0 (1 + K[\beta\text{-CD}]) \quad (1)$$

where $t'_{\beta\text{-CD}}$ and $t'_0$ are, respectively, the adjusted retention times of a solute on the column containing β-CD in a given solvent and on the reference column containing pure matrix solvent; $K$ is the stability constant of a 1:1 β-CD complex with DMCH.

Special attention was paid to maintaining constant values of the inlet pressure (2.75 ± 0.05 atm) and the helium flow-rate (50 ± 0.5 ml/min). Under these conditions it was possible to evaluate and compare stability constants of CD complexes, although there was no other means of determining their exact values[14,15].

## RESULTS AND DISCUSSION

Fig. 1 shows four chromatograms of a mixture of *cis*- and *trans*-1,2-, 1,3-, and 1,4-DMCH, which demonstrate how their separation is influenced by β-CD and α-CD in formamide. The corresponding chromatograms obtained from the column containining β- and α-CD in ethylene glycol are presented in Fig. 2. At 50°C, for each DMCH isomer, $t'_{CD}$ is greater than $t'_0$. This means, according to eqn. 1, that under these experimental conditions both α- and β-CD form inclusion complexes.

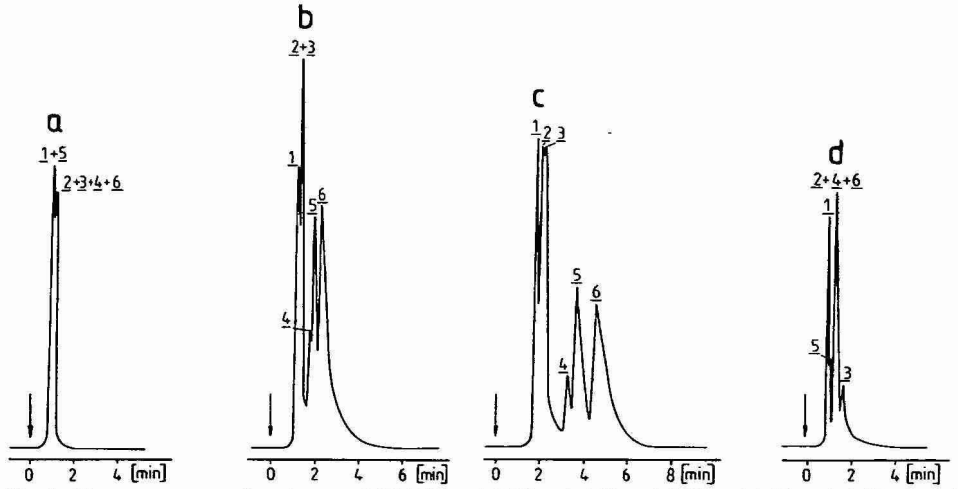



Fig. 1. Chromatograms of a mixture of DMCH: c-1,2 (6), t-1,2 (3), c-1,3 (1), t-1,3 (4), c-1,4 (2), t-1,4 (5), performed at 50°C on a column (2 m × 4 mm I.D.) packed with: (a) 0.0, (b) 0.10 and (c) 0.30 mol. % of β-CD and (d) 0.31 mol. % of α-CD in formamide, coated on Celite (4.54 g of formamide per 20 g Celite).

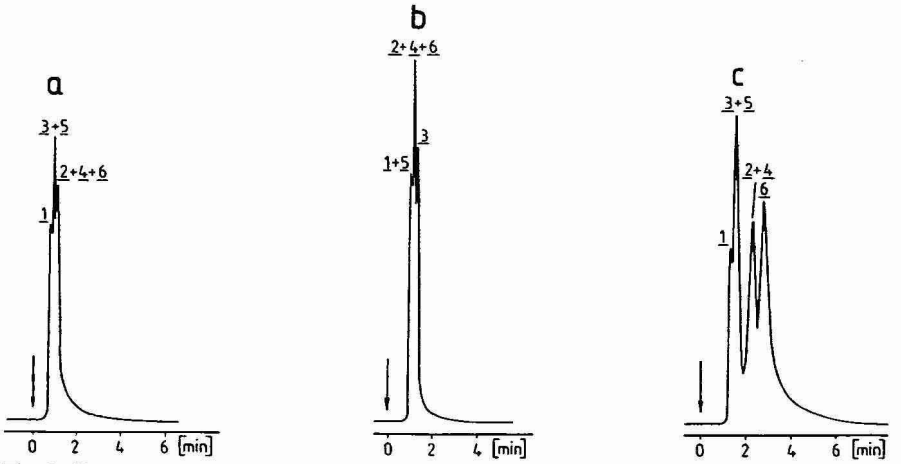



Fig. 2. Chromatograms of a mixture of DMCH: c-1,2 (6), t-1,2 (3), c-1,3 (1), t-1,3 (4), c-1,4 (2), t-1,4 (5), performed at 50°C on a column (2 m × 4 mm I.D.) packed with: (a) 0.0 and (b) 0.42 mol. % of α-CD and (c) 0.36 mol. % of β-CD in ethylene glycol solution, coated on Celite (4.54 g of ethylene glycol per 20 g Celite).

However, the selectivities that they impart to the liquid stationary phase towards DMCH are different. Thus, different elution orders are observed for α- and β-CD stationary phase solutions (*cf.* Figs. 1 and 2).

Separation factors (α) for neighbouring peaks, which illustrate the influence of β-CD complexation, are collected in Table I. For almost inseparable pair of isomers, *i.e.* t-1,2 and c-1,4 in the chromatograms shown in Figs. 1 and 2, α attains a value suitable for analysis (1.11) only in a solution of β-CD at very high concentration (1.48 mol. %).

TABLE I

SEPARATION FACTORS FOR NEIGHBOURING PEAKS OF DMCH ON COLUMNS WITH DIFFERENT CONCENTRATIONS OF β-CD IN FORMAMIDE

| *β-CD concentration in formamide solution (mol. %)* | *Temp. (°C)* | $\alpha_{c\text{-}1,4/c\text{-}1,3}$ | $\alpha_{t\text{-}1,2/c\text{-}1,4}$ | $\alpha_{t\text{-}1,3/t\text{-}1,2}$ | $\alpha_{t\text{-}1,4/t\text{-}1,3}$ | $\alpha_{c\text{-}1,2/t\text{-}1,4}$ |
|---|---|---|---|---|---|---|
| 0.0 | 50 | $\alpha_{c\text{-}1,2;t\text{-}1,2;t\text{-}1,3;c\text{-}1,4/c\text{-}1,3;t\text{-}1,4} = 1.24$ | | | | |
| 0.0 | 60 | $\alpha_{c\text{-}1,2;t\text{-}1,2;t\text{-}1,3;c\text{-}1,4/c\text{-}1,3;t\text{-}1,4} = 1.24$ | | | | |
| 0.10 | 50 | 1.16 | 1.00 | 1.74 | 1.11 | 1.25 |
| 0.10 | 60 | 1.17 | 1.00 | 1.94 | 1.00 | 1.28 |
| 0.30 | 50 | 1.21 | 1.03 | 1.92 | 1.13 | 1.30 |
| 0.30 | 60 | 1.27 | 1.00 | 1.83 | 1.13 | 1.29 |
| 1.48 | 45 | 1.21 | 1.11 | 1.78 | 1.19 | 1.31 |

The selectivities arising from α-CD complexation are smaller, *e.g.* α values for neighbouring peaks, determined with a column containing 0.31 mol. % α-CD in formamide, are as follows: $\alpha_{t\text{-}1,4/c\text{-}1,3} = 1.3$; $\alpha_{t\text{-}1,3;c\text{-}1,4;c\text{-}1,2/t\text{-}1,4} = 1.4$; $\alpha_{t\text{-}1,3/t\text{-}1,4} \approx \alpha_{c\text{-}1,4/t\text{-}1,4} \approx \alpha_{c\text{-}1,2/t\text{-}1,4} \approx 1.0$; $\alpha_{t\text{-}1,2/t\text{-}1,3;c\text{-}1,4;c\text{-}1,2} = 1.6$.

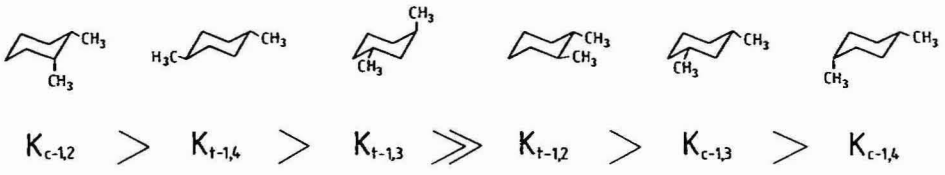
In general, β-CD complexes of DMCH are more stable than the corresponding complexes of α-CD. It should also be pointed out that β-CD inclusion processes are more stereoselective towards DMCH, *i.e.* more sensitive to changes in their structure. On account of this, the initial two peaks, corresponding to the DMCH resolution on a pure formamide-containing column (Fig. 1a) became five peaks (and a less marked sixth) on 0.30 mol. % of β-CD in formamide (Fig. 1c) and only three (and a less marked fourth) on 0.31 mol. % of α-CD (Fig. 1d).

Comparison of Figs. 1 and 2 leads to the conclusion that the matrix solvent influences not only capacity factors but also separation factors. The more polar solvent, formamide, seems to make a more efficient medium for CD inclusion processes than ethylene glycol; in formamide the effects of inclusion are more noticeable.

CD complexation equilibria are strongly affected by changes of temperature, as exemplified in Fig. 3 by the resolution of a DMCH mixture at 50, 60 and 70°C; at the highest temperature the inclusion effects are almost indistinguishable (Fig. 3c).

We have attempted to predict the stability constants of (β-CD · DMCH) complexes from the data obtained for more dilute solutions (up to 0.30 mol. %) of β-CD in formamide, where a 1:1 stoichiometry of complexes seems to be valid.

The predicted sequence is presented in Scheme 1.




Scheme 1.

The predicted stability constants are divided into two groups of distinctly different (about four times) values. Within each group the stability constants are similar. The division does *not* correspond to the *cis/trans* isomerization of DMCH molecules.

The most difficult resolution was between c-1,4 and t-1,2. On the columns with

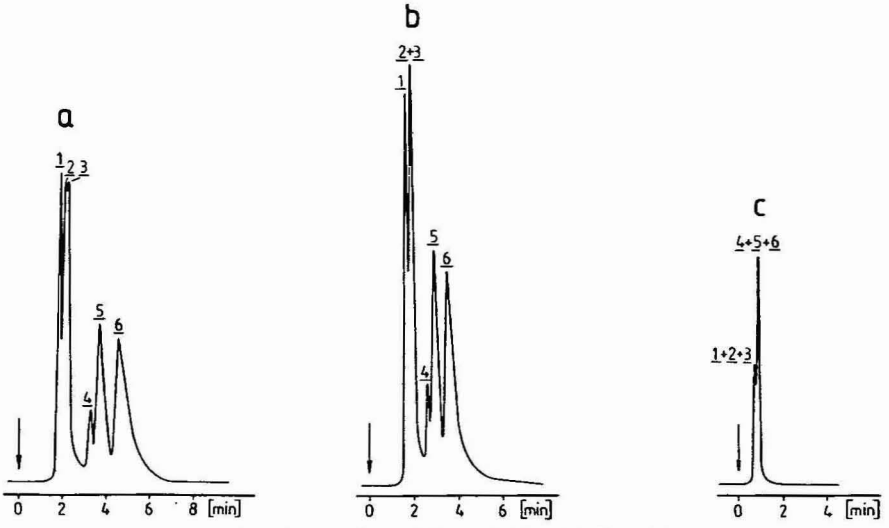



Fig. 3. Chromatograms of a mixture of DMCH: c-1,2 (6), t-1,2 (3), c-1,3 (1), t-1,3 (4), c-1,4 (2), t-1,4 (5) on a column (2 m × 4 mm I.D.) packed with 0.30 mol. % of β-CD in formamide, coated on Celite, at the following temperatures: (a) 50°C; (b) 60°C; (c) 70°C.

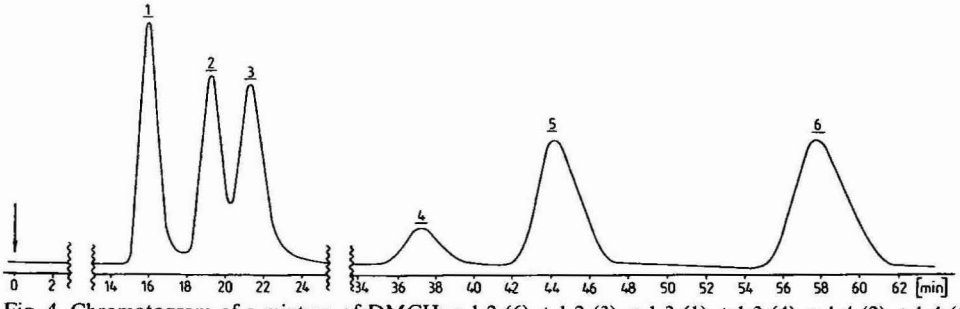



Fig. 4. Chromatogram of a mixture of DMCH: c-1,2 (6), t-1,2 (3), c-1,3 (1), t-1,3 (4), c-1,4 (2), t-1,4 (5) obtained on a column (5 m × 4 mm I.D.), packed with 1.48 mol. % of β-CD in formamide, coated on Celite. Temperature, 45°C.

low percentages of β-CD in formamide their peaks almost completely overlapped. Only on the columns with high percentages of β-CD (1.48 mol. %) was an adequate selectivity factor attained and complete separation achieved (Fig. 4).

The separation of c-1,4, c-1,2 and t-1,3 mixtures, which was earlier found to be inadequate[8], is easily accomplished on columns containing β-CD in formamide.

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REFERENCES

1 J. Szejtli, *Cyclodextrins and Their Inclusion Complexes*, Akadémiai Kiadó, Budapest, 1982.
2 W. L. Hinze, *Sep. Purif. Methods*, 10 (1981) 159.
3 E. Smolková-Keulemansová, *J. Chromatogr.*, 251 (1982) 17.
4 D. Sybilska and T. Kościelski, *J. Chromatogr.*, 261 (1983) 357.
5 T. Kościelski, D. Sybilska, L. Feltl and E. Smolková-Keulemansová, *J. Chromatogr.*, 286 (1984) 23.
6 T. Kościelski, D. Sybilska and J. Jurczak, *J. Chromatogr.*, 280 (1983) 131.
7 N. L. Soulages and A. M. Brieva, *J. Chromatogr.*, 101 (1974) 365.
8 W. Engewald, J. Pörschmann, T. Welsch and K. D. Ščerbakova, *Z. Chem.*, 17 (1977) 375.
9 A. S. Bojkova and K. D. Shcherbakova, *Neftekhimiya*, 7 (1967) 451.
10 R. I. Sidorov and M. P. Iwanova, *Neftekhimiya*, 7 (1967) 640.
11 S. S. Berman, W. A. Zakharenko and A. A. Petrov, *Neftekhimiya*, 7 (1967) 703.
12 V. M. Nabivach and A. V. Kirilenko, *Chromatographia*, 13 (1980) 29.
13 V. M. Nabivach and A. V. Kirilenko, *Chromatographia*, 13 (1980) 93.
14 J. H. Purnell, in A. B. Littlewood (Editor), *Gas Chromatography 1966*, Institute of Petroleum, London, 1967, p. 3.
15 C. Eon, C. Pommier and G. Guiochon, *J. Phys. Chem.*, 75 (1971) 2632.

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CHROMSYMP. 685

# OLIGO-OXAALKANOYL TETRAAMIDES DERIVED FROM L-PHENYLALANINE AS STATIONARY PHASES IN CAPILLARY GAS CHROMATOGRAPHIC RESOLUTION OF D,L-AMINO ACIDS

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## SUMMARY

The resolution of enantiomers of (±)-α-amino acid derivatives by capillary gas chromatography was studied using a series of optically active tetraamide stationary phases derived from L-phenylalanine, $[(CH_3)_3\ CNHC\ (O)\ CH\ (CH_2C_6H_5)\ NHC\ (O)\ CH_2]_2\ X$ with X = O, $O(CH_2CH_2O)_n$ and $n$ = 1–3. The effects of the structure of X on the separation factors are reported; the tetraamide phases were stable at high temperature (200°C) and allowed the separation of amino acid derivatives of high and low volatility in one run.

## INTRODUCTION

The use of chiral amide stationary phases for the gas chromatographic resolution of (±)-α-amino acid derivatives is a well known method and has recently been reviewed.[1] The technique is successful in many instances also for the separation of chiral hydroxy acid derivatives[2], amines[3], amino alcohols and sugars[4]. The mechanism of resolution has been studied, and stereoselective hydrogen bonding associations have been suggested to be responsible for the separation[5,6]. However, the known non-bonded amide phases could not be used at the high temperatures required for the resolution of amino acids of low volatility as they lack thermal stability; to solve the problem, binding processes of chiral amides to silicone phases have been developed, resulting in stable derivatives, such as the commercially available Chirasil-Val[7].

As a contribution to the understanding of the phenomenon of chiral resolution via hydrogen bonding, we utilized a series of dicarboxylic chiral ligands, containing L-phenylalanine, which had been previously synthesized to study the selective complexation and extraction of ions from aqueous into organic solutions[8]. We derivatized these ligands with *tert.*-butylamine and tested the corresponding tetraamides (Fig. 1) as a new chiral stationary phase for the resolution of D,L-amino acids by capillary gas chromatography.

We also studied the influence of the bridge binding the two amide chains and determined the dimensions corresponding to optimal resolution. Tetraamides with L-valine and diamides with an oxaalkane chain were also synthesized and compared with the corresponding L-phenylalanine derivatives.

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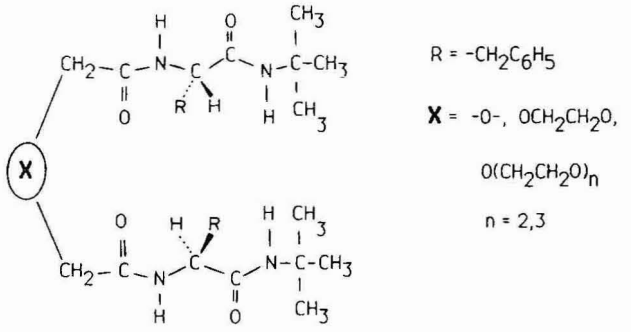


Fig. 1. Structure of the oligo-oxaalkanoyl tetraamides used as stationary phases.

## EXPERIMENTAL

### *Instruments*

IR spectra were recorded with a Perkin-Elmer 298 instrument in liquid films (KBr for ligand 5). $^1$H NMR spectra were recorded on a Varian 360 instrument at a frequency of 60 MHz. $[\alpha]_D$ values were measured on a Rudolph Research Polarimeter III at 20°C in 95% ethanol. Melting points are uncorrected (Büchi apparatus). Preparative HPLC was carried out with a Waters Model 440 liquid chromatograph, equipped with a UK-6 septumless injector, a 6000 A pump and a dual-wavelength (254 and 280 nm) UV detector, using a Perkin-Elmer $C_{18}$ reversed-phase column (25 × 2.5 cm I.D.).

### *Gas–liquid chromatography (GLC)*

Capillary GLC was carried out with a Dani 3900 instrument, equipped with a flame-ionization detector, using glass columns (25 m × 0.3 mm I.D.) with injector and detector temperatures of 250°C and helium at a pressure of 0.6 atm. The glass columns were leached with 20% HCl at 180°C, deactivated with barium carbonate[9] and statically wall-coated with a dichloromethane solution of the chiral amide phase (1–8) (0.15%) and of a commercial silicone gum (OV-101) (0.15%). The columns were conditioned for 10 h at 180°C (phases 1–6) or at 160°C (phases 7 and 8).

### *Materials*

The starting compounds N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), amino acids, trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), oxalyl chloride, glycols and suberic acid were commercial products.

*Synthesis of the tetraamide ligands 1–4 and 6.* Ligands were synthesized by allowing L-phenylalanine (ligands 1–4) or L-valine (ligand 6) sodium salts to react with the bis-acid chlorides $X(CH_2COCl)_2$, obtained from the corresponding $X(CH_2CH_2OH)_2$ glycols by reaction with nitric acid and then with oxalyl chloride, as previously described[8]. The dicarboxylic chiral ligands were purified by preparative reversed-phase high-performance liquid chromatography and then dissolved (10 mmol) in dry dioxane (50 ml) and treated at 20°C with DCC (22 mmol) and NHS (20 mmol). The solution was stirred overnight. Dicyclohexylurea was filtered off and

the solution containing the active bis-succinimidic esters was added directly to a cooled solution of *tert.*-butylamine (20 mmol) dissolved in dioxane (20 ml). After 10 h the solution was concentrated under vacuum, poured into ice water (300 ml) at pH 2.0 and extracted twice with 50-ml volumes of dichloromethane. The organic solution was dried, concentrated and flash-chromatographed on a silica gel column (20–40 μm; 20 × 3.5 cm I.D.), with *n*-hexane–ethyl acetate (7:3, v/v) as the eluent. The pure tetraamides thus obtained (total yield 50–60%) were characterized by IR and NMR spectroscopy and microanalysis.

*Synthesis of the tetraamide 5.* Tetraamide 5 was synthesized by allowing L-phenylalanine sodium salt to react with suberic acid bis-chloride, and proceeding as described for the synthesis of ligands 1–4.

*Synthesis of the diamides 7 and 8.* The ligands were synthesized by condensing the L-phenylalanine or L-valine sodium salt with $CH_3OCH_2CH_2OCH_2CH_2OCH_2COCl$ obtained by oxidation of the triethyleneglycol monomethyl ether with nitric acid and subsequent treatment with thionyl chloride. The chiral carboxylic acid so obtained was transformed into the active ester by reaction with equimolecular amounts of NHS and DCC, proceeding as reported above for the synthesis of tetramides.

*Properties.* The main properties of the chiral amides 1–8 obtained are reported in Table I. The $^1H$ NMR chemical shifts ($CDCl_3$, $\delta$ ppm down field from TMS, 60 MHz) are as follows: (1) 1.15 [18H, s, $C(CH_3)_3$], 3.0 (4H, d, $CH_2C_6H_5$), 4.10 (4H, s, $CH_2CO$), 4.8 (2H, m, CH), 6.0 (2H, s, broad, NH), 7.1–7.2 (10H, s, broad, arom.), 7.5 (2H ,d, broad, NH); (2) 1.15 [18H, s, $C(CH_3)_3$], 3.0 (4H, d, $CH_2C_6H_5$), 3.5 (4H, s, $-CH_2CH_2-$), 3.9 (4H, s, $CH_2CO$); 4.5 (2H, m, CH); 5.5 (2H, s, broad, NH), 7.1–7.2 (10H, s, broad, arom.), 7.3 (2H, d, NHJ); (3) 1.20 [18H, s, $C(CH_3)_3$], 3.0 (4H, d, $CH_2C_6H_5$), 3.4 (8H, s, $-CH_2CH_2-$), 3.8 (4H, s, $CH_2CO$), 4.8 (2H, m, CH), 6.4 (2H, s, broad, NH), 7.1–7.2 (10H, s, broad, arom.), 7.5 (2H, d, NH); (4) 1.15 [18H, s, $C(CH_3)_3$], 3.0 (4H, d, $CH_2C_6H_5$), 3.65 (12H, s, broad, $-CH_2CH_2-$), 4.6 (2H, m, CH), 5.90 (2H, s, broad, NH), 7.1–7.2 (10H, s, broad, arom.), 7.4 (2H, d, NH); (5) 1.2 [18H, s, $C(CH_3)_3$], 1.0–1.7 [8H, m, $-(CH_2)_4-$], 1.9–2.1 (4H, m, $CH_2CO$), 3.2 (4H, d, $CH_2C_6H_5$), 4.7 (2H, m, CH), 5.5 (2H, m, NH), 7.2–7.3 (2H, d, broad, NH), 7.3 (10H, s, broad, arom.); (6) 0.6–0.9 [12H, d, broad, $C(CH_3)_2$]; 1.35 [18H, s, $C(CH_3)_3$], 1.6–2.1 [2H, m, $CH(CH_3)_2$], 3.75 (8H, s, $-CH_2CH_2-$); 4.2 (4H, s, $CH_2CO$), 5.8 (2H, s, broad, NH), 7.6 (2H, d, broad, NH); (7) 1.25 [9H, s, $C(CH_3)_3$], 3.1 (2H, d, $CH_2C_6H_5$), 3.45 (3H, s, $OCH_3$), 3.7 (8H, s, $-CH_2CH_2-$), 4.07 (2H, s, $CH_2CO$), 4.65 (1H, m, CH), 5.75 (1H, s, broad, NH), 6.3 (1H, s, broad, NH); (8) 0.6–0.9 [6H, d, broad, $C(CH_3)_2$], 1.33 [9H, s, $C(CH_3)_3$], 1.6–2.1 [1H, m, $CH(CH_3)_2$], 3.45 (3H, s, $OCH_3$); 3.65–3.75 (8H, s, broad, $-CH_2CH_2-$), 4.10 (2H, s, $-CH_2CO$), 4.35 (1H, m, CH), 5.85 (1H, s, broad, NH), 6.8 (1H, d, broad, NH).

*Synthesis of solutes.* N-TFA- and N-HFB-amino acid isopropyl and *n*-butylesters were synthesized as described previously[10].

## RESULTS AND DISCUSSION

The characteristics of the capillary columns and the resolution factors $r$(L/D) obtained for N-TFA-amino acid *n*-butyl esters on stationary phases 1–4 are given in Table II. Fig. 2 shows a chromatogram of several amino acid derivatives, recorded in 30 min by programming the temperature from 100 tot 200°C. The best resolution

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF THE STATIONARY PHASES STUDIED (1-8)

| *No.* | *Name* | *X* | *R* | *Mol. wt.* | *M.p. (°C)* | $[\alpha]_D^{20}$ *(ethanol)* | *Main IR bands ($cm^{-1}$)* |
|---|---|---|---|---|---|---|---|
| 1 | Phe-1-O | –O– | $CH_2C_6H_5$ | 538.6 | Wax | +7.6 ($c=5$) | 3280, 2960–2900, 1645, 1540, 1220, 740, |
| 2 | Phe-2-O | $OCH_2CH_2O$ | $CH_2C_6H_5$ | 582.7 | Wax | +15.7 ($c=7$) | 3280, 2960–2900, 1660–1640, 1540, 1220, 1110, 740–730, 695 |
| 3 | Phe-3-O | $O(CH_2CH_2O)_2$ | $CH_2C_6H_5$ | 626.7 | Wax | +24.3 ($c=8$) | 3300–3290, 2980–2880, 1645, 1545–1520, 1220, 1110, 700 |
| 4 | Phe-4-O | $O(CH_2CH_2O)_3$ | $CH_2C_6H_5$ | 670.8 | Wax | +20.8 ($c=3$) | 3300–3280, 2960–2860, 1660–1640, 1540–1520, 1220, 1110, 740, 700 |
| 5 | Phe-2-C | $(CH_2)_4$ | $CH_2C_6H_5$ | 578.7 | 218–219 | −2.4 ($c=5$) | 3240, 2900, 1730, 1630, 1540 |
| 6 | Val-3-O | $O(CH_2CH_2O)_2$ | $CH(CH_3)_2$ | 530.7 | Wax | −10.9 ($c=3$) | 3300, 2960–2860, 1740, 1650–1520, 1450, 1360, 1210 |
| 7* | Phe-diamide | – | $CH_2C_6H_5$ | 370.4 | Wax | +3.9 ($c=4$)** | 3280, 2920–2860, 1660–1640, 1520, 740, 700 |
| 8* | Val-diamide | – | $CH(CH_3)_2$ | 332.4 | Wax | −3.6 ($c=2$) | 3300, 2960–2860, 1740, 1650, 1520 |

* 7 = $CH_3OCH_2CH_2OCH_2CH_2OCH_2CONHCH(CH_2C_6H_5)CONHC(CH_3)_3$;
8 = $CH_3OCH_2CH_2OCH_2CH_2OCH_2CONHCH[CH(CH_3)_2]CONHC(CH_3)_3$.

** Ethyl acetate.

TABLE II

COLUMN CHARACTERISTICS AND RESOLUTION FACTORS FOR TFA-AMINO ACID *n*-BUTYL ESTERS

| *Column* | *Length (m)* | *I.D. (mm)* | *HEPT* $\times 10^3$ | *Film thickness (μm)* | *Resolving power, r(L/D)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *150°C* | | | | | | | *190°C* | | | |
| | | | | | *Ala* | *Thr* | *Val* | *Ile* | *Nva* | *Leu* | *Nleu* | *Met* | *Asp* | *Phe* | *Glu* |
| Phe-1-O | 24 | 0.33 | 17 | 0.2 | 1.038 | 1.027 | 1.038 | 1.036 | 1.044 | 1.054 | 1.050 | 1.029 | 1.00 | 1.023 | 1.026 |
| Phe-2-O | 25 | 0.30 | 24 | 0.2 | 1.036 | 1.022 | 1.035 | 1.029 | 1.034 | 1.040 | 1.037 | 1.024 | 1.00 | 1.020 | 1.022 |
| Phe-3-O | 25 | 0.30 | 18 | 0.23 | 1.047 | 1.036 | 1.046 | 1.040 | 1.048 | 1.057 | 1.049 | 1.034 | 1.01 | 1.027 | 1.031 |
| Phe-4-O | 25 | 0.30 | 24 | 0.2 | 1.035 | 1.030 | 1.035 | 1.029 | 1.039 | 1.040 | 1.037 | 1.024 | 1.00 | 1.018 | 1.024 |
| Phe-2-C | 24 | 0.29 | 15 | 0.2 | 1.000 | – | – | – | – | 1.00 | – | – | – | – | – |

* Ratio of the corrected retention times of the L-enantiomer over that of the D-isomer.

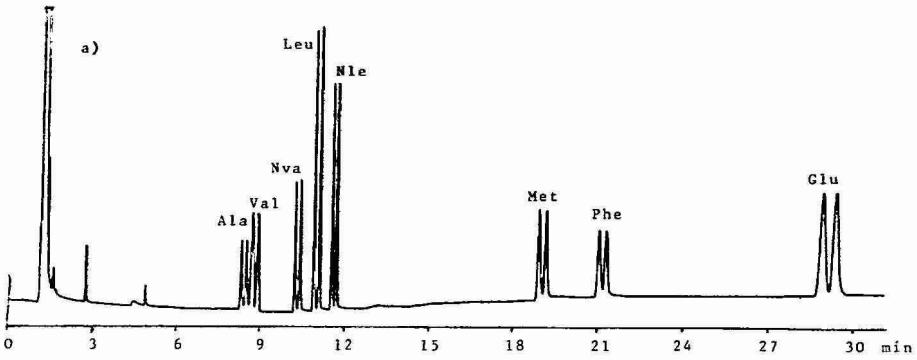


Fig. 2. Enantiomeric separation of N-TFA-amino acid *n*-butyl esters on a 25 m × 0.30 mm I.D. glass column wall-coated with the Phe-3-O (3) phase.

factors were obtained with the Phe-3-O ligand (3); the phases with a shorter (1,2) or longer bridge X (4) gave inferior results.

The resolving powers of stationary phases 1–5 were compared with those of the well established N-lauroyl-L-valyl-*tert.*-butylamide[11], which showed better resolution factors ($R$ = 1.09–1.10 for Ala, Thr, Val, Ile, Nva, Leu, Nleu at 130°C). However, $R$ factors for Met, Asp, Phe and Glu could not be measured because above 140°C the N-lauroyl phase bled to much.

By substituting the oxaalkane bridge X with the less polar $-(CH_2)_4-$ group (5), the resolution factors decreased dramatically. We think that this phenomenon is connected with the lower solubility of this ligand in the solvent used to fill the column, which is probably due to the formation of strong intramolecular hydrogen bonds between the two diamide chains. This reduces the chiral interactions and favours the formation of crystals along the column, thus decreasing the ability to cover the glass surface.

The oxaalkane bridge X seems to play an important role in chiral resolution, as it confers to the phase the waxy character necessary for a good dispersion during the column coating. The lower efficiency in chiral resolution shown by stationary

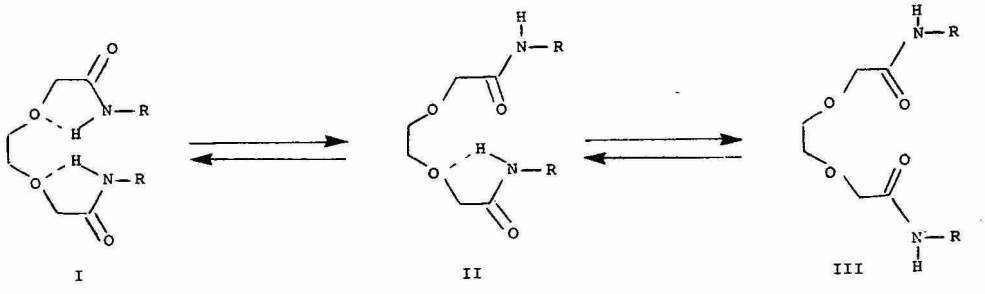


Fig. 3. Conformational behaviour expected for the Phe-2-O tetraamide ligand.

phases 1, 2 and 4 compared with 3 could be connected with the conformational equilibria that ligands undergo in solution. Intramolecular hydrogen bonding between the amide NH and the ethereal oxygens of the bridge X can maintain the two chains in a rigid conformation and at a certain distance from one another, thus influencing the formation of the chiral D–L and L–L associates between the stationary phase and the amino acid derivatives. Conformational studies by NMR of the dicarboxylic acid Phe-2-O provided evidence for the presence of an equilibrium between "open" and "closed" forms determined by intramolecular hydrogen bonding[8]. The tetraamide Phe-2-O, which gives the lowest resolution factors, could show similar equilibria (Fig. 3), with a favoured closed form I in less polar solvents, less available for chiral D–L or L–L associates, an intermediate form II with only one intramolecular hydrogen bond and an open form III, in which both the NH groups are well exposed to give chiral association with the solute.

Glass capillary wall-coated columns carrying the chiral stationary phase Phe-3-O (3) have been used at high temperatures (190–200°C) for long periods (100 h) without evidence of bleeding or reduction in the resolving power. Aspartic acid was only partially resolved and proline not at all; the detection of tryptophan required derivatization with 2-propanol and pentafluoropropionic anhydride, as the retention time of the N-TFA *n*-butyl ester derivative was too high, even at 200°C. GLC experiments performed with a maximum temperature of 220°C for several hours produced only a slow loss of resolution and a slight increase of the baseline.

The characteristics of the Phe-3-O phase were compared with those of the corresponding tetraamide ligand Val-3-O (6), synthesized from L-valine, usually considered to give more effective chiral ligands in amino acid resolution. However, Val-3-O gave resolutions of the same order as Phe-3-O and showed a lower resistance to temperature; the maximum operating temperature had to be reduced to 180°C. Diamide phases with L-Phe, L-Val and an oxadecanoyl chain were synthesized (ligands 7 and 8, Table I) to study the influence of the number of amide chains on the resolution factors. Although diamides 7 and 8 and tetraamides 3 and 6 gave similar resolution factors, the former required lower temperature limits, 180°C for ligand 7 and 160°C for ligand 8.

We also tried to prepare a bonded stationary phase starting from ligand 8 by deactivating the glass surface with Carbowax 1500, adding dicumyl peroxide (5%) to the stationary phase and slowly conditioning from 100 tot 160°C. The stability of the chiral phase towards higher temperatures actually increased, but the resolution slowly decreased to coalescence, and bleeding gradually became significant.

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REFERENCES

1 R. H. Liu and W. W. Ku, *J. Chromatogr.*, 271 (1983) 309.
2 B. Koppenhoefer, H. Allmendinger, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 260 (1983) 63.
3 W. A. König, W. Francke and I. Benecke, *J. Chromatogr.*, 239 (1982) 227.
4 W. A. König, I. Benecke and S. Sievers, *J. Chromatogr.*, 238 (1982) 427.
5 B. Feibush and E. Gil-Av, *Tetrahedron*, 26 (1970) 1361.

6 C. H. Lochmüller and R. W. Souter, *J. Chromatogr.*, 113 (1975) 283.
7 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr. Sci.*, 15 (1977) 174.
8 T. Lodi, R. Marchelli, A. Dossena, E. Dradi and G. Casnati, *Tetrahedron*, 38 (1982) 2055.
9 K. Grob, G. Grob and K. Grob, Jr., *Chromatographia*, 10 (1977) 181.
10 S. Nakaparksin, P. Birrel, E. Gil-Av and J. Orò, *J. Chromatogr. Sci.*, 8 (1970) 177.
11 B. Feibush, *J. Chem. Soc. Chem. Commun.*, (1971) 544.

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CHROMSYMP. 678

# DETERMINATION OF PERMANENT GASES AND LIGHT HYDROCARBONS BY SIMULTANEOUS OPERATION ON PACKED AND CAPILLARY COLUMNS WITH THERMAL CONDUCTIVITY DETECTION AND FLAME-IONIZATION DETECTION ON A SINGLE COMPUTER INTEGRATOR

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## SUMMARY

Simple instrumentation, based on a commercial gas chromatograph equipped with one packed and one capillary column channel and two different detectors leading to a single computer integrator, is described. The simultaneous injection into both channels provides the means to perform the determination of inorganic and hydrocarbon gases of the same sample.

Methane, used as an internal standard, allows correlation between the two chromatograms, recorded on two files of the same computing integrator. Data reduction is performed by means of BASIC program. The instrumentation is suitable for routine laboratory analysis or on-lined and closed-loop application.

## INTRODUCTION

The determination of the single components of a gaseous mixture containing oxygen, nitrogen, methane, carbon monoxide, carbon dioxide and hydrocarbons from $C_1$ to $C_8$, is of great interest in petrochemical plants and refineries, and in the production of natural gas.

Several gas chromatographic (GC) methods have been developed for this type of analysis[1–3]. Most of them are suitable for analysing all the components of the mixture, making use of the newest microprocessor control, multi-column switching, automatic valves, and different types of capillary column. Nevertheless, all the methods described suffer from some limitations. For example, in one case[1] the sample injection is performed in two steps, in other cases the quantitation requires special calibration and high sample volume reproducibility[2] or demands high instrumentation cost for a second integrator channel in case simultaneous introduction is desired[3].

The GC system described here permits the analysis of both inorganic gases and hydrocarbons up to $C_8$ with a single, unmodified commercial gas chromatograph and with a single computer integrator. The sample is injected simultaneously into two different GC channels. The single final report summarizes all qualitative and quantitative data.

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## EXPERIMENTAL

The GC system consists of a DANI 8500 gas chromatograph with a flame ionization detector at the end of the capillary column channel for hydrocarbon analysis, and a thermal conductivity detector at the end of a channel including three packed column and two switching column valves for the inorganic gases. Two gas-sampling valves with different sample loops are mounted. All valves are installed outside the column oven and are automatically operated by means of compressed air. The switching time programme is set on the GC control module.

The two detectors are connected to a single computer integrator, Shimadzu C-R3A, via a switching relay, timed by the computer. All analytical parameters, *e.g.* temperature programme, detector temperatures, sample injection, and column switching, are set on the GC control module. Analysis files, *e.g.* integrator parameters, calibration tables, analysis report, detector outputs, switching time, and BASIC programs, are stored in the computing memory without need for a cassette tape unit. The schematic diagram of the chromatographic system is shown in Fig. 1.

The gas-sampling loop of the capillary column channel sampling valve is 50 $\mu$l. This small amount of sample permits the use of a low split ratio (1:50). The gas-sampling loop of the packed column sampling valve is 0.5 ml. The sample volume may be optimized independently in order to ensure the correct linear detector response and to avoid column overload effects. On the first column switching valve a short Porapak column is mounted where $C_2$ and higher hydrocarbons are retarded and backflushed just after methane and carbon dioxide enter the main Porapak column.

When methane and other inorganic gases enter the molecular sieve column, mounted on the second switching valve, this column is isolated. Carbon dioxide is now eluted from the main Porapak column. After carbon dioxide elution, the mo-

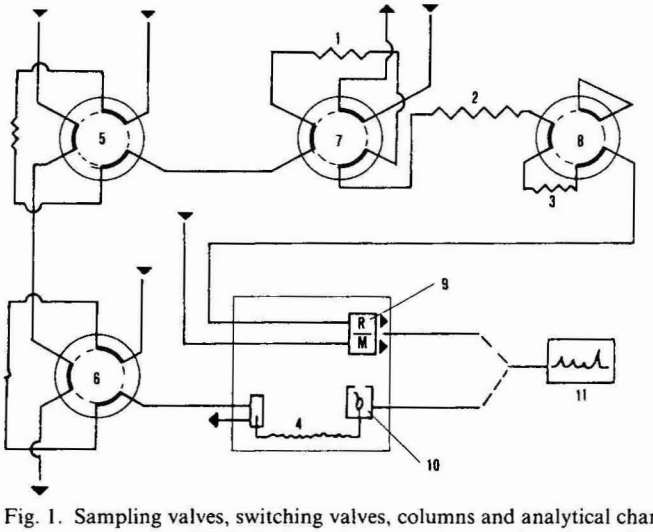


Fig. 1. Sampling valves, switching valves, columns and analytical channels of DANI 8500 GC system for permanent gases and light hydrocarbon analysis. 1 = Precolumn; 2 = Porapak Q; 3 = molecular sieve 5A; 4 = capillary column; 5 and 6 = sampling valves; 7 and 8 = column switching valves; 9 = thermal conductivity detector; 10 = flame ionization detector; 11 = computer integrator.

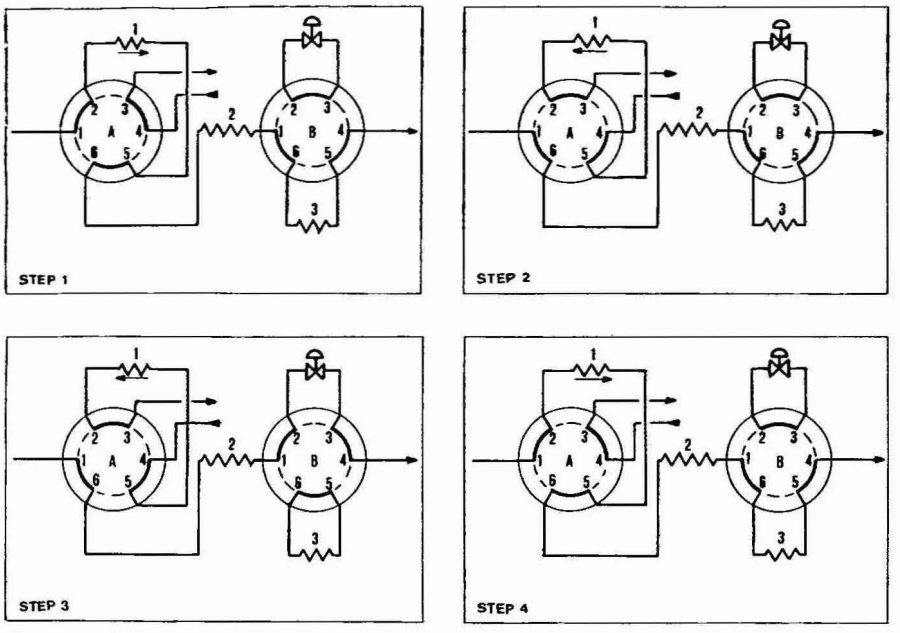


Fig. 2. Configuration of the column switching valves and organization of the columns during the different steps of a complete analytical run.

lecular sieve column is again included in the analytical cycle for the elution of oxygen, nitrogen, methane and carbon monoxide. A schematic diagram of the valves actuation and column organization during the analytical cycle sequence is shown in Fig. 2.

At the beginning of the analysis, the sample, which purged ahead of both gas-sampling loops, is injected into both analytical channels by simultaneous switching of the sampling valves. At the starting time the column oven is kept at 50°C and the integrator input is connected to the thermal conductivity detector. The carrier gas flow-rate of capillary column is adjusted to produce elution of methane 20 s after the carbon monoxide peak. After the elution of carbon monoxide, the column oven is programmed to 200°C at a rate of 10°C/min, and the input of the integrator is switched to the flame ionization detector.

Fig. 3 shows a complete chromatogram of a mixture containing inorganic gases and hydrocarbons up to $C_6$.

The detector response is ingegrated on two different files of the computing integrator, and at the end of the analysis a BASIC program produces the correlation of data obtained from both the capillary flame ionization detection (FID) and packed thermal conductivity detection (TCD) column systems. Methane, which appears in both channels, is used as internal standard.

The sum of components is normalized to 100% by volume. The calculation is expressed by the following formula:

$$\Sigma\left(\frac{A_i}{C_i} + \frac{C_x}{A_x} - 1\right) \cdot K = 100$$

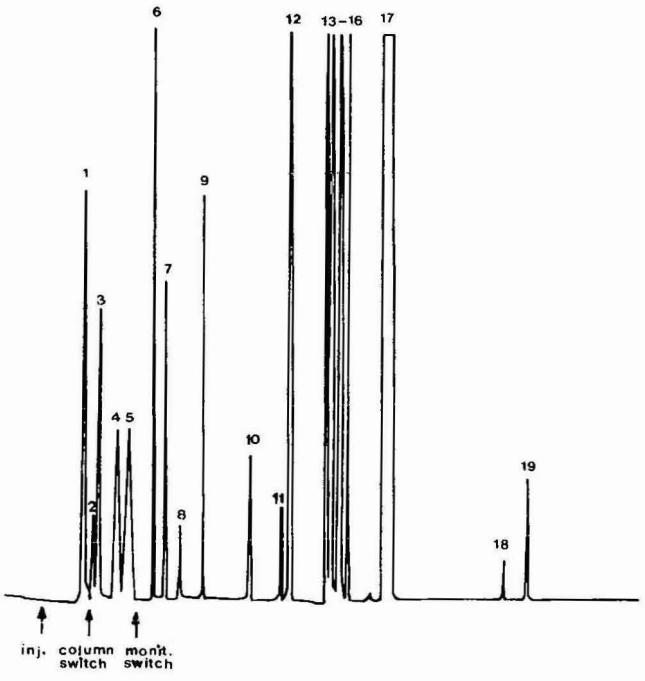


Fig. 3. Typical chromatogram of permanent and hydrocarbon gases, with a single recording computer, obtained from a DANI 8500 gas chromatograph. Columns: 1 = stainless steel, 50 cm × 3 mm I.D., Porapak Q; 2 = stainless steel, 1.50 m × 3 mm I.D., Porapak Q; 3 = stainless steel, 1 m × 3 mm I.D., molecular sieve 5A; 4 = fused silica, 50 m × 0.32 mm I.D., $Al_2O_3$, KCl (Chrompack). Carrier gas, hydrogen. Flow-rate: packed columns, 25 ml/min; capillary column, 2 ml/min; split, 50 ml/min. Oven temperature programme: 50°C for 4 min, then 10°C/min to 200°C, then 200°C for 7 min. Peaks: 1 = $CO_2$; 2 = $O_2$, 3 = $N_2$; 4 = $CH_4$(TCD); 5 = CO; 6 = $CH_4$(FID); 7 = $C_2H_6$; 8 = $C_2H_4$; 9 = $C_3H_8$; 10 = $C_3H_6$, 11 = *iso*-$C_4H_{10}$; 12 = *n*-$C_4H_{10}$; 13–16 = $C_4H_8$ isomers; 17 = 1,3-butadiene; 18 = *iso*-$C_5H_{12}$, 19 = *n*-$C_5H_{12}$.

where $A_i$ is the TCD area of component $i$, $C_i$ is the TCD area of methane, $A_x$ is the FID area of component $x$, $C_x$ is the FID area of methane, $K$ is the normalization constant. This procedure allows quantification even if different amounts of sample are injected.

The analytical cycle is automatic from the injection up to the final report, and the instrument is suitable for on-line operation or in a closed-loop system, provided the sample is available at the input of the sampling valves as a continuous stream.

## RESULTS AND DISCUSSION

The capillary column used (Chrompack, 50 m × 0.32 mm I.D., $Al_2O_3$, KCl) described by De Nijs and De Zeeuw[4] has two main advantages.

(1) The retention time of methane at the initial temperature programme is long enough to permit the previous elution of all inorganic gases coming from the packed column.

(2) It provides the separation of all saturated and unsaturated $C_4$ hydrocar-

TABLE I

STATISTICAL DATA OBTAINED FROM TEN RUNS

Calculated by the internal normalization method.

| *Compound* | $t_R$ | *R.S.D. (%)* | *Concentration* | |
|---|---|---|---|---|
| | | | % | *R.S.D. (%)* |
| $CO_2$ | 2.11 | 0.5 | 5.3 | 0.8 |
| $O_2$ | 2.58 | 0.3 | 1.1 | 0.7 |
| $N_2$ | 2.58 | 0.3 | 4.4 | 0.6 |
| $CH_4$ | 3.77*/5.54** | 0.25*/0.15** | 5.0 | 0.8 |
| CO | 4.33 | 0.25 | 4.9 | 0.7 |
| $C_2H_6$ | 6.91 | 0.15 | 2.2 | 0.6 |
| $C_2H_4$ | 8.05 | 0.12 | 0.6 | 0.7 |
| $C_3H_8$ | 9.05 | 0.10 | 2.1 | 0.8 |
| $C_3H_6$ | 10.28 | 0.12 | 1.9 | 0.6 |
| *iso*-$C_4H_{10}$ | 11.73 | 0.10 | 0.4 | 0.8 |
| *n*-$C_4H_{10}$ | 12.13 | 0.10 | 7.4 | 0.5 |
| *iso*-$C_4H_8$ | 13.05 | 0.10 | 6.7 | 0.5 |
| $C_4H_8$ | 13.95 | 0.10 | 4.83 | 0.5 |
| 2-*trans*-$C_4H_8$ | 14.27 | 0.10 | 10.80 | 0.4 |
| 2-*cis*-$C_4H_8$ | 14.66 | 0.10 | 4.7 | 0.7 |
| 1,3-Butadiene | 16.28 | 0.09 | 38.8 | 0.8 |
| *iso*-$C_5H_{12}$ | 21.05 | 0.08 | 0.4 | 0.6 |
| *n*-$C_5H_{12}$ | 22.18 | 0.08 | 1.0 | 0.8 |

* Packed column.
** Capillary column.

bons. This separation is not required in the case of a natural gas analysis, but it is of paramount importance in the petrochemical industry. However, this column is not suitable for the determination of hydrocarbons with more than eight carbon atoms.

The simultaneous injection into both analytical channels as already stated[4] provides two advantages.

(1) There is a guarantee that the quantitative data refer to a homogeneous sample, resulting in greater reliability, not only when standard or calibration mixtures are injected, but also when actual samples are analysed.

(2) It is possible to perform the quantification by using the relative response factor, thus eliminating the need for repeatable sample volume injections and frequent recalibration.

The use of a single computing integrator saves money and simplifies the reading of the analytical data and results.

It is clear that from such a detailed report, a simple post-chromatographic calculation may provide the calorific value, the specific gravity, and the Wobbe index of the analysed gas mixture.

Statistical data from ten consecutive automatic analyses are given in Table I.

## CONCLUSION

The instrumentation described may be used for the analysis of gases, oil con-

densate and complex gas mixtures coming from pilot plants without any change.

The final report provides all the required physical and chemical parameters of the analysed gas mixtures.

REFERENCES

1 L. Huber, *J. Chromatogr. Sci.*, 21 (1983) 519.
2 H. Osjord and D. M. Sorensses, *J. Chromatogr. Sci.*, 297 (1983) 219.
3 P. Coleman and L. Ettre, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 112.
4 R. C. M. de Nys and J. de Zeeuw, *J. Chromatogr. Sci.*, 279 (1983) 41.

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CHROMSYMP. 670

# DETERMINATION OF PYRROLIZIDINE ALKALOIDS IN *SENECIO INAEQUIDENS* D.C. BY CAPILLARY GAS CHROMATOGRAPHY

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## SUMMARY

A study of the toxic pyrrolizidine alkaloids contained in *Senecio inaequidens* D.C., an infestant species of the *Senecio* genus, widespread in the North East of Italy, is reported. Five of these compounds, senecivernine, senecionine, integerrimine, retrorsine and an analogue of retrorsine, were identified by means of capillary gas chromatography and capillary gas chromatography–mass spectrometry.

## INTRODUCTION

Pyrrolizidine alkaloids constitute a class of more than 200 compounds generated through an ornithine biogenetic pathway. Comprehensive reviews and textbooks describing this class of compounds[1–5], their chemotaxonomic significance[6] and general pyrrolizidine chemistry[7] are available.

Pyrrolizidine alkaloids are well known for their toxicological characteristics, inducing serious chronic or acute intoxication in both man and cattle. In fact, these compounds show marked hepatotoxic activity, producing liver necrosis and cirrhosis in animals and man[5,8,9] and, even in small amounts, they can induce pulmonary arterial hypertension[10,11]. Some of them are also hepatocarcinogenic[12–14] and chronic intoxication has been considered responsable for the unusual incidence of cancer of the liver in some geographic areas[15,16]. Sporadic episodes of human intoxication and loss of life from pyrrolizidine alkaloids have been reported[17]. Human contact is generally due either to chance contamination of foods, such as flour, because several *Senecio* or *Heliotropium* species can infest corn and maize crops, or to swallowing herbal "medicines" (generally teas) containing plants in which such compounds are present[18,19]. Pyrrolizidine alkaloids have also been detected in honey[20,21] and in milk[22,23].

The toxicological risks related to pyrrolizidine alkaloids are widespread, because plants containing these compounds occur in all parts of the world. The plant species most frequently responsible for poisoning belong to the genera *Senecio* (Compositae), *Heliotropium* (Boraginaceae) and *Crotalaria* (Leguminosae).

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Owing to the toxicity of these compounds and their chemotaxonomic importance, it is of great importance to have rapid and sensitive methods available for their analysis So far, analytical techniques involving thin-layer chromatography (TLC)[24–26], high-performance liquid chromatography[27–29], gas–liquid chromatography and gas chromatography–mass spectrometry (GC–MS)[24,30,31–33] and NMR spectroscopy[34] have been reported.

In this paper, a capillary gas chromatographic (CGC) method for the determination of pyrrolizidine alkaloids in *Senecio inaequidens* D.C. is described. *S. inaequidens* is a *Senecio* species, native to South Africa, naturalized in Italy after the Second World War, and so widely diffused in Eastern Italy as to be considered potentially dangerous, both indirectly as a food contaminant and directly for cattle. Wiedenfeld *et al.*[32] identified senecionine and retrorsine from a sample of *S. inaequidens*. At least five compounds belonging to this class were detected by CGC and CGC–MS analysis of a sample of *S. inaequidens* from Eastern Italy.

## EXPERIMENTAL

### *Plant material*

Flowering plants of *Senecio inaequidens* D.C., collected in June 1984 along the edges of the road on the outskirts of Padua, were utilized.

### *Reagents*

All the chemicals used were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Authentic samples of senecionine and retrorsine were kindly provided by Dr. C. C. J. Culvenor, Parkville, Australia.

### *Sample preparation*

A 25-g amount, exactly weighed, of the aerial parts was extracted in a Soxhlet apparatus with methanol for 4 h. The extract was evaporated to dryness under reduced pressure and the residue was treated with 2.5% hydrochloric acid and washed with diethyl ether and chloroform to remove chlorophylls and lipids, respectively. The aqueous layer was made alkaline with 25% ammonia solution and extracted with dichloromethane. The organic layer (dichloromethane) was again treated with 2.5% hydrochloric acid, 25% ammonia solution and dichloromethane. The resulting solution, containing the free alkaloids, was dried over anydrous sodium sulphate and evaporated to dryness. To investigate the presence of pyrrolizidine alkaloid N-oxides, an aliquot of the solution resulting after washing with diethyl ether and chloroform was reduced with zinc dust overnight, filtered and subsequently treated as described above. The dried residues were weighed on an analytical balance and dissolved in appropriate amounts of dichloromethane to produce suitable concentrations for TLC, CGC and CGC–MS analysis. Decreasing amounts (25, 10, 5, 2 and 1 g) were extracted to investigate the minimum amount of dried plant material necessary to obtain reliable results. The composition of the methanol extract of 25 g of dried plant material after different extraction times (0.5, 1, 2, 4 and 8 h) was also investigated to evaluate the minimum time necessary for complete extract of the pyrrolizidine alkaloids.

*CGC and CGC–MS analysis*

CGC analyses were performed by introducing 1 μl of pyrrolizidine alkaloid extract into a glass capillary column, installed in a Carlo Erba 4160 instrument, equipped with a flame-ionization detector. The following conditions were used: carrier gas, hydrogen; flow-rate, 3 ml/min; injection system, splitting ratio 1:30; injector temperature, 250°C; detector temperature, 280°C; column temperature, programmed from 120°C (1 min) to 230°C (20 min) at 5°C/min; columns, 20 m × 0.32 mm I.D. soda-lime and Duran-50 glass capillary columns, pre-treated by high-temperature silylation, coated with OV-1, then immobilized (film thickness 0.1 μm).

Quantitation of the alkaloids was carried out with respect to a suitable amount of $C_{24}$ hydrocarbon in hexane solution as internal standard, added to the alkaloid solution. The peak areas were calculated with a Carlo Erba Mega 2 integrator.

CGC–MS analyses were carried out on a Finnigan-MAT Model 4021 GC–MS system, equipped with a Data General Nova 3 computer with helium as carrier gas. The CGC conditions were the same as above. Identification was based on comparison of the mass spectral data with data obtained from the literature, and on comparison with retention data and mass spectra of pure compounds.

TLC analyses were performed by applying the pyrrolizidine alkaloid extract to silica gel pre-coated plates (DC Alufolien Kieselgel 60 F254; Merck). The plates were developed with dichloromethane–methanol–25% ammonia solution (85:14:1) for a distance of approximately 15 cm. The plates were dried and either observed under UV light at 254 nm or sprayed with Dragendorff reagent and heated for 1 min.

## RESULTS AND DISCUSSION

Fig. 1 shows the GC pattern of the fraction containing pyrrolizidine alkaloids of *Senecio inaequidens*. CGC–MS analysis showed the presence of five different alkaloids of this class. In particular, senecivernine (a), senecionine (b), integerrimine (c), retrorsine (d) and a retrorsine analogue (e), molecular weight (MW) 351, were separated and identified. Two other peaks, not yet identified and with probable MW 354 (f) and 396 (g) were detected. Fig. 2 shows the structures of senecivernine, senecionine, integerrimine and retrorsine. Dried samples of *Senecio inaequidens* gave a yield of total alkaloids (free base plus N-oxides) in the range 0.3–0.4% of the dry weight.

In Table I, molecular weights, percentage compositions and standard deviations for five analyses of each compound present in the sample are reported.

The identification of senecionine (b) and retrorsine (d) was carried out by comparing the retention times, mass spectra and TLC $R_F$ values of the peaks of the sample with those of pure standards. The mass spectra of senecivernine and integerrimine reported in the literature[31,33] were found to be identical with those of peaks a and c of the sample. The mass spectrum of the retrorsine analogue (e) is reported in Fig. 3. Compounds f and g have presumably even molecular ions (354 and 396 a.m.u., respectively); the very similar fragmentation patterns and intensities of the peaks in their mass spectra led us to conclude that compound g is an acetyl derivative of f. The elucidation of the structures of f and g is in progress.

The CGC pattern of the aliquot treated with zinc dust was very similar to that of the untreated sample; therefore, it may be concluded that only small amounts of

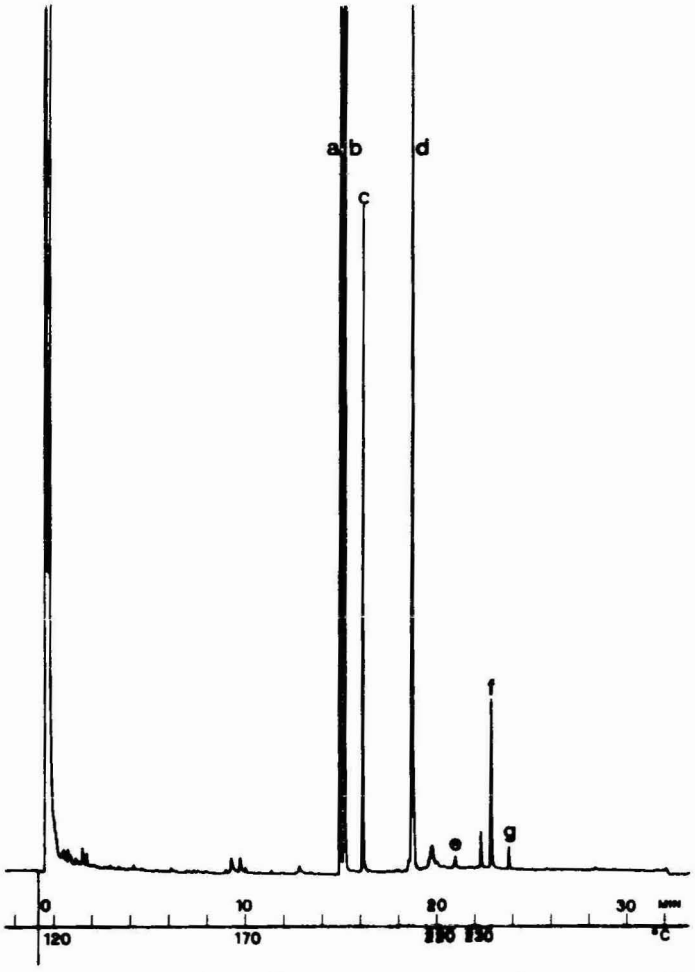


Fig. 1. GC pattern of pyrrolizidine alkaloid fraction of *Senecio inaequidens* D.C.

pyrrolizidine alkaloid N-oxides are present. Pyrrolizidine alkaloid N-oxides are thermally labile and partially decomposed under CGC temperature conditions, hence this technique will provide only qualitative information on the free base/N-oxide ratio in a sample[33]; these results were confirmed by TLC analysis.

The experiments designed to evaluate the minimum amount of plant material required showed that 1 g is sufficient to obtain a reliable and significant GC pattern of the pyrrolizidine alkaloid fraction. Parallel tests to check the yield showed that more than 85% of each pyrrolizidine alkaloid was extracted after extraction for 4 h with methanol in the Soxhlet apparatus.

TLC data were used to confirm the results obtained by CGC. In fact, the separation of the MW 335 and 351 analogues was not as good as that in CGC. The $R_F$ values of senecionine and retrorsine (0.62 and 0.29, respectively) agreed with those reported in the literature[33].

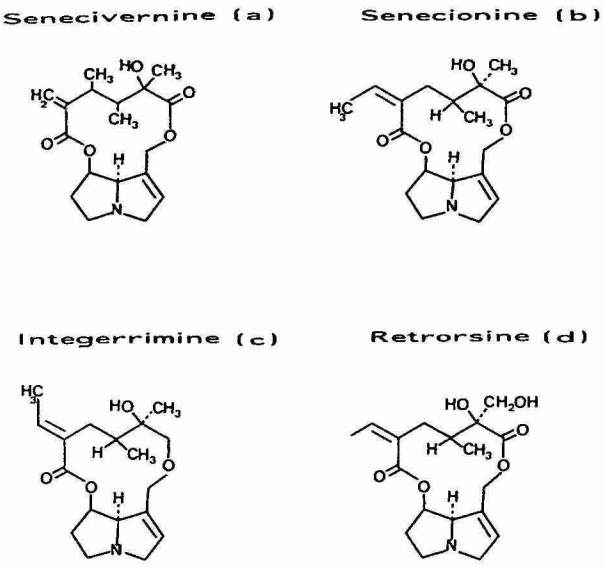


Fig. 2. Structures of the identified pyrrolizidine alkaloids: senecivernine, senecionine, integerrimine and retrorsine.

The results reported here show that CGC furnishes complete and reliable fingerprints of the pyrrolizidine alkaloid fraction. CGC allowed us to separate and identify at least five compounds of this class in *Senecio inaequidens*, compared with the two compounds previously identified by packed-column GLC and TLC[33]. The use of thin-film capillary columns (0.1 $\mu$m) allowed a more rapid analysis than packed columns. The presence of a basic "active" centre in the structure of pyrrolizidine alkaloids requires the injection port and column to be as inactive as possible in order to avoid both loss of compounds through absorption and severe tailing of the peaks. Probably for the same reasons, the CGC analyses carried out on a soda-lime glass column gave less tailing than those on a column prepared with Duran-50 glass.

TABLE I

COMPOSITION OF THE PYRROLIZIDINE ALKALOID FRACTION OF *SENECIO INAEQUIDENS* D.C.

| *Compound identification* | *Name* | *MW* | *Concentration (%)* | *Standard deviation (%)* |
|---|---|---|---|---|
| a | Senecivernine | 335 | 16.4 | 1.4 |
| b | Senecionine | 335 | 21.3 | 1.5 |
| c | Integerrimine | 335 | 4.7 | 0.8 |
| d | Retrorsine | 351 | 27.6 | 1.1 |
| e | Retrorsine analogue | 351 | 1.2 | 0.3 |
| f | Unidentified | 354* | 2.9 | 0.4 |
| g | Unidentified | 396* | 1.4 | 0.5 |

* Assumed.

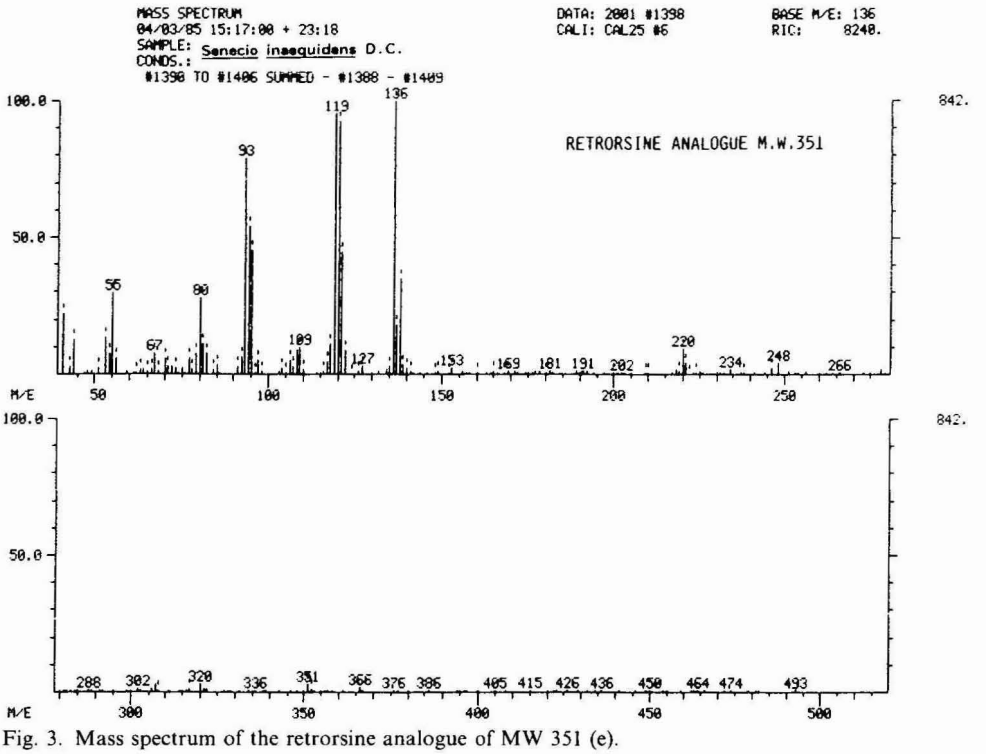


Fig. 3. Mass spectrum of the retrorsine analogue of MW 351 (e).

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REFERENCES

1 F. L. Warren, *Fortschr. Chem. Org. Naturst.*, 12 (1955) 198.
2 F. L. Warren, *Fortschr. Chem. Org. Naturst.*, 24 (1966) 329.
3 D. J. Robins, *Fortschr. Chem. Org. Naturst.*, 42 (1981) 116–202.
4 F. L. Warren, in R. H. F. Manske (Editor), *The Alkaloids*, Vol. XII, Academic Press, New York, 1978, pp. 246–324.
5 L. B. Bull, C. C. J. Culvenor and A. T. Dick, *The Pyrrolizidine Alkaloids*, North-Holland, Amsterdam, 1968.
6 C. C. J. Culvenor, *Bot. Not.*, 131 (1978) 473.
7 D. J. Robins, *Adv. Heterocycl. Chem.*, 24 (1969) 247.
8 A. R. Mattocks, in J. B. Harborne (Editor), *Phytochemical Ecology*, Academic Press, New York, 1972, Ch. 11, p. 179.
9 O. H. Muth, *J. Am. Vet. Assoc.*, 153 (1968) 310.
10 R. Huxtable, D. Ciaramitaro and D. Eisenstein, *Mol. Pharmacol.*, 14 (1978) 1189.
11 C. N. Gilles, R. J. Huxtable and R. A. Roth, *Br. J. Pharmacol.*, 63 (1978) 435.
12 J. R. Allan, I. C. Hsu and L. A. Carstens, *Cancer Res.*, 35 (1975) 997.
13 R. Schoental, *Cancer Res.*, 35 (1975) 2020.

14 R. Schoental, *Biochem. Soc. Trans.*, 3 (1975) 292.
15 R. J. Huxtable, *Trends Pharmacol. Sci.*, (1980) 299.
16 R. Schoental, *Cancer Res..*, 28 (1968) 2337.
17 A. E. Stillman, R. Huxtable, P. Consroe, P. Kohnen and S. Smith, *Gastroenterology*, 73 (1977) 349.
18 C. C. J. Culvenor, M. Clarke, J. A. Edgar, J. L. Frahn, M. V. Jago, J. E. Peterson and L. W. Smith, *Experientia*, 36 (1980) 377.
19 J. N. Roitman, *Lancet*, i (1981) 944.
20 M. L. Deinzer, P. A. Thomson, D. M. Burgett and D. L. Isaacson, *Science*, 195 (1977) 497.
21 C. C. J. Culvenor, J. A. Edgar and L. W. Smith, *J. Agric. Food Chem.*, 29 (1981) 958.
22 J. O. Dickinson, M. P. Cooke, R. R. King and P. A. Mohamed, *J. Am. Vet. Med. Assoc.*, 169 (1976) 1192.
23 M. L. Deinzer, B. L. Arbogast, D. R. Buhler and P. R. Cheeke, *Anal. Chem.*, 54 (1982) 1811.
24 A. H. Chalmers, C. C. J. Culvenor and L. W. Smith, *J. Chromatogr.*, 20 (1965) 270.
25 A. R. Mattocks, *J. Chromatogr.*, 27 (1967) 505.
26 R. J. Molyneux and J. N. Roitman, *J. Chromatogr.*, 195 (1980) 412.
27 C. W. Qualls Jr. and H. J. Segall, *J. Chromatogr.*, 150 (1978) 202.
28 G. P. Dimenna, T. P. Krick and H. J. Segall, *J. Chromatogr.*, 192 (1980) 474.
29 H. S. Ramsdell and D. R. Buhler, *J. Chromatogr.*, 210 (1981) 154.
30 M. Deinzer, P. Thomson, D. Griffin and E. Dickinson, *Biomed. Mass Spectrom.*, 3 (1978) 175.
31 E. Roeder, H. Wiedenfeld and U. Pastewka, *Planta Med.*, 37 (1979) 131.
32 H. Wiedenfeld, U. Pastewka, P. Stengl and E. Roeder, *Planta Med.*, 41 (1981) 124.
33 J. Luethy, U. Zweifel, B. Karlhuber and C. Schlatter, *J. Agric. Food Chem.*, 29 (1981) 302.
34 R. J. Molyneux, A. E. Johnson, J. N. Roitman and M. E. Benson, *J. Agric. Food Chem.*, 27 (1979) 494.

*Journal of Chromatography*, 349 (1985) 31–38
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CHROMSYMP. 640

# USE OF COMPUTERIZED PATTERN RECOGNITION IN THE STUDY OF THE CUTICULAR HYDROCARBONS OF IMPORTED FIRE ANTS

## I. INTRODUCTION AND CHARACTERIZATION OF THE CUTICULAR HYDROCARBON PATTERNS OF *SOLENOPSIS INVICTA* AND *S. RICHTERI*

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SUMMARY

A method is described in which gas chromatographic (GC) data obtained from cuticular hydrocarbons are treated by methods of pattern recognition. Based on a recently described sample preparation procedure, GC data are normalized to eliminate slight variations in chromatographic conditions and converted into the proper format for discriminant analysis by computer. The results of several methods of data treatment and display are discussed, based upon the chemometric system package, ARTHUR. The approach has the advantage of largely removing operator bias.

## INTRODUCTION

Recently the cuticular hydrocarbons of insects have been receiving much attention in the literature. Besides being involved in preventing desiccation, the cuticular hydrocarbons also play a significant role in chemical communication[1–4]. The semiochemical functions of the cuticular hydrocarbons include territory marking, recruitment and alarm pheromones, kairomones and defensive secretions, as well as sex pheromones[5].

The two species of imported fire ants, *Solenopsis invicta* (red imported fire ant) and *S. richteri* (black imported fire ant), are serious pests in the southern United States. The cuticular hydrocarbons of these two species have been investigated and characterized[3,6]. It has been suggested that the cuticular hydrocarbons of insects are also involved in species and caste recognition[5,7]. When ants encounter each other, recognition occurs by the one ant brushing its antennae over the cuticle of the other ant[8]. This suggests that the cuticle acts as a source of semiochemicals which are species- and colony-specific. Besides the use of different substances as chemical messengers, different species or colonies may also use the same compounds, but in different mixtures, to communicate chemical messages[9]. If this is the case, the cuticular hydrocarbon profiles could become important for understanding ant communication.

Variations in the cuticular hydrocarbon patterns between different samples,

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*i.e.* individual ants, colonies, or species, have to be investigated by statistical procedures, which take into consideration the variations within the same sample and between samples from different sources. Such methods are termed pattern recognition and are considered to fall into the domain of chemometrics[10]. Properties of different samples can be related to each other basically in two ways. In certain cases it is necessary to investigate how a particular property changes as a function of some external variable. This method is termed continuous property analysis. The change in profile in the cuticular hydrocarbons of an insect as a function of geographic location or season is an example. In cases where it is important to point out systematic differences between different types of samples, discontinuous property analysis is applied. The desired result is a clustering of the most distinguishing properties of the sample sets, and the task essentially is treated by principles of information theory[11].

Pattern recognition is a process whereby a hidden property of a collection of objects (in this case species, colonies, etc.) can be detected and/or predicted by using indirect measurements on the individual objects[12].

In most cases, a single, discriminating measurement cannot be found. Only a combination of measurements provides sufficient information. When dealing with a small number of measurements (three or less), the human perception is the best pattern recognizer. However, when the number of objects and measurements greatly exceeds three or four, the problem can only be handled successfully by using computerized pattern recognition procedures.

Pre-packaged computer programs are available for such purposes. The program used in this application, termed ARTHUR (available from Kowalski*, at nominal charge), consists of several subprograms, some of which are particularly suitable for cluster analysis. Although ARTHUR runs on main frame computers, software designed for microcomputers is currently being introduced, bringing these techniques well within the range of small, applications-oriented laboratories.

In pattern recognition, patterns of profiles such as gas chromatograms are examined. The data measured, *i.e.* gas chromatographic (GC) peaks, are called features. The same measurements are performed on each individual sample, giving rise to a series of chromatograms which differ by the magnitude and/or the nature of the peaks. Samples which are closely related usually share the same peaks and only differ in their relative magnitudes. Pattern recognition studies are usually conducted in a series of steps. In the first step, known as preprocessing, a calibration set containing objects of known class are characterized numerically. This is done by converting the GC data (*i.e.* retention times and peak areas) into ordered vectors, called data vectors or pattern vectors[13,14]. The resulting data matrix is known as a training set. The objects of unknown class(es) are then characterized in a similar fashion, to form a test set. The second step involves deriving a mathematical model from each of the training sets. This then allows the test set to be classified. The whole process is called supervised learning.

The basic goal of all pattern recognition analyses is to associate or recognize unobservable properties of samples with a set of observable properties provided by

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the data vectors[12]. In this case, the unobservable property is the unknown identity or class of an ant and the observable properties are the data vectors resulting from the GC profile of the insect.

It is advantageous in this process to favor the features which carry the largest amount of information. If additional features are used, noise or information unrelated to the problem of interest is introduced into the system. This increases the difficulty of the analysis and may also detract from its reliability. The process of choosing which features to use is called feature selection[10,13,14]. The principal feature selection methods used for this work were autoscaling and Fisher weighting. Autoscaling prevents the data set from being biased by the average sizes or magnitudes of the features. Fisher weighting assigns greater importance to those features which vary little within a given category, but vary a great deal over the entire data set[13].

It is practically impossible for a person to visualize the data, when displayed in $n$-dimensional space (where $n > 3$). Therefore, computers are used to project an approximation of the points from $n$-space into two-dimensional space, to permit visual inspection of the data. This procedure is known as non-linear mapping and is achieved by taking non-linear combinations of the $n$ coordinates of the $n$ data vectors[15].

The ARTHUR package provides the four classifications methods used in this application. These methods are the Bayes method, the $K$-nearest neighbor (KNN) method, the linear learning machine (LLM) method and the SIMCA method (named for statistical isolinear multicomponent analysis).

In the Bayesian classification, for each class, as well as over all objects in the training set, the frequency distributions of each feature are determined. This allows a probability measure, which describes the fit of an object to a class, to be estimated, based upon how well the data vector elements of the object fit the class frequency distributions. The probability of each object is calculated for each class and the object is assigned to the class with the highest probability.

In the KNN procedure, classification is based upon the distance of a sample to its $K$-nearest neighbors. The objects are considered as points in an $n$-dimensional hyperspace, where $n$ is equal to the number of measurements made on each object. This procedure is based on the assumption that nearness in space between two points is a good measure of similarity between the corresponding objects[12].

The LLM procedure determines $(Q-1)$ hyperplanes with a dimensionality of $(M-1)$, using a feedback procedure in such a way that the different classes fall on different sides of the hyperplanes[16].

The SIMCA method is related to methods of factor analysis and principal components analysis. Additional details are available elsewhere[10,14].

## EXPERIMENTAL

### *Samples*

The fire ants used in these studies were collected from nests located in Pickens county, in west central Alabama, U.S.A.

Samples were prepared using a recently described dynamic headspace analysis procedure. The details of this method are described elsewhere[17]. In each study, a single ant was placed in a quartz sample tube in a Pyroprobe® (CDS 100, Chemical

Data Systems, Oxford, PA, U.S.A.), which was then inserted into the inlet of a gas chromatograph. The cuticular hydrocarbons were desorbed from the specimen by rapid heating of the Pyroprobe to 300°C and maintaining that temperature for 5 s. Besides the cuticular hydrocarbons, other compounds (such as the venom alkaloids) are desorbed as well. However, these components either lie outside the diagnostic region of the chromatogram, or they can be eliminated from the chromatogram by using a selective detector such as a mass spectrometer[17].

*Gas chromatography*

The gas chromatograph used was a Hewlett-Packard 5830A, fitted with an injector port suitable to accept the Pyroprobe insert. The gas chromatograph was controlled by a 18850A GC terminal. The column used was a 16 m × 0.25 mm I.D. WCOT glass capillary, coated with a 0.25-$\mu$m film of immobilized OV-1. The column was temperature-programmed from 80 to 300°C at 8°C/min. The carrier gas was helium, at a flow-rate of *ca.* 1.0 ml/min. The split ratio was set to *ca.* 100:1. Each sample was treated with 250 ng of dotriacontane ($C_{32}H_{66}$) as an internal standard.

*Data analysis*

The retention time and area of each hydrocarbon peak was encoded onto a computer card. All of the data were then transferred to a UNIVAC mainframe computer for data handling. This process, however, can (and has been) done on-line[18]. At present, we are in the process of adapting our system so that the data can be collected directly from the gas chromatograph by a microcomputer and then transferred to the mainframe computer for data handling.

The data were entered in a format compatible with the SETUP GC transducing program[19], which also served to adjust chromatographic retention times for proper feature assignment. The card-image data were saved on disk and magnetic tape. Seven marker peaks were assigned from peaks which occurred in all of the chromatograms. Marker peaks are a form of internal standardization. Peaks designated as markers may be internal standards, or peaks which are common to all the chromatograms. The data were transduced into a multivariate form by the SETUP program using the conditions given in Table I. The resulting data set consisted of 49 data vectors, each containing 52 features. *S. richteri* was represented by 29 data vectors, obtained from 4 queens, 6 alate males, and 19 workers. This species is designated category 1 in the pattern recognition treatment. *S. invicta* was represented

TABLE I

ADJUSTABLE PARAMETERS USED FOR DATA SET TRANSDUCTION BY SETUP

| *Parameter* | |
|---|---|
| Maximum allowed retention time error for matching peaks | 0.10 min |
| Number of marker peaks per chromatogram | 7 |
| Minimum retention time distance between non-redundant features* | 0.05 min |
| Minimum frequency of occurrence of acceptable features* | 0% |

* Features are based on normalized peak areas.

by 20 data vectors, obtained from 1 queen and 19 workers. *S. invicta* was designated category 2.

This data set was submitted to the ARTHUR chemometric package for analysis[20]. The features were autoscaled, and an investigation was made to determine the number of features which gave the best classification results, as judged by the separation of the two clusters. Fisher weighting was used as a basis for the selection of data sets consisting of the two, three, four and six most discriminating features of the original data set. From each of these data sets non-linear maps were generated, in order to visualize the separations in the resulting data spaces. The maps generated from the three most discriminating features that had the best separation and were chosen for further processing.

The ability of this three-dimensional data space to classify properly chromatographic patterns of species/classes unknown to the computer, but known to the investigator, was examined using four discrete category classification methods contained in the ARTHUR chemometrics package. The four methods used were: the Bayes method, the KNN method, the LLM method and the SIMCA method.

## RESULTS AND DISCUSSION

The optimum number of features was determined by plotting non-linear maps containing the two, three, four and six most significant features. The optimum was observed to be at around three or four features. The effect of including more features was that the noise level apparently increased without concomitant improvement in information. On the other hand, two features did not carry as much information as three or four. A non-linear map of the three-dimensional set, shown in Fig. 1, shows fairly tight clustering. It should be noted that the cluster formed by the *S. richteri* data set is more diffuse than that of the *S. invicta* set. This can be attributed to the

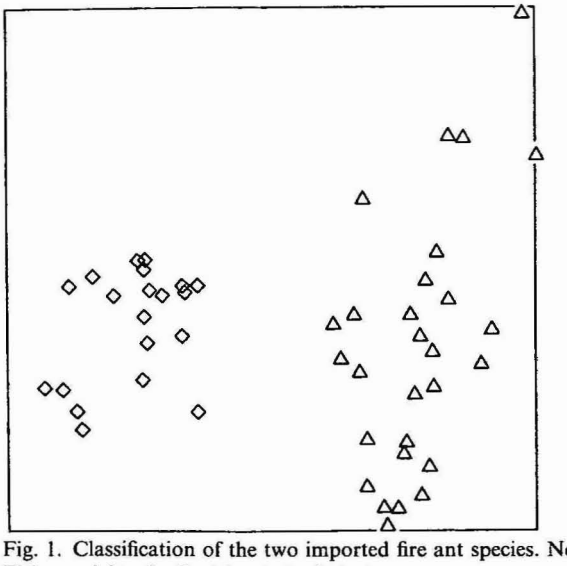

Fig. 1. Classification of the two imported fire ant species. Non-linear map of the three best features, by Fisher weight. △, *S. richteri*; ◇, *S. invicta*.

inclusion of males, queens, and workers, which clearly results in a more heterogeneous distribution.

The ability of this three-dimensional set to classify correctly an unknown sample was tested by dividing the original data set into five training set–test set combinations. The computer was given the proper classifications in the training sets. It was then given test patterns from samples known only to the observer and asked for a classification. All data from the original data set were used as test set patterns at least once. Table II reports on classification probabilities using the Bayes, KNN, LLM and SIMCA methods. No errors were encountered with Bayes, KNN and SIMCA methods, but a few misclassifications occurred with the LLM procedure. The overall results are quite acceptable.

Fig. 2 shows a typical chromatogram for each of the two species. The three

TABLE II

RESULTS OF CLASSIFICATION TEST METHODS

Number of runs: category 1 = 29; category 2 = 20. Category 1 = *S. richteri*; category 2 = *S. invicta*.

| *Method* | *Category* | *Training set* | | *Test set* | | *Overall percent correct* |
|---|---|---|---|---|---|---|
| | | *No. of misses* | *Percent correct* | *No. of misses* | *Percent correct* | |
| KNN | 1 | 0 | 100 | 0 | 100 | 100 |
| ($K$ = 10) | 2 | 0 | 100 | 0 | 100 | 100 |
| LLM | 1 | 0 | 100 | 3 | 89.6 | 97.9 |
| | 2 | 0 | 100 | 0 | 100 | 100 |
| SIMCA | 1 | 0 | 100 | 0 | 100 | 100 |
| | 2 | 0 | 100 | 0 | 100 | 100 |
| Bayes | 1 | 0 | 100 | 0 | 100 | 100 |
| | 2 | 0 | 100 | 0 | 100 | 100 |

most discrininating features are labelled. The chemical identities of these compounds have not yet been established, but are under study.

The particular application presented here may not necessarily require a computer, since the difference between the cuticular hydrogen profiles of *S. invicta* and *S. richteri* are easily discernable by visual inspection. Rather, it is used to illustrate a principle and method which is generally applicable. There are, however, many applications examples where the computerized system clearly is necessary and superior to human perception. In cases where the different classes have very similar patterns, computerized pattern recognition procedures are required. The identification of discriminating features between different colonies of the same species, the examination of any seasonal variation with a colony, and the study of the profiles of hybrid species are examples of such situations.

We have proceeded further with these studies. Part II of these studies examines the cuticular hydrocarbon profiles of different colonies of the black imported fire ant, *Solenopsis richteri*.

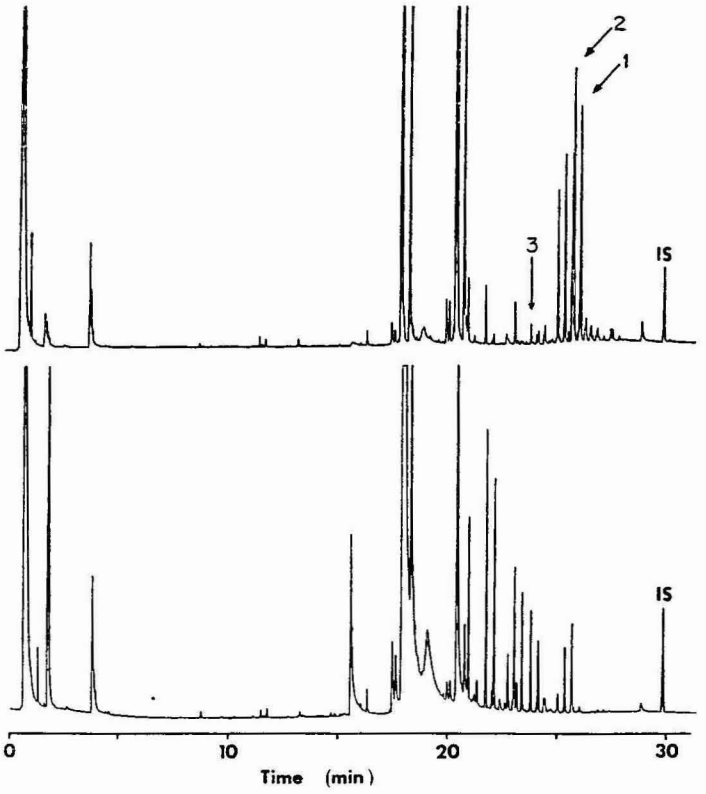


Fig. 2. Top, cuticular hydrocarbon profile of *S. invicta*; bottom, cuticular hydrocarbon profile of *S. richteri*; IS, internal standard. Peaks 1, 2 and 3 are the three most discriminating features, by Fisher weight, between the hydrocarbon profiles of the two species/classes. Note: peaks between 15 and 21 min represent venom alkaloids.

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REFERENCES

1 N. F. Hadley, G. L. Blomquist and U. N. Lanham, *Insect Biochem.*, 11 (1981) 173.
2 G. L. Blomquist and L. L. Jackson, *Prog. Lipid Res.*, 17 (1979) 319.
3 J. B. Lok, E. W. Cupp and G. L. Blomquist, *Insect Biochem.*, 5 (1975) 821.
4 D. R. Nelson, J. W. Dillwith and G. L. Blomquist, *Insect Biochem.*, 11 (1981) 187.
5 R. W. Howard and G. L. Blomquist, *Ann. Rev. Entomol.*, 27 (1982) 149.
6 D. R. Nelson, C. L. Fatland, R. W. Howard, C. A. McDaniel and G. L. Blomquist, *Insect Biochem.*, 10 (1980) 409.
7 R. K. Vander Meer and D. P. Wojcik, *Science (Washington, DC)*, 218 (1982) 806.
8 E. O. Wilson, *The Insect Societies*, Harvard University Press, Cambridge, MA, 1971, p. 272.
9 M. S. Blum, *Bull. Entomol. Soc. Amer.*, 20 (1974) 30.
10 B. R. Kowalski, *ACS Symp. Ser.*, 52 (1977) 14.

11 E. Shannon and W. Weaver, *The Mathematical Theory of Information*, University of Illinois Press, Urbana, IL, 1947.
12 B. R. Kowalski and C. F. Bender, *J. Amer. Chem. Soc.*, 94 (1972) 5632.
13 D. L. Duewar, J. R. Koskinen and B. R. Kowalski, *Documentation for ARTHUR, Version 1-8-75, Chemometrics Society Report No. 2,* Laboratory for Chemometrics, Department of Chemistry, University of Washington, Seattle, WA, 1975.
14 P. C. Jurs and T. L. Isenhour, *Chemical Applications of Pattern Recognition,* Wiley, New York, 1975.
15 B. R. Kowalski and C. F. Bender, *J. Amer. Chem. Soc.*, 95 (1973) 686.
16 N. B. Nilsson, *Learning Machines*, McGraw-Hill, New York, 1965.
17 J. H. Brill and W. Bertsch, *Insect Biochem.*, 15 (1985) 49.
18 H. Engman, H. T. Mayfield, W. Bertsch and T. Mar, *J. Anal. Appl. Pyrol.*, 6 (1984) 137.
19 H. T. Mayfield and W. Bertsch, *Computer Applications in the Laboratory*, 2 (1983) 130.
20 D. L. Duewar, J. R. Koskinen and B. R. Kowalski, *ARTHUR*, obtained from B. R. Kowalski, Laboratory for Chemometrics, Department of Chemistry, University of Washington, Seattle, WA, 1975.

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# USE OF COMPUTERIZED PATTERN RECOGNITION IN THE STUDY OF THE CUTICULAR HYDROCARBONS OF IMPORTED FIRE ANTS

## II. COMPARISON OF THE CUTICULAR HYDROCARBON PATTERNS BETWEEN DIFFERENT COLONIES OF *SOLENOPSIS RICHTERI*

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SUMMARY

Gas chromatography (GC) data obtained from the cuticular hydrocarbons of the black imported fire ants are treated by methods of pattern recognition. Based on a recently described sample preparation procedure, GC data are normalized to eliminate slight variations in chromatographic conditions, and converted to the proper format for discriminant analysis by computer. The results of several methods of data treatment and display are discussed, based on the chemometrics system package, ARTHUR.

## INTRODUCTION

The cuticular hydrocarbons of insects have been shown to be important compounds in the chemical communication of insects, besides their major function of preventing desiccation[1–3]. The cuticular hydrocarbons of the imported fire ants have been characterized. They have been shown to be complex mixtures containing homologous series of monomethyl and dimethyl alkanes[4,5]. It has been suggested that the cuticular hydrocarbons are involved in species and caste recognition[3,6]. Another function of the cuticular hydrocarbons might be in colony recognition. Worker and soldier ants are extremely sensitive to alien colony odors. Alien intruders in a colony appear to be detected by contact chemoreception[3,7,8], which would indicate that the cuticle plays a role in recognition. If the cuticular hydrocarbons do play a role in intraspecific colony recognition, then it can be expected that there might be differences in the cuticular hydrocarbon profiles from different colonies.

The cuticular hydrocarbon profiles from a number of different colonies of the black imported fire ant, *Solenopsis richteri*, were investigated using a number of chemometric data treatment methods, in order to determine if there are significant differences between the profiles from different colonies. These methods have been discussed in an earlier publication[9].

Initially, a relatively small data set incorporating the hydrocarbon profiles from three different colonies was used. After examining the results, it was then de-

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cided to expand the data set to contain the profiles of ten different colonies in order to determine whether similar results could be obtained with a relatively large data set.

## EXPERIMENTAL

### *Samples*

The black imported fire ants used were collected from nests in Pickens county, west central Alabama, U.S.A. In the first part of these studies, samples from three different nests were collected. Two of the nests were nearest neighbors (located *ca.* 5 m apart) and the third nest was located *ca.* 24 km away. For convenience, the two nearest neighbor colonies will be designated A and B, while the third colony will be designated Y.

In the second part, 10 colonies were collected in Pickens and Lamar counties, Alabama, as well as in east-central Mississippi (Moxube and Lowndes counties). These included four pairs of nearest neighbor colonies.

Samples were prepared using a recently described solventless procedure. Details of this method have been described elsewhere[9,10].

### *Gas chromatography*

The samples were examined by gas chromatography (GC), using a Hewlett-Packard 5830A gas chromatograph, fitted with an injector port suitable to accept the Pyroprobe insert. The column used was a 16 m × 0.25 mm I.D. glass capaillary, coated with a 0.25-$\mu$m film of immobilized OV-1 (prepared according to the method described by Grob and Grob[11]). The column was temperature-programmed from 80° to 300°C at 8°C/min. The carrier gas was helium, at a flow-rate of *ca.* 1.0 ml/min. The split ratio was set to *ca.* 100:1. Each sample was treated with an internal standard, containing 250 ng each of tricosane ($C_{23}H_{48}$) and dotriacontane ($C_{32}H_{66}$).

GC–mass spectrometry (GC–MS) was also performed on a number of the samples. The system used was a Hewlett-Packard 5985A instrument, operated in the electron impact (EI) mode. The column was a 15 m × 0.32 mm I.D. fused-silica capillary, coated with a 0.10-$\mu$m film of DB-5 phase (J&W Scientific, Rancho Cordova, CA, U.S.A.). Other chromatographic conditions were as above.

### *Data analysis*

From each of the three colonies, 10–15 workers of roughly the same size were selected. These workers were analyzed individually by Pyroprobe dynamic headspace analysis[10]. The retention time and absolute area for each peak in the diagnostic (hydrocarbon) region of each chromatogram were encoded onto computer cards and the data, together with the marker peaks for the SETUP program[12], were transferred to the mainframe computer. The SETUP parameters for transducing the data set into multivariate form are listed in Table I. The resulting data set consisted of 33 data vectors, comprising 40 features. Colony A was represented by thirteen data vectors and was designated as category 1. Colony B was represented by ten data vectors and was assigned category 2, while colony Y consisted of ten data vectors and was designated category 3.

The raw data set (designated as data set 1a) was submitted to the ARTHUR

TABLE I

ADJUSTABLE PARAMETERS USED FOR DATA SET TRANSDUCTION BY SETUP

| *Parameter* | |
|---|---|
| Maximum allowed retention time error for matching peaks | 0.10 min |
| Number of marker peaks per chromatogram | 7 |
| Minimum retention time distance between non-redundant features* | 0.05 min |
| Minimum frequency of occurrence of acceptable features* | 0% |

* Features are based on normalized peak areas.

program for chemometric analysis. Various preprocessing and feature selection procedures were examined, in an attempt to enhance the discriminating ability of the data in the raw data set, 1a. A new data set, designated 2a, was obtained by first autoscaling the data in 1a, then weighting all the data vectors, using the Fisher weighting method. Finally, a third data set, designated 3a, was obtained from data set 2a by determining the number of weighed features which gave the greatest discrimination between the categories. After visual inspection of the non-linear maps, four features were found to be the most significant.

Various classification methods were tested, to determine the discriminating ability of the data in set 3a. Table II lists the training set–test set combinations used in the classification tests. Classification testing was performed using the *K*-nearest neighbor method (KNN), the linear learning machine method (LLM), the SIMCA method and the Bayes method.

The data from the GC profiles of the ten colonies were treated in the same manner as that for the three colonies, with the following changes: the classification tests were omitted, since the non-linear maps showed discernible clustering of the different categories. The data set resulting from the SETUP treatment consisted of

TABLE II

TRAINING SET–TEST SET COMBINATIONS FOR THE CLASSIFICATION METHODS TESTED

$R$ = (No. of training set patterns)/(No. of features).

| *Method* | *Category 1 (colony A)* | | *Category 2 (colony B)* | | *Category 3 (colony Y)* | |
|---|---|---|---|---|---|---|
| | *Training set* | *Test set* | *Training set* | *Test set* | *Training set* | *Test set* |
| KNN ($K$ = 1) | 52 ($R$ = 13) | 13 | 40 ($R$ = 10) | 10 | 40 ($R$ = 10) | 10 |
| LLM | 104 ($R$ = 26) | 26 | 80 ($R$ = 20) | 20 | 80 ($R$ = 20) | 20 |
| SIMCA | 52 ($R$ = 13) | 13 | 40 ($R$ = 10) | 10 | 40 ($R$ = 10) | 10 |
| Bayes | 52 ($R$ = 13) | 13 | 40 ($R$ = 10) | 10 | 40 ($R$ = 10) | 10 |

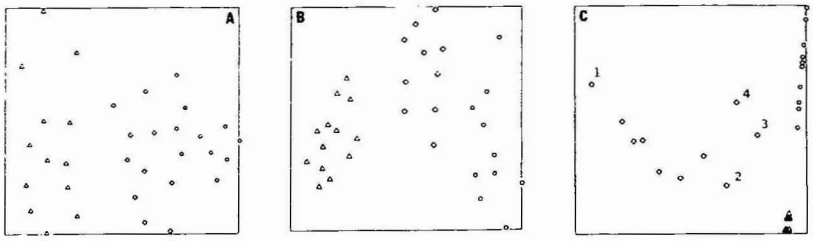


Fig. 1. Non-linear maps of hydrocarbon profiles from three colonies of the black imported fire ant. A, raw data set 1a; B, Fisher weighted data set 2a; C, best four features by Fisher weight data set 3a. 1–4 in C are outliers (see text). △ = Colony A; ○ = colony B; ◇ = colony Y.

160 data vectors, comprising 52 features. Each category (colony) was represented by an average of 16 data vectors.

## RESULTS AND DISCUSSION

### *Comparison of the profiles of three colonies*

The clustering of the categories is improved with the various prepocessing procedures. The non-linear map (NLM) of the raw data for the three colonies, 1a, shows incomplete separation of the three categories (see Fig. 1A). The data for the two nearest neighbor nests appear to be separated, but the pattern vectors for category 3 are interspersed between them. Thus, there is a poor separation among the three categories.

After the first preprocessing step, in which the data vectors were weighed using the Fisher method, there was a marked improvement in the separation in data set 2a. Fig. 1B shows a separation beginning to appear between all three categories, as the clustering becomes tighter.

The final preprocessing step was feature selection. It was determined that four features (by Fisher weight) gave the greatest discrimination among the categories. Fig. 1C shows the NLM for data set 3a, in which the three colonies were compared, using the four best features as the basis for discrimination. The two nearest neighbor nests are well separated, colony A showing a very tight clustering. Colony Y, which was located some 24 km from the other two colonies, shows a rather scattered clustering.

Fig. 2 shows the cuticular hydrocarbon profiles for the three colonies. The four most discriminating features are indicated by the arrows. Feature 1 appeared to be a branched hydrocarbon eluted between heptacosane and octacosane. Feature 2 was identified as *n*-hexacosane, while feature 3 proved to be *n*-heptacosane. Feature 4 appeared to be a branched hydrocarbon, eluted between pentacosane and hexacosane.

The clustering of the data for colony Y is rather scattered. This is due to a number of factors. There are differences in the cuticular hydrocarbon profiles of individual ants from the same colony[13]. This would preclude the data vectors in a colony profile from overlapping completely, even if the experimental errors were negligible. The GC profiles for colony Y were examined visually. Sometimes, small and apparently insignificant peaks (features) have a great diagnostic value in discrim-

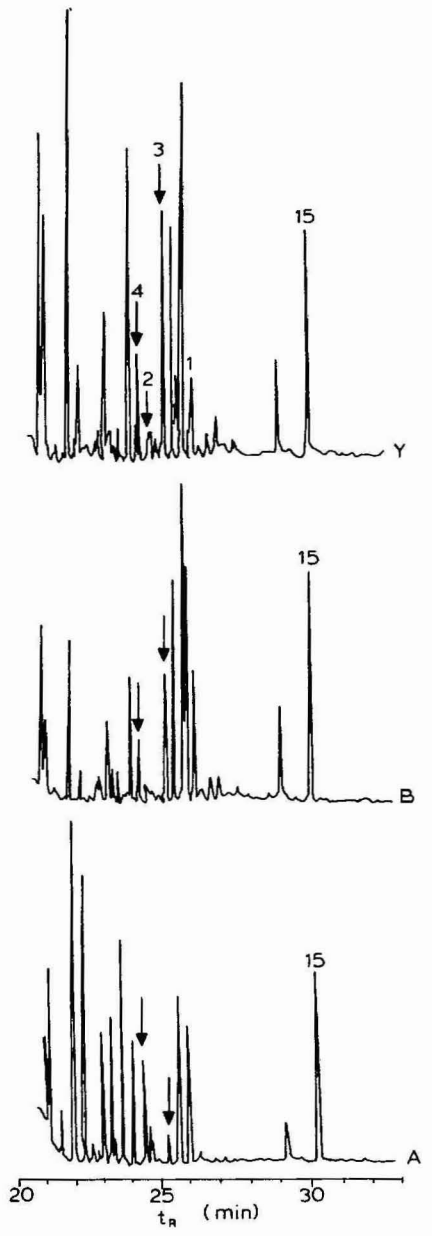


Fig. 2. Comparison of the cuticular hydrocarbon profiles of three colonies of the black imported fire ant. Colonies A and B are nearest neighbors (5 m apart). Colony Y was located *ca.* 24 km away. The four best features (by Fisher weight) are indicated by 1–4. IS, internal standard (dotriacontane).

inating between two or more patterns. This is the case in these studies. Feature 1 in the profile of colony Y is a small peak on the shoulder of a large peak (see Fig. 2Y). This presents an integration problem. In a few of the chromatograms, the integrator

TABLE III

RESULTS OF CLASSIFICATION TEST ON THREE COLONIES OF THE BLACK IMPORTED FIRE ANT (DATA SET No. 3a)

Number or runs: category 1 = 13; category 2 = 10; category 3 = 10. Category 1 = colony A, category 2 = colony B, category 3 = colony Y.

| *Method* | *Category* | *Training set* | | *Test set* | | *Overall percent correct* |
|---|---|---|---|---|---|---|
| | | *No. of misses* | *Percent correct* | *No. of misses* | *Percent correct* | |
| KNN | 1 | 0 | 100 | 0 | 100 | 100 |
| ($K$ = 1) | 2 | 0 | 100 | 0 | 100 | 100 |
| | 3 | 6 | 85 | 1 | 90 | 86 |
| LLM | 1 | 0 | 100 | 0 | 100 | 100 |
| | 2 | 0 | 100 | 0 | 100 | 100 |
| | 3 | 0 | 100 | 2 | 90 | 98 |
| SIMCA | 1 | 0 | 100 | 0 | 100 | 100 |
| | 2 | 0 | 100 | 0 | 100 | 100 |
| | 3 | 9 | 77.5 | 2 | 80 | 78 |
| Bayes | 1 | 0 | 100 | 0 | 100 | 100 |
| | 2 | 0 | 100 | 0 | 100 | 100 |
| | 3 | 0 | 100 | 4 | 60 | 87.9 |

did not detect the peak as a separate peak. As a result, it was considered to be missing from these chromatograms by the pattern recognition procedure. Feature 2 in the Colony Y profile is a small peak located in the middle of a group of five small peaks which are very close together (see Fig. 2Y). Once again, this situation presents integration problems. In addition, in some of the chromatograms the peaks were fused, and it was difficult to determine whether there were three, four or five peaks in the group.

Four outliers were identified in the colony Y data cluster (see Fig. 1C). The chromatograms of these outliers were examined carefully in order to determine why they were possibly outliers. For example, the chromatogram for outlier 1 showed a problem with feature 1, which was too small a shoulder peak to be integrated and was thus considered to be missing by the pattern recognition routine. In addition, feature 2 was larger than average. In the other outliers, similar problems were observed, in which one or more of the features differed significantly from the average for the category profile.

Table III shows the results of the various classification tests performed on data set 3a. The results show excellent classification for colonies A and B (nearest neighbors). Classification was less than 100% for colony Y. This is perhaps due to the rather scattered clustering of the data, the reasons for which have been discussed above. The overall results are quite acceptable.

*Comparison of the profiles of ten colonies*

Fig. 3 shows a comparison of NLM for the $n$ best features, by Fisher weight (where $4 \leqslant n \leqslant 8$). Fig. 3A shows the NLM of the raw data set, before weighing.

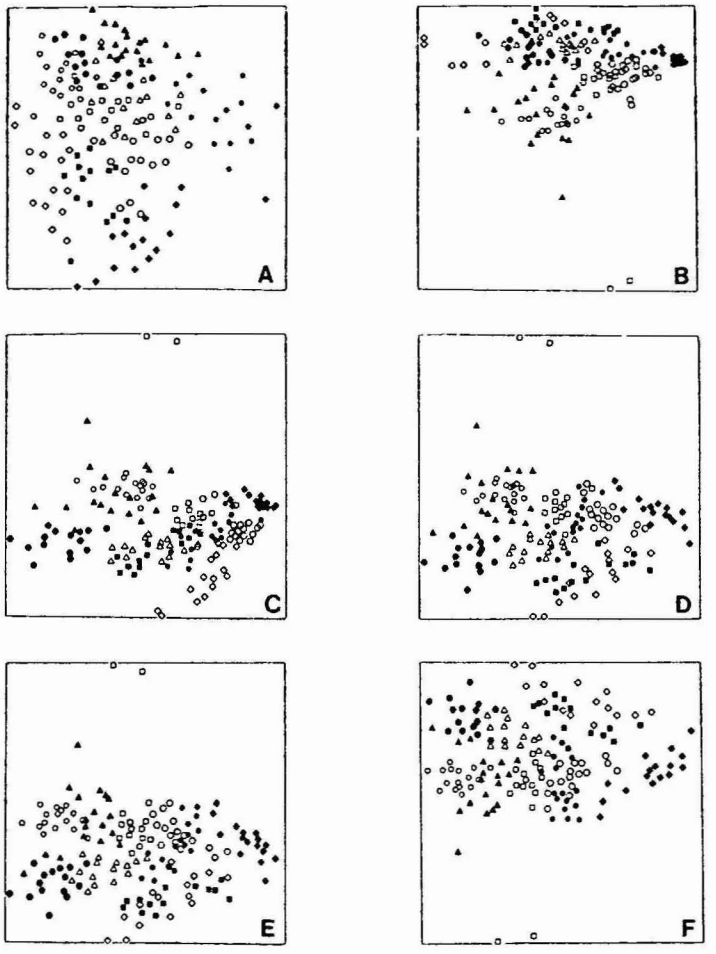


Fig. 3. Non-linear maps of the hydrocarbon profiles of 10 colonies of the imported fire ant. A, raw data; B, best four features; C, best five features; D, best six features; E, best seven features; F, best eight features (by Fisher weight). Nearest neighbor nests are ● and △; ◆ and ○; ■ and □; ○ and ◇. The category assignments were as follows: 1, ◆; 2, △; 3, ◆; 4, ○; 5, ■; 6, □; 7, ▲; 8, ○; 9, ◇; 10, ⬢.

The separation between the ten categories is poor in this plot. After Fisher weighting, feature selection shows an improvement as the number of features selected is increased. An optimum number of features is reached, where the separation among the different categories is maximized. Thereafter, increasing the number of features results in a deterioration in the category separations, due to an increase in noise in the system. This can be seen by comparing Fig. 3B through F. The optimum number of features was *ca.* 5 (see Fig. 3C). However, the categories are not completely separated from one another. This is possibly due to there being a relatively large number (10) of categories. Moreover, the ten profiles are from the same species and are not greatly different from one another. Nonetheless, as was observed in the comparison of three colonies, the nearest neighbor colony profiles showed the greatest separation (see Fig. 3C).

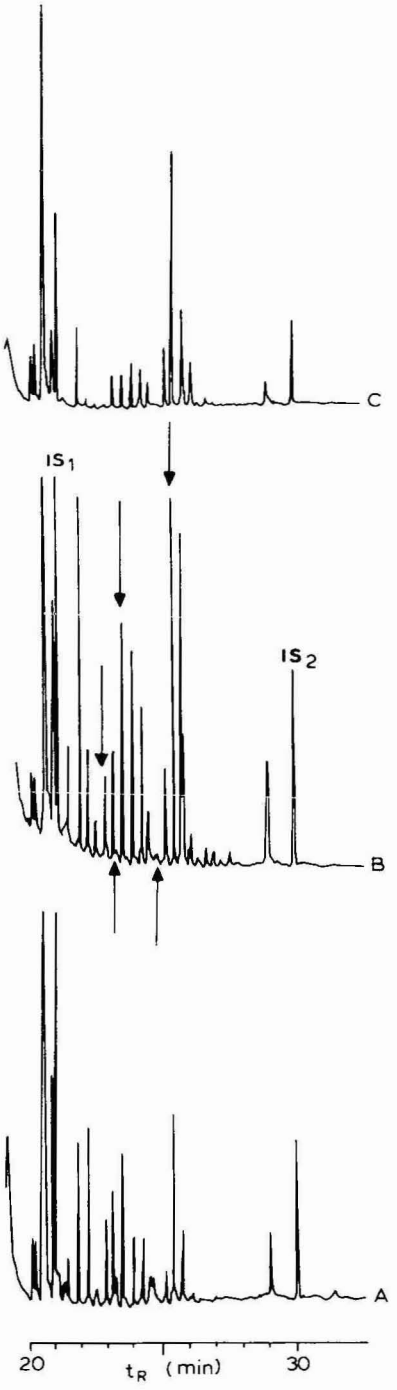


Fig. 4. Hydrocarbon profiles from three of the ten colonies of the black imported fire ant. Arrows indicate the best five features, by Fisher weight. B and C were nearest neighbor colonies.

In the NLM's (see Fig. 3, category 6) it appeared that some outliers were present. The profiles corresponding to these outliers were examined more closely. The same problems as discussed in the comparison of the three colonies were observed here, *i.e.*, in the chromatogram of each of the outliers there were some peaks which were larger and/or smaller than average. In addition, there were also small peaks located on the shoulders of much larger peaks, which created integration problems (see Fig. 4).

An additional consideration concerns the NLM procedure itself. NLM is a method whereby a multi-dimensional data space is reduced to a two-dimensional space for display purposes. This reduction can be accomplished only approximately. Every data point in the multi-dimensional data space (where $n \gg 3$) has a distance from every other data point. In NLM, these distances are calculated and then considered to be constants. Upon reduction to two dimensions, NLM attempts to preserve the interpoint distances by minimizing an error function, using a non-linear minimization method[14,15]. It must be remembered that NLM is a method for displaying multi-dimensional data in a form which is easily perceived by the human observer. If classes overlap in the display, they may still be separated in multi-dimensional space by anyone of a number of classification methods.

Fig. 4 shows the hydrocarbon profiles from three of the ten colonies. The five most discriminating features are indicated by the arrows. The profiles from the nearest neighbor colonies show marked differences from each other (compare Fig. 4B and C). This is also the case in the comparison of the three colonies (compare Fig. 2A and B).

These studies show that there are distinct and significant differences between the hydrocarbon profiles of different colonies of the black imported fire ant. These differences appear to be most pronounced between colonies which are nearest neighbors. Workers from neighboring colonies are much more likely to encounter one another while out foraging than workers from more distant nests. Therefore, if the cuticular hydrocarbons are involved in colony recognition, it is expected that differences in the hydrocarbon profiles would be observed between neighboring colonies. However, the degree of territoriality depends on the number of queens in the colony[7,16]. Nests which contain only a single egg-laying queen display a well-defined territoriality and react aggressively towards towards other colonies of the same species. In contrast, polygynous colonies show little aggressiveness and are quite amicable toward neighboring colonies of the same species. It was not determined whether the colonies sampled in these studies were monogynous or polygynous.

Workers and brood from two neighboring colonies were collected and maintained in the laboratory for a while. The hydrocarbon profiles of the two colonies were very different from each other. It was observed that workers (especially the foragers) from these two colonies quickly recognized and reacted aggressively toward each other. The queens from these two colonies wsere not taken; thus it was not possible to determine whether these colonies were monogynous or polygynous.

ACKNOWLEDGEMENTS

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## REFERENCES

1 N. F. Hadley, G. L. Blomquist and U. N. Lanham, *Insect Biochem.*, 11 (1981) 173.
2 G. L. Blomquist and L. L. Jackson, *Prog. Lipids Res.*, 17 (1979) 319.
3 R. W. Howard and G. L. Blomquist, *Ann. Rev. Entomol.*, 27 (1982) 149.
4 J. B. Lok, E. W. Cupp and G. L. Blomquist, *Insect Biochem.*, 5 (1975) 821.
5 D. R. Nelson, C. L. Fatland, R. W. Howard, C. A. McDaniel and G. L. Blomquist, *Insect Biochem.*, 10 (1980) 409.
6 R. K. Vander Meer and D. P. Wojcik, *Science* (Washington, D.C.), 218 (1982) 806.
7 J. W. S. Bradshaw and P. E. Howse, in J. W. Bell and R. T. Carde (Editors), *Chemical Ecology of Insects*, Sinauer Assoc., Sunderland, MA, 1984, p. 443.
8 E. O. Wilson, *The Insect Societies*, Harvard University Press, Cambridge, MA, 1971, p. 272–278.
9 J. H. Brill, T. Mar, H. T. Mayfield and W. Bertsch, *J. Chromatogr.*, in press.
10 J. H. Brill and W. Bertsch, *Insect Biochem.*, 15 (1985) 49.
11 K. Grob and G. Grob, *J. Chromatogr.*, 213 (1981) 211.
12 H. T. Mayfield and W. Bertsch, *Comput. Appl. Lab.*, 2 (1983) 130.
13 J. H. Brill, *Ph.D. Dissertation*, 1985, in preparation.
14 B. R. Kowalski and C. F. Bender, *J. Am. Chem. Soc.*, 94 (1972) 5632.
15 B. R. Kowalski and C. F. Bender, *J. Am. Chem. Soc.*, 95 (1973) 686.
16 D. J. C. Fletcher, personal communication.

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CHROMSYMP. 669

# MICROCOMPUTER PROGRAMMING IN BASIC FOR THE EVALUATION OF CAPILLARY GAS CHROMATOGRAPHY IN THE ANALYSIS OF PESTICIDE RESIDUES

## I. MATRIXCOMP—A PROGRAM THAT FACILITATES THE RECOGNITION OF INTERFERING PEAKS FROM THE BIOLOGICAL MATRIX

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SUMMARY

Chromatograms of pesticide residues in food include peaks produced by pesticides and matrix compounds. Pesticide peaks are recognized by means of relative retention times and response factors; two detectors are used and internal standard methods are applied. Chromatograms of reference samples for all types of food are stored as raw data in a reduced format, together with tables of all chromatographic data for the matrix compounds. MATRIXCOMP provides the analyst with the chromatograms of the actual sample and the reference in parallel on a visual display screen for visual comparison. Simultaneously, the relevant chromatographic data for the sample, the reference and the calibration tables are displayed on a second screen page in a condensed form.

## INTRODUCTION

In the analysis of pesticide residues in food, chromatograms include peaks produced by pesticides and peaks resulting from compounds from the biological matrix. The background chromatograms, representing the substances passing through the clean-up together with the pesticides, vary considerably with the variety of food analysed. Although the provenance might be different, background chromatograms produced by the same type of food show sufficient resemblance.

In this paper we describe a computer program designed to assist the analyst in evaluating the actual chromatogram. Chromatographic peaks from the biological matrix can be distinguished from those produced by pesticides simply by comparing the chromatogram of the actual sample with one of a food of the same type and similar origin, which was known to be free of pesticide contamination.

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## METHODS

After a standardized clean-up[1], analysis of pesticide residues in food was performed by using a gas chromatograph (Sichromat 2, Siemens, Karlsruhe, F.R.G.), capillary columns, and effluent splitting to two selective detectors, an electron-capture detector and a flame photometric detector[2]. The signals from the detectors were transferred via an analog-to-digital converter to the microcomputer system (Trilab 2500, Trivector, Niederolm, F.R.G.), automatically processed by the manufacturer's software package, and stored as raw data and result files on a floppy disk.

### *Computer configuration*

Our Trilab 2500 is a chromatographic data system, incorporating a visual display unit (VDU), 288 kbyte RAM, twin floppy disk drives (each diskette with 640 kbyte) and one 10 mbyte hard disk unit. The system includes a software package for evaluating all kinds of chromatographic data files and a BASIC interpreter.

### *Program*

With the help of our program, MATRIXCOMP, actual chromatograms can be compared with the background chromatograms from foods of the same type and from the same region. These reference samples have been carefully checked to be free of residues of those pesticides, available as standards. In our laboratory a mass spectrometer is coupled to a gas chromatograph to enable us to detect amounts in the parts per billion range. The chromatograms of the reference samples are catalogued and stored on the hard disk unit in a reduced format. Identification of the pesticides that might contaminate the sample is performed by means of a table with retention times and response factors of nearly 200 substances.

The MATRIXCOMP program covers 32 kbyte and works with four internal memory files for the chromatographic raw data of the actual sample and reference sample, as well as for the corresponding result tables.

Program parts, generally used for automated processing of chromatographic data, are combined with subprograms designed to apply the analyst with tools for the visual comparison of the actual and reference sample on the visual display screen.

The analyst is conducted through the program by menus, which offer the following functions:

(1) Table of the chromatograms, stored on the hard disk, with name, variety, origin, and date of input;

(2) Table of the retention times and ratios of response factors for the actual sample;

(3) Corresponding table for the reference sample;

(4) Corresponding table for nearly 200 calibrated pesticides;

(5) The two chromatograms for the visual comparison are displayed in parallel;

(6) Expansion of critical parts of each chromatogram;

(7) Cursor-controlled call-up of chromatographic data of significance to the analyst;

(8) Manual input and actualization of the chromatographic data for the pesticides in the calibration table.

TABLE I

COMPILATION OF CHROMATOGRAPHIC DATA CALLED UP BY THE CURSOR, ACTIVATED IN THE SAMPLE CHROMATOGRAM

Abbreviations: RT = retention time; RRT = retention time relative to the internal standard aldrin; ECD = response relative to the internal standard aldrin; FPD = response relative to the internal standard O-2-naphthyldimethylthiophosphinate; ECD/FPD = ratio of the two response facors. Peaks with similar retention times. Retention time at the position of the cursor: 16.18.

| | *RT* | *RRT* | *ECD* | *FPD* | *ECD/FPD* |
|---|---|---|---|---|---|
| Actual sample | | | | | |
| | 16.18 | 0.9304 | 0.640 | 0.003 | 213.3 |
| | 16.34 | 0.9396 | 0.221 | 0 | 0 |
| Reference sample | | | | | |
| | 16.49 | 0.9396 | 0.094 | 0 | 0 |
| Calibration table | | | | | |
| Metribucin | | 0.9197 | 0.642 | 2.788 | 0.231 |
| Vinclozolin | | 0.9303 | 1.057 | 0.005 | 211.3 |
| Alachlor | | 0.9448 | 0.120 | 0 | 0 |

A special help-function offers the analyst a list of the available orders with a short explanation of their functions. The handling of all numerical and graphical data is very convenient, because the analyst has a choice between three screen pages. All outputs can be examined on the screen or may be printed with a plotter.

A normal raw-data file consists of 3000 to 4000 points, representing a chromatogram of 30 to 40 min. This high resolution is necessary for the accurate calculation of peak areas, for baseline corrections, and other manipulation routines. Reference chromatograms destined for the visual comparison on the screen can usually be catalogued as 1000 data points. This reduction minimizes the memory space necessary for the chromatogram library on the hard disk. Another aspect is that the screen resolution in the horizontal direction is limited to 1000 points. Therefore, the reduction does not influence the visual information supplied on the screen for comparing chromatograms.

## RESULTS

How the program is used was demonstrated by applying it to an analysis of pesticide residues in a real food sample. The cleaned sample was injected into the gas chromatograph and the effluent was split to the two selective detectors. The signals were recorded in parallel and processed by applying the "Trilab" software. By means of the calibration table, small amounts of vinclozolin and procymidone were identified at retention times of 16.18 and 19.30 min in the actual sample (indicated by arrows in Fig. 1). In the MATRIXCOMP program the corresponding electron-capture chromatogram of a reference sample was searched and loaded from the hard disk. The two chromatograms were displayed together on the screen (Fig. 1).

The lower half shows a chromatogram of an actual sample of strawberries from Italy: the upper half the corresponding reference. The two chromatograms show

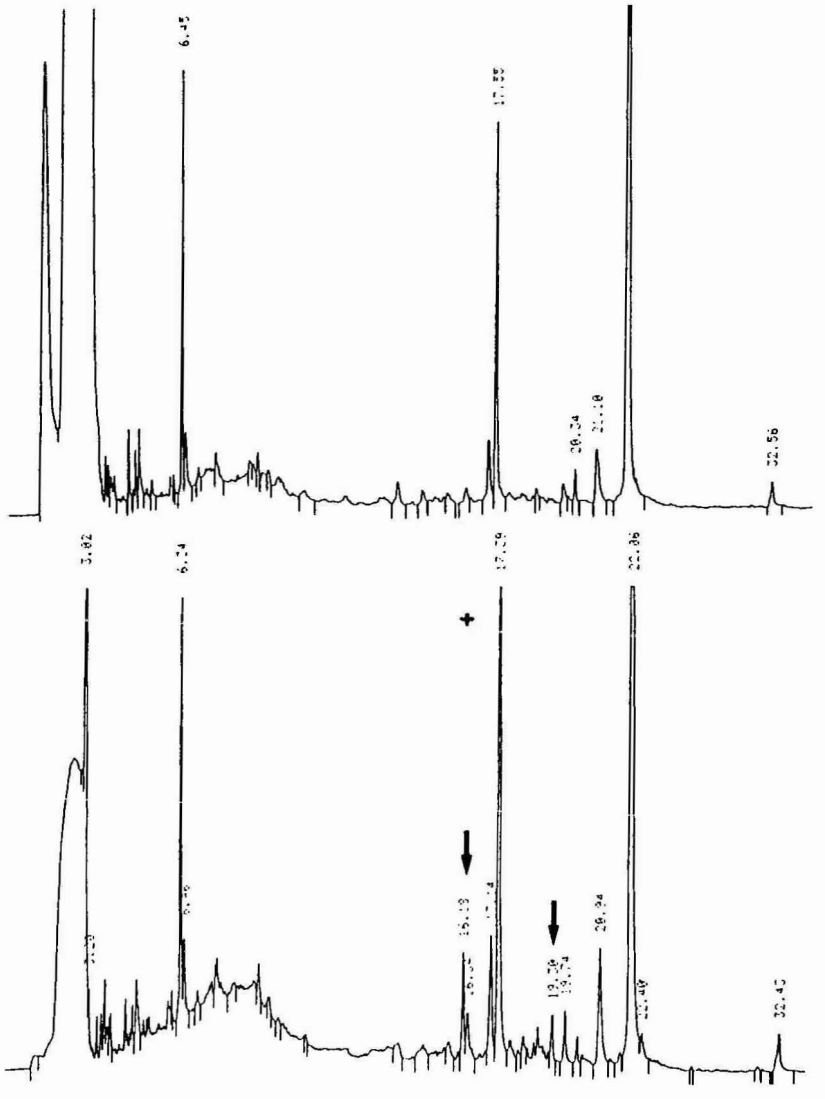

Fig. 1. Electron-capture chromatograms from strawberries from Italy. Top, reference sample; bottom, actual sample with activated cursor and two peaks from pesticides, indicated by arrows.

a characteristic resemblance in their pattern of the major peaks. Two of them are plasticizers, which are typical contaminants in pesticide residue analysis. The peak at a retention time of 6.34 min as well as the smaller peak at 32.43 min are permanent contaminants in our chemicals. The internal standard aldrin appears at 17.39 min with a smaller satellite at 17.14 min.

These four peaks were found in all our analyses and form a typical background frame for all chromatograms. The largest peak in both chromatograms is another plasticizer, which contaminates all packed strawberries from Italy this year. In ad-

dition to this significant peak pattern, a number of smaller peaks, similar in retention time and appearance, can be found in both chromatograms.

By means of the subprogram "cursor", a small cross (Fig. 1) is activated. It can be moved all over the screen by pressing the cursor keys. The cross is used to indicate individual peaks. By pressing the return key the data listed in Table I are displayed on the second screen page. Simultaneously, this information is prepared for peaks in a specified retention window for the reference sample and for all calibrated pesticides in the same range.

Provided with the relevant information in a very condensed format, the analyst must decide whether the indicated peak is produced by a calibrated pesticide, an unknown matrix compound, a common environmental contaminant or perhaps an unexpected pesticide. The confirmatory procedure for calibrated pesticides by use of effluent splitting and two-dimensional capillary GC was described elsewhere[2–4]. If an unknown pesticide cannot be ruled out, gas chromatography–mass spectrometry must be used for identification. An additional tool for handling chromatograms is the expansion procedure, which enables the analyst to study selected parts of the chromatogram in more detail.

## DISCUSSION

Multi-residue pesticide analysis in a whole range of foods is performed by standardized extraction and clean-up procedures. In most laboratories the vast majority of samples is divided into just two groups: food samples with low fat content and food samples with high fat content. This means that the clean-up procedures must remove a variety of matrix compounds in nearly 100 types of food. At the same time, the clean-up procedures must not remove any of the more than 200 pesticides that can be analysed by GC. It is surprising to what a great extent the clean-up procedures now in use in connection with selective detection in GC fulfil this requirement. However, several peaks produced by matrix compounds are found in all chromatograms.

The aim of our MATRIXCOMP program is to facilitate the recognition of such interfering peaks in screening for pesticide residues. The evaluation follows the same line as that used in routine analysis, where experienced analysts collect data on interfering substances in the biological matrix in order to avoid wasting their time in hunting chimeras. The advantage of our program is that the information is as complete as possible. Not only retention times, but the entire background chromatograms from the two detectors and all interesting chromatographic data are available for the evaluation of actual chromatograms.

Although the MATRIXCOMP program was developed for pesticide analysis in food, it also might be useful for other environmental samples, the determination of the provenance of mineral oil or standardization in quality control.

## REFERENCES

1 G. Becker, *Dtsch. Lebensm.-Rundsch.*, 75 (1979) 148–152.
2 H.-J. Stan and D. Mrowetz, *J. Chromatogr.*, 279 (1983) 173–187.
3 H. Goebel and H.-J. Stan, *J. Chromatogr.*, 279 (1983) 523–532.
4 H.-J. Stan and H. Goebel, *J. Automat. Chem.*, 6 (1984) 14–20.

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CHROMSYMP. 662

# DETERMINATION OF TRACE CHLORINATED BENZENES IN FUEL OIL BY ON-LINE MULTIDIMENSIONAL CHROMATOGRAPHY USING PACKED-CAPILLARY LIQUID CHROMATOGRAPHY AND CAPILLARY GAS CHROMATOGRAPHY

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## SUMMARY

The application of a multidimensional chromatography system with a packed-capillary liquid chromatographic (LC) column, coupled on-line with a capillary gas chromatograph, for the determination of trace chlorinated benzenes in fuel oil is described.

The liquid chromatograph is utilized as a highly efficient clean-up step prior to introduction of the sample into the gas chromatograph. This significantly reduces sample preparation times and demonstrates that an on-line high-resolution LC–gas chromatography (GC) system is superior to either LC or GC alone, since the analyses described could not be accomplished by either of the two techniques used independently. The detection limits obtained ranged from 8.0 to 17 $\mu$g/g for the various chlorinated benzenes.

## INTRODUCTION

The determination of minor components in complex hydrocarbon matrices can be accomplished by techniques that involve extensive preseparation procedures, such as liquid–liquid extraction[1], derivatization prior to gas chromatographic (GC) separation, and mass spectrometric detection[2], or by the use of element-selective detectors, such as the nitrogen–phosphorus detector[3] or Hall conductivity detector[4] if the components of interest contain detectable functional groups.

Multidimensional open tubular GC systems, in which a selected cut, eluted from one column is switched into a second column of different selectivity (heart cutting) have already been described[5,6]. More recently, the use of a multidimensional process, consisting of liquid chromatography (LC) and GC was reported for the characterization of gasoline[7]. However, using conventional LC columns, only small fractions of a peak could be introduced into the gas chromatograph. The present work describes the use of an on-line multidimensional system, consisting of highly efficient, packed-capillary LC columns, coupled with a capillary gas chromatograph[8] and its application to the determination of trace chlorinated benzenes in fuel oil. The use of this system allows complete fractions of the LC effluent to be introduced

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reproducibly into the capillary gas chromatograph without loss in resolution or detrimental effects on peak shape.

## EXPERIMENTAL*

The system used is essentially the same as that described previously[8], where the liquid chromatograph consisted of a Jasco Uvidec II detector with a modified cell with an illuminated volume of 6 nl. Injections were made with a Valco Model N14W injection valve (Valco, Houston, TX, U.S.A.). Injection volumes were 60 nl. The solvent delivery system consisted of a Waters Model M-45 pump, equipped with a micro-flow module (Waters Assoc., Milford, MA, U.S.A.); the flow-rate was 10.6 $\mu$l/min. The columns were constructed of fused-silica capillaries with an internal diameter of 250 $\mu$m (Spectran, Sturbridge, MA, U.S.A.), 105 cm long. Columns were packed with Zorbax (DuPont, Wilmington, DE, U.S.A.) silica (particle diameter, 7 $\mu$m) at 6000 p.s.i. in a 20% (w/v) slurry in methyl iodide–pentane (1:1). The LC mobile phase used through this work was heptane (distilled-in-glass, Burdick & Jackson, Muskegon, MI, U.S.A.).

The GC system consisted of a Hewlett-Packard Model 5790 chromatograph, equipped with a flame-ionization detector. Connection of the micro liquid chromatograph to the gas chromatograph was made with a ten-port valve (Valco Model 4NI10WT) to keep the dead-volume to a minimum. The valve was installed outside of the oven cabinet. An inlet section of the fused-silica capillary column, free of stationary phase, was connected between the switching valve and the analytical column. This retention gap, as previously described[9], effectively focused the components of interest at the head of the column and allowed the introduction of large volumes into the capillary GC column without detrimental effects on the peak shape of the components. A glass-lined stainless-steel butt connector (Scientific Glass Engineering, Austin, TX, U.S.A.) was used to couple the retention gap to the analytical column with essentially no dead-volume.

The analytical column used was a 30 m × 0.25 mm I.D. column, coated with Supelcowax 10 of 0.25 $\mu$m thickness (Supelco, Bellefonte, PA, U.S.A.). The carrier gas was helium at a linear velocity of 70 cm/s. Nitrogen at 30 ml/min was used as the make-up gas for the flame-ionization detector, operated at 275°C. The oven temperature was 105°C for 7 min, with a temperature programme to 245°C at 5°C/min.

The resolution obtained with packed-capillary LC was compared to that obtained with a conventional system, which consisted of a Altex Model 110A pump, a Rheodyne Model 7125 injection valve, equipped with a 20-$\mu$l loop, a Perkin-Elmer LC-75 variable-wavelength ultraviolet detector, a Hewlett-Packard Model 3380 integrator, and a 250 × 4.6 mm I.D. Partisil-10 silica column (Whatman, Hillsboro, OR, U.S.A.). The eluent consisted of heptane, at a flow-rate of 2.0 ml/min. The detector was set at 214 nm.

## DISCUSSION

A standard, containing the chlorinated benzenes of interest (chlorobenzene,

* The method and apparatus described in this publication are the subjects of pending patent applications.

1,2-dichlorobenzene, 1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, and hexachlorobenzene) at concentrations of 50–100 μg/10 ml, was prepared and injected into the conventional LC system described in the Experimental section. A sample of the fuel oil, prepared by dissolving 1.00 g in 10.0 ml of heptane, was analyzed in the same manner. Fig. 1 shows the chromatograms obtained. As can be observed, only hexachlorobenzene could be marginally separated from the sample matrix. A standard solution of the chlorobenzenes (500–1000 μg/10 ml) and the solution of fuel oil were then injected into the packed-capillary LC system. Fig. 2 shows

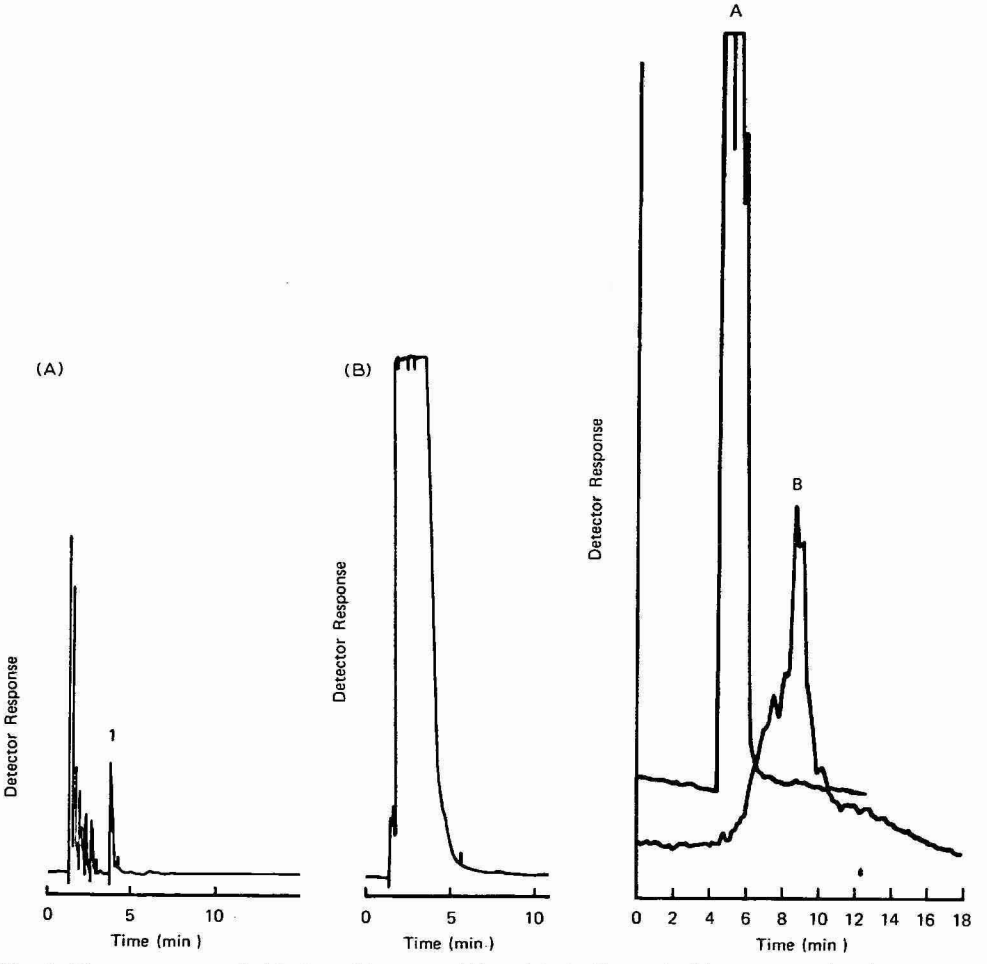


Fig. 1. Chromatograms of chlorinated benzenes (A) and fuel oil sample (B) on conventional system. 1 = Hexachlorobenzene. Column, Whatman Partisil-10 (250 × 4.6 mm I.D.); mobile phase, heptane; flow-rate, 2.0 ml/min; UV at 214 nm; pressure, 1000 p.s.i.; standard, 50–100 μg/10 ml; sample, 1.00 g/10 ml heptane.

Fig. 2. Chromatograms of chlorinated benzenes (A) and fuel oil sample (B) on a packed-capillary column. Column, 105 cm × 250 μm I.D., packed with Zorbax silica (particle diameter, 7 μm); mobile phase, heptane; flow-rate, 10.6 μl/min; detector, Jasco Uvidec II at 214 mm; pressure, 3800 p.s.i.; standard, 500–1000 μg/10 ml.

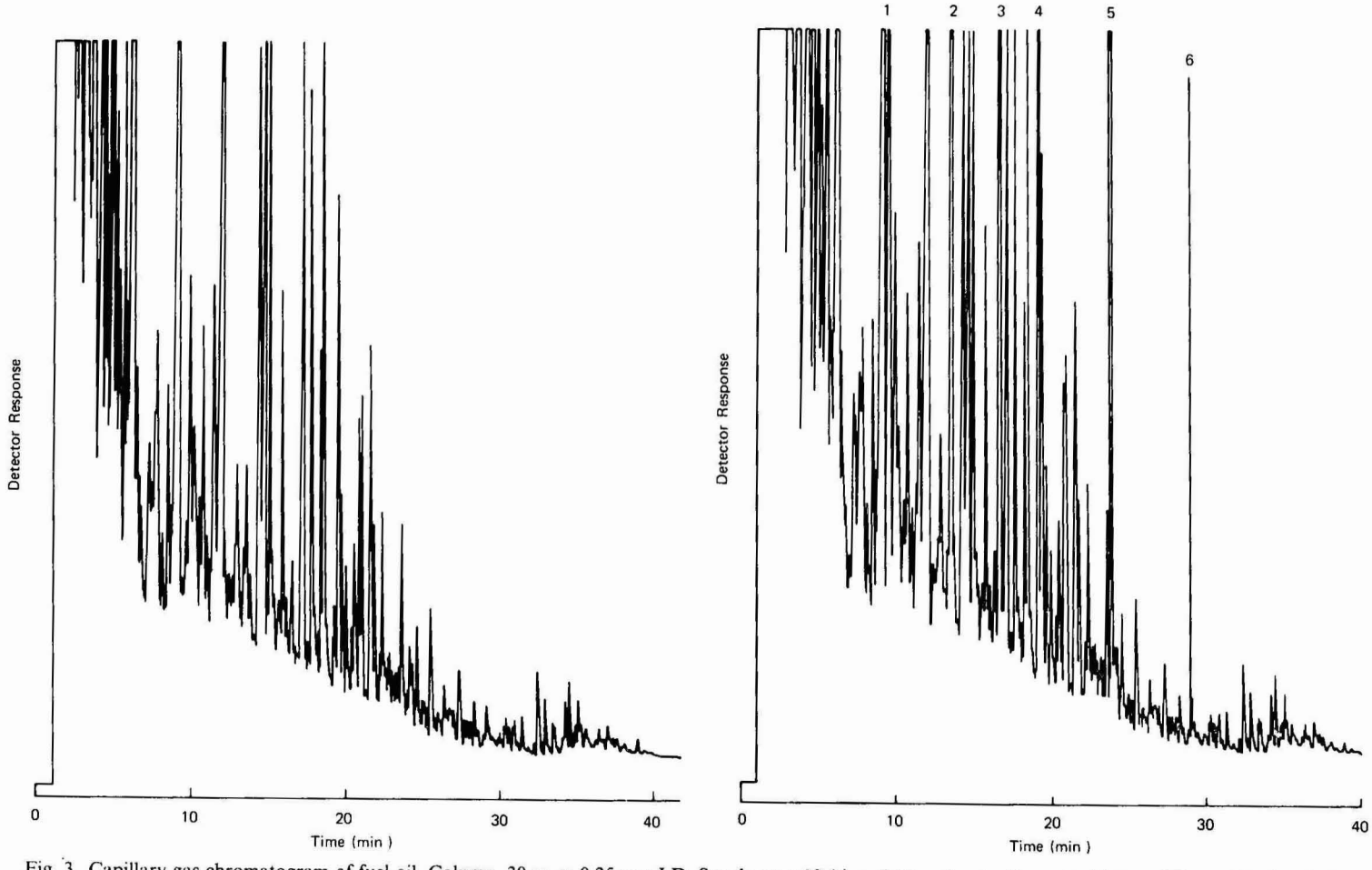


Fig. 3. Capillary gas chromatogram of fuel oil. Column, 30 m × 0.25 mm I.D. Supelcowax 10 ($d_f$ = 0.25 μm); retention gap, 15 m × 250 μm I.D. fused silica; oven temperature, 105°C for 9 min, programmed to 245°C at 5°C/min; carrier, helium at 70 cm/s; make-up gas, nitrogen at 30 ml/min; detector, flame-ionization detector at 275°C; sample size, 10 μl.

Fig. 4. Capillary gas chromatogram of fuel oil, spiked with chlorobenzenes. Conditions as in Fig. 3. Peaks: 1 = chlorobenzene; 2 = 1,2-dichlorobenzene; 3 = 1,2,4,5-tetrachlorobenzene; 4 = 1,2,3,4-tetrachlorobenzene; 5 = pentachlorobenzene; 6 = hexachlorobenzene.

the chromatograms obtained. The chlorobenzenes were separated from the majority of the components in the fuel oil matrix.

A 10-$\mu$l injection of the fuel oil solution was made into the gas chromatograph, using the switching valve described above. The resulting chromatogram is presented in Fig. 3. The fuel oil was then spiked with the chlorobenzene standard, and the resulting solution was chromatographed in the same manner. As can be observed in Fig. 4, the chlorobenzenes of interest are eluted together with various components in the sample matrix when analyzed by capillary GC alone. The liquid chromatograph was then connected to the switching valve, and the selected fraction of the effluent was introduced into the gas chromatograph.

Fig. 5A represents the chromatogram obtained when the chlorobenzene standard was analyzed by packed-capillary LC and indicates the fraction introduced in the gas chromatograph. Fig. 5B is the resulting capillary gas chromatogram, obtained from this LC effluent fraction.

Fig. 6A represents the chromatogram of the fuel oil solution obtained with the LC system. The indicated fraction was introduced into the gas chromatograph, and Fig. 6B shows the resulting chromatogram. The retention times for the chlorinated

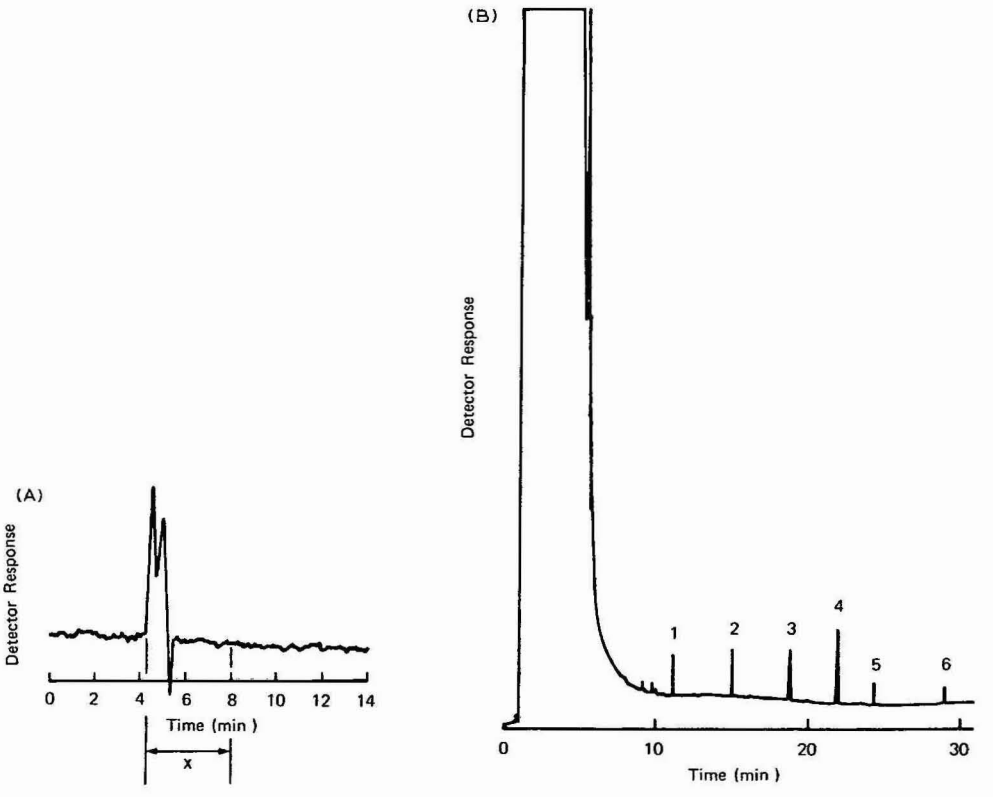


Fig. 5. Chromatograms of chlorobenzene standard. (A) Packed-capillary liquid chromatogram. Conditions as in Fig. 2. Sample, 100 mg/10 ml in heptane. X = Cut introduced into the gas chromatograph. (B) Capillary gas chromatogram. Conditions as in Fig. 3. Sample volume, 22 $\mu$l. Retention times of chlorobenzenes of interest are indicated. Peak identification as in Fig. 4.

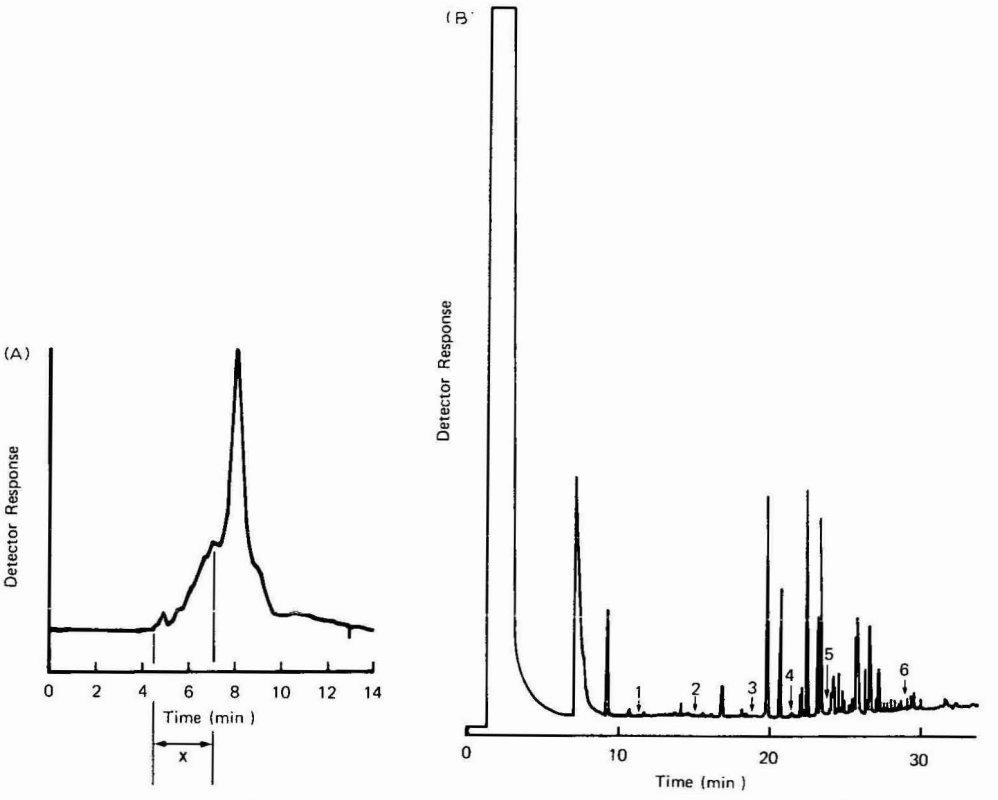


Fig. 6. Chromatograms of fuel oil sample. (A) Packed-capillary liquid chromatogram. Conditions as in Fig. 2. Sample, 1.00 g/10 ml in heptane. X = Cut introduced into the gas chromatograph. (B) Capillary gas chromatogram. Conditions as in Fig. 3. Sample volume, 22 $\mu$l. Retention times of chlorobenzenes of interest are indicated. Peak identification as in Fig. 4.

benzenes of interest are also shown. The volume injected into the gas chromatograph was 22 $\mu$l, and the chlorinated benzenes were resolved from the interferences in the fuel oil matrix without detrimental effects on peak shape.

## CONCLUSIONS

A multidimensional chromatographic system, in which highly efficient packed-capillary LC columns are coupled with a capillary GC column, has been utilized to determine minor components in a complex hydrocarbon matrix without prior sample clean-up or preconcentration steps.

The chlorinated benzenes of interest were found to be absent in the sample analyzed at detection limits of 8.0 to 17 $\mu$g/g, calculated as three times baseline random noise level. On-line coupling of packed-capillary LC and capillary GC offers the advantages of a highly efficient clean-up or chemical class fractionation without prior separation steps, and thus significantly reduces sample preparation time in many applications. The speed of analysis and high degree of resolution obtained demonstrates the superiority on the on-line multidimensional LC–GC system over the use of LC or GC alone.

## REFERENCES

1 J. M. Schmitter, Z. Vajta and P. J. Arpino, *Advances In Organic Geochemistry — 1979*, Pergamon, Oxford, 1980, p. 67.
2 F. P. DiSanzo, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 649.
3 M. L. Lee, K. D. Bartle and M. V. Novotny, *Anal. Chem.*, 47 (1975) 540.
4 L. V. McCarthy, E. B. Overton, M. A. Mayberry, S. A. Antoine and S. L. Laseter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 164.
5 D. R. Deans, *J. Chromatogr.*, 203 (1981) 19.
6 P. A. Schenk and C. H. Hall, *Anal. Chim. Acta*, 38 (1967) 65.
7 J. A. Apffel and H. McNair, *J. Chromatogr.*, 279 (1983) 139.
8 H. J. Cortes, C. D. Pfeiffer and B. E. Richter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, (1985) in press.
9 K. Grob, Jr., *J. Chromatogr.*, 237 (1982) 15.

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CHROMSYMP. 652

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NAPHTHOLS AS 4-AMINOANTIPYRINE DERIVATIVES

## APPLICATION TO CARBARYL

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## SUMMARY

The high-performance liquid chromatographic determination of 1- and 2-naphthol after derivatization with 4-aminoantipyrine is described. The chromatographic analysis of the reaction mixtures showed two main coloured products for each naphthol. In order to investigate the nature of the reaction products a spectroscopic study (UV–VIS, IR, NMR) of the products separated by chromatography was performed. On the more stable 1-naphthol reaction product the quantitative determination of 1-naphthol was performed in the 0.05–10 ppm concentration range. Instability of the 2-naphthol products made their quantitative determination difficult; detection at two different wavelengths (460 and 412 nm) gave a very reliable criterion of qualitative identification. As 1-naphthol is the major degradation product of the insecticide carbaryl, this determination reaction of 1-naphthol was applied to carbaryl. The applicability and performance of the method was checked by determining the carbaryl content in commercial powder formulations and residues in apples.

## INTRODUCTION

The determination of monohydric phenols by high-performance liquid chromatography (HPLC) after derivatization with 4-aminoantipyrine (4-AAP) has been discussed previously[1]. The aim of this study was to examine the derivatization reaction and its analytical utility when the method is applied to 1-and 2-naphthol. The direct determination of naphthols by HPLC with UV detection has been reported[2–4], and the reaction of naphthols with 4-AAP is known[5–9]. As for monohydric phenols[1], the combination of the specificity of the derivatization reaction with the sensitivity and separation power of HPLC seemed to be promising.

1-Naphthol is a degradation product of carbaryl (1-naphthylmethylcarbamate): in fact, both the metabolic processes and alkaline hydrolysis yield 1-naphthol. Carbaryl is a broad-spectrum insecticide, extensively used because of its effectiveness and low mammalian toxicity. Carbaryl may contain 2-naphthylcarbamate as a contaminant, derived from impure 1-naphthol containing 2-naphthol[10]. The determination of trace amounts of 2-naphthol is very important, as it has been found to produce cancerous tumours in rats[11].

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Numerous HPLC methods have been developed for the analysis of carbaryl and 1-naphthol with either UV[12–14] or fluorescence detection[15–17]. In this work, the determination of 1-naphthol as the 4-AAP derivative was employed for carbaryl analysis. The applicability and performance of this method was checked by determining carbaryl in powder formulations and residues in apples.

## EXPERIMENTAL

The analyses were performed with a Spectra-Physics SP 8700 solvent delivery system with an SP 8750 organizer module. A Model 770 spectrophotometric detector at wavelengths variable from 200 to 600 nm and an SP 4270 computing integrator were used. A μBondapak-phenyl column (300 × 3.9 mm I.D.; Waters Assoc.) with methanol–water (65:35) as the mobile phase was chosen for HPLC determinations. Unless otherwise specified, the experimental conditions and derivatization procedures were as previously described[1].

## RESULTS AND DISCUSSION

*Chromatographic study of reaction variables in derivatization*

The reaction variables in the previously described procedure[1] were investigated in order to select the optimum conditions for the naphthol derivatization reaction. The pH range 8.5–10.5 was studied; 1-naphthol gave the maximum yield at pH 9.5 and 2-naphthol at pH 8.5, so a compromise value of 9 was chosen. The Britton–Robinson buffer was then replaced with $NaHCO_3$–$Na_2CO_3$ buffer because of the blank interference at the detection wavelength of the 2-naphthol derivative (412 nm). According to Svobodova and Gasparic[18], the buffer capacity and the reaction yield were the same with the carbonate buffer. A suitable proportion of 4-AAP to oxidizing agent seemed to be 1:3, as previously determined; a greater proportion of 4-AAP increased both the naphthol response and the blank interference.

*Chromatographic study of the reaction mixtures*

Fig. 1 illustrates the chromatographic analysis of the chloroform extracts from reaction mixtures and shows two derivative products for each naphthol under the chromatographic conditions reported above. Other reaction by-products were negligible. Table I reports the capacity factors, $k'$, for naphthol derivatives.

In order to investigate the nature of these coloured products, the chloroform extracts were separated by thin-layer chromatography (TLC). Concentrated solutions containing 5 mg of each naphthol were examined. According to Aly[7], mixtures of methylene chloride and ethyl acetate and of chloroform and ethyl acetate were investigated on silica gel plates. Methylene chloride–ethyl acetate (70:30) gave the best separation of the reaction products. The 2-naphthol chloroform extracts were not sufficiently stable for TLC analysis. Therefore, only the 1-naphthol products were examined.

A red band with $R_F$ 0.3 and an orange band with $R_F$ 0.5 were evident. Their HPLC analysis confirmed the two chromatographic peaks with $k'$ values of 1.96 and 2.59, respectively. The stability of 1-naphthol products was determined. The chromatographic response of the product at $k' = 2.59$ was stable for three days, whereas

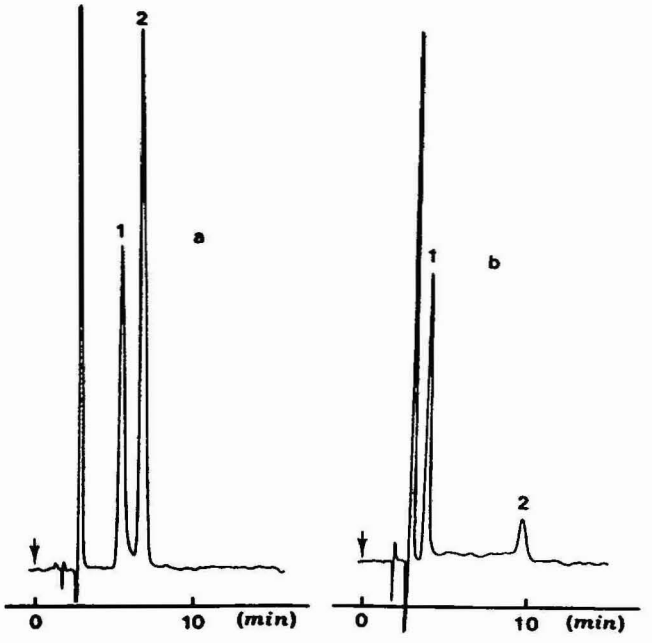


Fig. 1. Chromatographic analysis of (a) 1-naphthol and (b) 2-naphthol derivatives. Conditions as under Experimental; flow-rate, 1.5 ml/min; detector, visible, 460 nm, 1.6 × 0.04 a.u.f.s. Sample: (a) 1-naphthol, 3.30 ppm; (b) 2-naphthol, 10 ppm.

TABLE I

CAPACITY FACTORS ($k'$) FOR NAPHTHOL DERIVATIVES

| *Compound* | *Peak* | $k'$ |
|---|---|---|
| 1-Naphthol | First | 1.96 |
| | Second | 2.59 |
| 2-Naphthol | First | 1.09 |
| | Second | 4.06 |

at the same time that at $k' = 1.96$ was halved. Therefore, the coloured product at $k' = 2.59$ was selected for the quantitative determination of 1-naphthol by HPLC. However, the first peak area, was constant for 3 h after the reaction.

The low stability of the 2-naphthol derivatives allowed their identification but not an accurate quantitative determination by HPLC.

*Spectroscopic study of the reaction products*

The chloroform extracts from the naphthol reaction mixtures, red for 1-naphthol and faint green for 2-naphthol, showed maximum absorption at 460 and 412 nm, respectively. Previous spectrophotometric determinations[5–9] had been carried out at the single wavelength of 500 nm. UV–VIS spectra of both the 1- and 2-naphthol coloured products, separated by HPLC, were recorded.

The 1-naphthol derivative corresponding to the first chromatographic peak ($k'$ = 1.96 and $R_F$ = 0.3) showed maximum absorption at 515 nm, whereas the more stable derivative corresponding to the second peak ($k'$ = 2.59 and $R_F$ = 0.5) showed maximum absorption at 460 nm. For 2-naphthol derivatives, the first eluate ($k'$ = 1.09) had a maximum at 460 nm and the second ($k'$ = 4.06) at 412 nm. Therefore, a wavelength of 460 nm was selected for the chromatographic detection of naphthols.

After separation by TLC, the more stable 1-naphthol derivative ($k'$ = 2.59) was investigated by IR and NMR spectroscopy. The IR spectrum of a chloroform solution showed only one significant band at 1660 $cm^{-1}$ relating to the amide C=O group. A study of the NMR spectrum permitted the identification of the 4-AAP coupling product in the *para* position of 1-naphthol[19].

*Detectability and linearity*

Under the above chromatographic conditions, the detection limit at 460 nm, *i.e.*, the amount injected that gives a peak height equivalent to twice the noise level, was calculated to be 2 ng for 1-naphthol. The calibration graph of peak area *versus* concentration of standard samples was determined following the experimental procedure described previously[1]. The calibration graph was linear in the concentration range 0.05–10 ppm.

For 2-naphthol, the detection limit was calculated to be about 30 ng, while the low stability of its derivatives provided an inaccurate calibration graph. It was possible to detect the presence of 2-naphthol up to a 500:1 ratio between 1- and 2-naphthol. The chromatographic detection at two different wavelengths (460 and 412

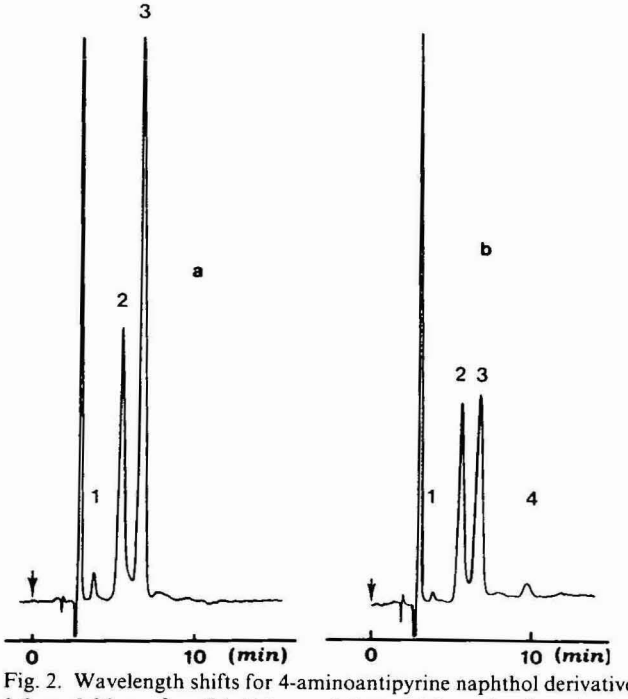


Fig. 2. Wavelength shifts for 4-aminoantipyrine naphthol derivatives. Conditions as in Fig. 1; (a) 460 nm, 1.6 × 0.04 a.u.f.s.; (b) 412 nm, 3.2 × 0.04 a.u.f.s. Sample: 1-naphthol, 3.9 ppm (peaks 2 and 3); 2-naphthol, 1.4 ppm (peaks 1 and 4).

nm) and the measurement of wavelength shifts further confirmed the presence of 2-naphthol. The bathochromic and hypsochromic shifts are shown in Fig. 2 for naphthol derivatives.

*Carbaryl determination*

A spectrophotometric method for carbaryl determination, based on the 4-AAP derivatization of 1-naphthol, has already been developed[9]. Here the same carbaryl hydrolysis conditions were applied. Complete hydrolysis was confirmed by HPLC. The detection limit was calculated to be 3 ng as carbaryl, consistent with the 1-naphthol detection limit. Following the derivatization procedure[1] and the separation conditions described above, carbaryl was determined in powder formulations and as residues in apples.

*Formulation analysis.* According to Appaiah *et al.*[9], 1–4 mg of powder formulation were dissolved in 10 ml of methanol, shaken and filtered. Hydrolysis, the derivatization reaction and chromatographic analysis were carried out as described previously. The features of the chromatograms, recorded at 460 and 412 nm, made it possible to exclude the presence of 2-naphthol in all of the formulations examined. An example is shown in Fig. 3.

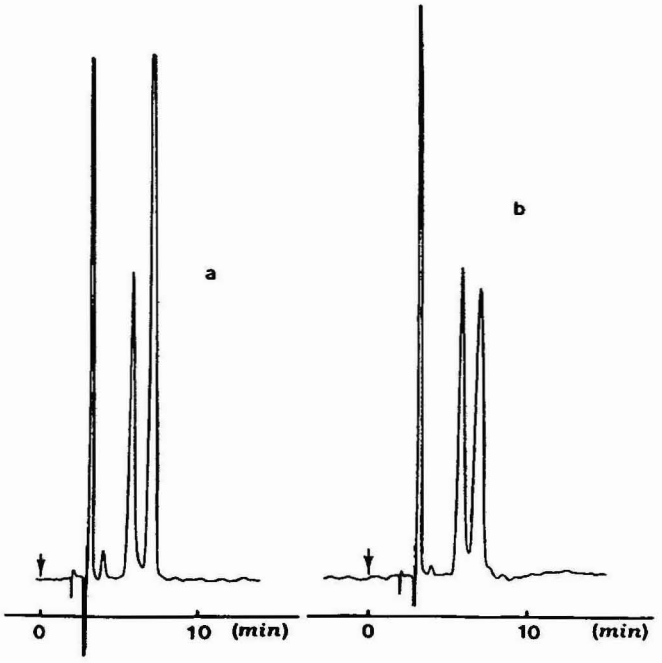


Fig. 3. Use of wavelength shifts of 2-naphthol derivatives for their identification in a carbaryl formulation. Conditions as in Fig. 1; (a) 460 nm, 1.6 × 0.04 a.u.f.s.; (b) 412 nm, 16 × 0.04 a.u.f.s.

*Recovery of residues in apples.* Apples (25 g), blended for 2 min, were extracted with two 50-ml portions of chloroform. The chloroform extracts, combined and filtered, were concentrated to 10 ml in a rotary vacuum evaporator. Chromatographic clean-up on a silica gel-$Na_2SO_4$ column[9] and on a $C_{18}$ Sep-Pak cartridge[3,14] allowed

TABLE II

RECOVERY OF CARBARYL FROM APPLES

| *Amount added (ppm)* | *Amount found* (ppm)* | *Difference (%)* |
|---|---|---|
| 0.20 | 0.19 | −5 |
| 0.50 | 0.52 | +4 |
| 0.60 | 0.60 | 0 |
| 1.00 | 0.95 | −5 |
| 2.00 | 2.03 | +2 |

* Average of three analyses. Repeatability: ± 12–15%.

"clean" apple blank chromatograms to be obtained for different samples. Because it is rapid, clean-up on the cartridge was preferred.

Apple samples were spiked with known amounts of carbaryl (0.2–2 ppm) and then analysed. Recoveries varied from 95 to 104%, as reported in Table II. The detection limit (0.1 ppm) makes this method useful for the determination of carbaryl residues in apples.

## CONCLUSIONS

The HPLC determination of 1-naphthol as 4-AAP derivative is a very selective and sensitive method. This procedure can be applied to the determination of carbaryl. Owing to its high selectivity and sensitivity, it is comparable to the other known methods. The low stability of 2-naphthol products permits only qualitative analysis. Wavelength shifts represent a reliable criterion for identification purposes.

## REFERENCES

1 G. Blo, F. Dondi, A. Betti and C. Bighi, *J. Chromatogr.*, 257 (1983) 69.
2 K. Bhatia, *Anal. Chem.*, 45 (1973) 1344.
3 A. S. Jones, L. A. Jones and F. L. Hastings, *J. Agric. Food Chem.*, 30 (1982) 997.
4 G. K. Chao and J. C. Suatoni, *J. Chromatogr. Sci.*, 20 (1982) 436.
5 M. B. Ettinger, C. C. Ruchhoft and R. J. Lishka, *Anal. Chem.*, 23 (1951) 1783.
6 S. D. Faust and E. W. Mikulewicz, *Water Res.*, 1 (1967) 509.
7 O. M. Aly, *Water Res.*, 2 (1968) 587.
8 J. Farino, G. Norwitz, W. J. Boyko and P. N. Keliher, *Talanta*, 28 (1981) 705.
9 K. M. Appaiah, R. Ramakrishna, K. R. Subbarao and O. Kapur, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 32.
10 R. J. Argauer and J. D. Warthen, *Anal. Chem.*, 47 (1975) 2472.
11 M. A. Zabezhinskii, *Vopr. Onkol.*, 16 (1970) 106.
12 C. M. Sparacino and J. W. Hines, *J. Chromatogr. Sci.*, 14 (1976) 549.
13 G. Blaicher, W. Pfannhauser and H. Woidich, *Chromatographia*, 13 (1980) 438.
14 R. J. Bushway, *J. Chromatogr.*, 211 (1981) 135.
15 R. W. Frei, J. F. Lawrence, J. Hope and R. M. Cassidy, *J. Chromatogr. Sci.*, 12 (1974) 40.
16 R. T. Krause, *J. Chromatogr. Sci.*, 16 (1978) 281.
17 R. T. Krause, *J. Chromatogr. Sci.*, 255 (1983) 497.
18 D. Svobodova and J. Gasparic, *Mikrochim. Acta*, (1971) 384.
19 P. F. Jones and K. E. Johnson, *Can. J. Chem.*, 51 (1973) 2860.

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CHROMSYMP. 674

# SEPARATION OF NATURAL POLAR SUBSTANCES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, CENTRIFUGAL THIN-LAYER CHROMATOGRAPHY AND DROPLET COUNTER-CURRENT CHROMATOGRAPHY

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## SUMMARY

Reversed-phase high-performance liquid chromatography, centrifugal thin-layer chromatography and droplet counter-current chromatography are complementary, highly efficient techniques for isolating natural, very polar, substances from plant sources. The choice of method can be made directly by a preliminary thin-layer chromatographic investigation. An application to the isolation of some new triterpenic glycosides in *Passiflora quadrangularis* L. is described.

## INTRODUCTION

*Passiflora* extracts have been wisely used in the folk medicine of Europe and America because of their sedative and antihypertensive properties. Many *Passiflora* spp. have already been investigated, and some indole alkaloids of the harman type, C- and O-glycosyl flavonoids, cyanogenic glycosides, and a cyclopropane triterpenic glycoside (passiflorine) have been identified[1–5]. In spite of the different classes of compounds found, the active principles of *Passiflora* spp. have not yet been recognized, and pharmacological tests are currently under investigation. Prior to biological testing, we have studied the applicability of a number of isolation methods to the purification of individual constituents of the aqueous soluble fraction of *Passiflora quadrangularis* L.

## EXPERIMENTAL

### *Gel-filtration column chromatography (GFCC)*

Sephadex LH-20 (130 g; Pharmacia) was packed by a slurry technique into a glass column (950 × 30 mm I.D.) and eluted with methanol (300 ml) at a flow-rate of 12 ml/h. A total of 120 2-ml fractions were collected and monitored by thin-layer chromatography (TLC) on silica gel with solvent A.

### *TLC*

Silica gel plates (5 × 10 cm), $F_{254}$ TLC (Merck, Darmstadt, F.R.G.), were

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used. Different eluting systems were applied: (A) chloroform–methanol–*n*-propanol–water (5:6:1:4) (lower layer); (B) ethyl acetate–ethanol–water (8:2:1); (C) chloroform–methanol–*n*-propanol–water (45:60:6:40) (lower layer); (D) chloroform–methanol–water (17:3:2) (lower layer); (E) ethyl acetate–ethanol–water (14:6:3).

For reversed-phase separation, preparative conditions were chosen on reversed-phase thin-layer plates: RP 18 (5 × 10 cm, Merck), eluted with methanol–water (9:1); RP 8 (5 × 10 cm, Merck), eluted with methanol–water (4:1).

*Droplet counter-current chromatography (DCCC)*

Separations were carried out on a Model-A instrument (Tokyo Rikakikai, Tokyo, Japan) using the solvent systems chloroform–methanol-*n*-propanol–water (5:6:1:4) and chloroform–methanol–*n*-propanol–water (45:60:6:40) in the ascending mode. Fractions of 2 ml were collected at a flow-rate of 0.25 ml/h.

*Reversed-phase high-performance liquid chromatography (RP-HPLC)*

A Miniprep Jobin-Yvon liquid chromatograph was used. A 40-g quantity of Lichroprep RP-18 (25–40 μm; Merck) was necessary to fill the column (500 × 20 mm I.D.). Two subsequent separations of *ca.* 430 mg of each sample were carried out on the same column. One litre of methanol–water (17:3) was used for each at a flow-rate of 15 ml/min. Fractions of 7 ml were collected and monitored by TLC on silica gel (solvent A) and RP-18 (methanol–water (9:1)).

*Centrifugal thin-layer chromatography (CTLC)*

Separation was carried out on a Chromatotron Model 7924 T (Harrison Research, Palo Alto, CA, U.S.A.). The starch-bound layer (1 mm) was prepared by mixing 1.2 g of starch (Merck, Art. 1252) with 130 ml of water and 3.0 g of $MgSO_4 \cdot 7H_2O$ (RP; Carlo Erba) and heating the mixture to boiling point. When the solution was clear, silica gel (40 g of $PF_{254}$ + $CaSO_4 \cdot \frac{1}{2}H_2O$) was added in portions, mixed by swirling, then heated for a further 10 min with occasional mixing. The hot mixture was rapidly poured onto the rotor, turning the rotor to avoid unsymmetric loading and tapping the rotor against the cloth-covered surface to release air bubbles. The layer was allowed to dry for at least 48 h at room temperature before drying it in an oven at 70°C for 2 h. After scraping the layer, the rotor was placed on the Chromatotron and repeatedly washed free of inorganic salts with methanol. Complete removal of the two sulphates was controlled with a barium chloride solution. During this operation, some care must be taken to avoid obstruction of the capillary tube. The layer was saturated with the solvents *ca.* 200 ml under our conditions: ethyl acetate–ethanol–water (16:3:2) and then separation could be carried out. The flow-rate was 1.5 ml/min (a faster rate caused too rapid an elution and vitiated resolution). About 20–200 mg of crude products could be applied without loss of resolution. Fractions of 1–2 ml were collected and monitored by TLC on silica gel with solvent B.

## RESULTS

A methanolic extract (13.2 g) of *Passiflora quadrangularis* L. leaves was par-

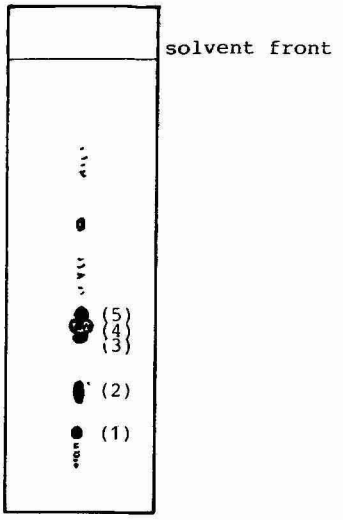
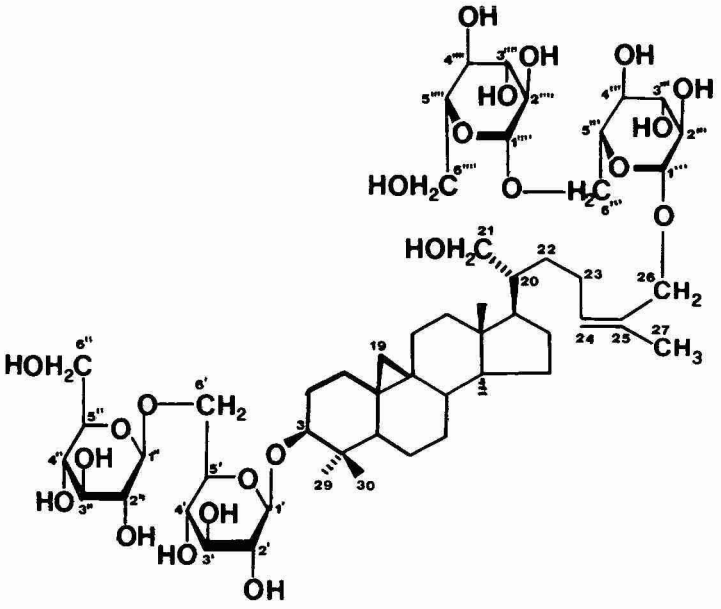



Fig. 1. Thin-layer chromatogram of an aqueous phase of *Passiflora quadrangularis* on Silica gel. Elution system, chloroform–methanol–*n*-propanol–water (5:6:1:4).

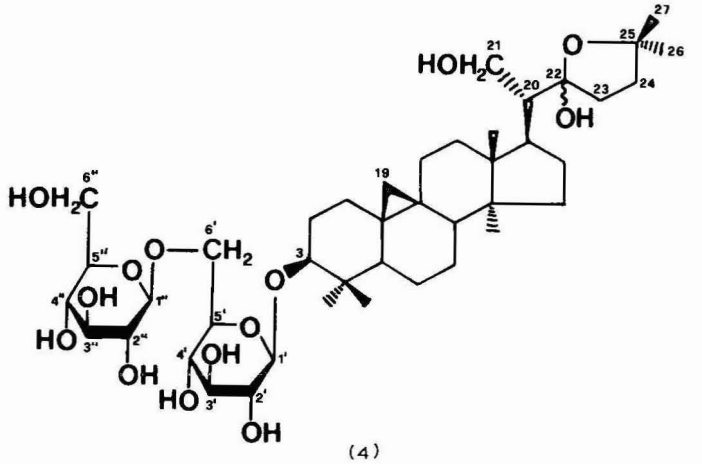
titioned between organic and aqueous phases. The latter (2.60 g) was first investigated by silica gel TLC and showed a series of plum-coloured spots, distributed in the $R_F$ range 0.1–0.4 (see Fig. 1).

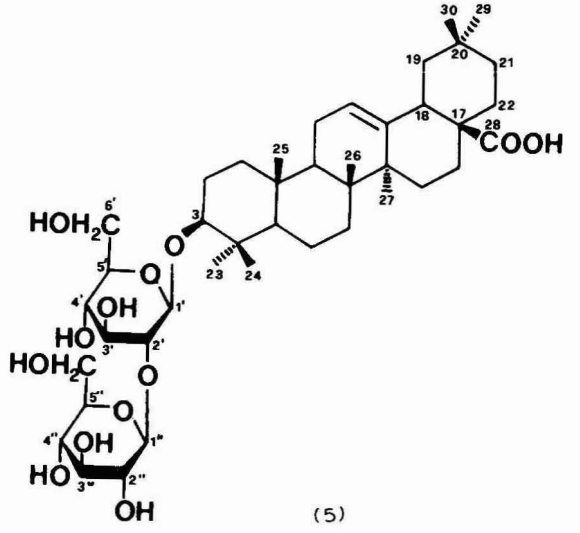
A first fractionation (1.590 g) was carried out by elution of a Sephadex LH-20 column with methanol. Three enriched fractions were obtained (79–98 = 566 mg;



QUADRANGULOSIDE (1)

99–130 = 863 mg; 131–150 = 71 mg). The first one was subjected to DCCC [chloroform–methanol–*n*-propanol–water (5:6:1:4), ascending mode], affording 157 mg of quadranguloside (1) and 58 mg of a mixture (2) (see Fig. 1), which was resolved into three substances by silica gel TLC with different eluting systems (solvents D and E). A preliminary investigation by $^{1}H$ NMR, $^{13}C$ NMR and mass spectroscopy indicated that (2) is related to quadranguloside, but further work is in progress to obtain a larger quantity of product.




The second fraction was subjected to RP-HPLC on the basis of RP-8 and RP-18 TLC. Using a derivatized silica packing (RP-18, 25–40 $\mu$m), 253 mg of compounds (3) + (4) and 400 mg of compounds (5) + (4) were obtained. The latter mixture was subjected to DCCC with chloroform–methanol–*n*-propanol–water (45:60:6:40; ascending mode) and 35 mg of (4) (fractions 155–172) and 150 mg of (5) (fractions 245–273) were isolated.

Repeated CTLC was used to purify 253 mg of (3) + (4) on a starch-bound silica plate, eluted with ethyl acetate–ethanol–water (16:3:2). This allowed us to isolate (3) (fractions 12–17 = 45 mg) and (4) (fractions 7–11 = 130 mg) in pure form.

The structures of the new compounds (1) and (4) isolated were elucidated and compound (5) was identified, both on the basis of acid and enzymatic hydrolysis and on spectral evidence ($^1$H NMR, $^{13}$C NMR and mass spectroscopy) of both hydrolysed and intact glycosides. They have been characterized as 9,19-cyclolanost-24*Z*-en-3$\beta$,21,26-triol,3,26-di-O-gentiobioside [quadranguloside (1)] and 9,19-cyclolanosta-22,25-epoxy-3$\beta$-OH,21,22-triol,-3-$\beta$-O-gentiobioside [22-hydroxy-isoquadranguloside (4)]. Compound (5) was identified as oleanolic acid 3-O-$\beta$-D-glucopyranosyl-(1 → 2)-$\beta$-D-glucopyranoside. It is the first time that (5) has been isolated from a natural source; it was only previously obtained by hydrolysis of natural precursors[6–8].

On submission of this paper, the structure of compound (3) had not been fully ascertained; it will be published elsewhere, together with details on the structure determination of (4) and (5). The structure elucidation of (1) was the subject of a previous paper[9].

Further work is in progress to resolve the minor components.

## DISCUSSION

DCCC is a powerful technique for separating natural, very polar, substances. A quick method for selecting a suitable system consists in subjecting the sample to TLC with the organic layer as eluent[10,11]. Sometimes this technique needs to be supplemented by other methods to improve the separation of complex mixtures.

It has been shown[12–29] that a direct extrapolation of the analytical conditions

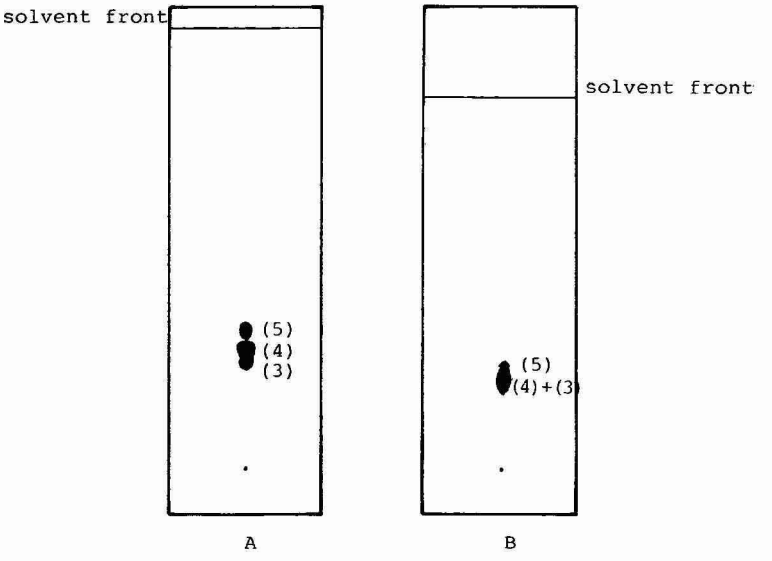



Fig. 2. (A) Separation of fractions 99–130 by RP-TLC on a RP-18 plate, eluted with methanol–water (9:1). (B) Separation of the same sample on a RP-8 plate, eluted with methanol–water (4:1).

in TLC on silica gel or RP layers to a preparative HPLC is possible, since identical or very similar retention mechanisms are responsible for the separation of the respective substances in both chromatographic methods. However, an essential precondition for this transferability is that the adsorbents used in both methods must have identical or at least very similar physical and chemical properties. An empirical rule that we follow to save time is to aim for a maximum $R_F$ of 0.3 in TLC, even if complete separation between the spots is not obtained (see Fig. 2). The separation obtained with an RP-18 packing shows that the highly hydrophobic RP-18 packing requires a slight decrease in solvent strength, in order to give complete separation of (3), (4) and (5).

The great advantages of CTLC have been demonstrated[30–35]; the technique combines good resolving power with low consumption of solvent (generally $< 150$ ml) and relatively short separation times (30–40 min). A few words on the preparation of a layer suitable for water-containing eluents are in order. The manufacturer recommends the use of a HF silica-gel packing and corn starch, with the addition of magnesium sulphate so as to inhibit cracking and to soften the layer. Our experience in the preparation of the layer led us to the conclusion that a better layer could be obtained by using a silica gel type PF + $CaSO_4 \cdot \frac{1}{2}H_2O$ with the addition of corn starch and magnesium sulphate. After drying (for least 48 h at room temperature, followed by 2 h at 70–80°C), the plate must be washed repeatedly with methanol to remove the inorganic salts.

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REFERENCES

1 J. Lutomski and E. Segiet-Kujawa, *Herba Pol.*, 26 (1980) 77.
2 H. Eskil, *Acta Chem. Scand.*, 19 (1965) 1431.
3 A. Ulubelen, T. J. Mabry, G. Dellamonica and J. Chopin, *J. Nat. Prod.*, 47 (1984) 384; and references cited therein.
4 K. C. Spencer and D. S. Seigler, *J. Agric. Food Chem.*, 31 (1983) 794; and references cited therein.
5 E. Bombardelli, A. Bonati, B. Gabetta, E. M. Martinelli, G. Mustich and B. Danieli, *Phytochemistry*, 14 (1975) 2661.
6 L. P. Vecherko, A. F. Sviridov, E. P. Zinchevic and L. M. Kogan, *Khim. Prir. Soedin.*, 11 (1975) 366.
7 G. Romussi, G. Ciarallo, G. Palsone and C. Schneider, *Liebigs Ann. Chem.*, 12 (1979) 2028.
8 E. S. Davidyants, Zh. M. Putieva, V. A. Bandyukova and N. K. Abubakirov, *Khim. Prir. Soedin.*, 6 (1984) 750.
9 F. Orsini, F. Pelizzoni and L. Verotta, *Phytochemistry*, (1985) in press.
10 K. Hostettmann, M. Hostettmann-Kaldas and K. Nakanishi, *J. Chromatogr.*, 170 (1979) 355.
11 K. Hostettmann, M. Hostettmann-Kaldas and O. Sticher, *Helv. Chim. Acta*, 62 (1979) 2079.
12 B. Coq, J. P. Nicolas, A. Lamotte and M. Porthault, *J. Chromatogr.*, 97 (1974) 137.
13 S. Hara and K. Mibe, *Chem. Pharm. Bull.*, 23 (1975) 2850.
14 E. Soczewinski and W. Golkiewicz, *J. Chromatogr.*, 118 (1976) 91.

15 W. Golkiewicz, *Chromatographia*, 9 (1976) 113.
16 E. Soczewinski, T. Dzido, W. Golkiewicz and K. Gazda, *J. Chromatogr.*, 131 (1977) 408.
17 A. M. Siouffi, A. Guillemonat and G. Guiochon, *J. Chromatogr.*, 137 (1977) 35.
18 S. Hara, *J. Chromatogr.*, 137 (1977) 41.
19 H. Jork, E. Reh and H. Wimmer, *GIT Fachz. Lab.*, 25 (1981) 566.
20 D. Nurok and M. J. Richard, *Anal. Chem.*, 53 (1981) 563.
21 B. Buglio and V. S. Venturella, *J. Chromatogr. Sci.*, 20 (1982) 165.
22 F. Eisenbeiss, *Chem.-Ztg.*, 95 (1971) 237.
23 W. Jost, H. E. Hauck and F. Eisenbeiss, *J. Chromatogr.*, 256 (1983) 182.
24 E. von Arx and M. Faupel, *J. Chromatogr.*, 154 (1978) 68.
25 C. Gonnet and M. Marichy, *Analusis*, 7 (1979) 204.
26 T. Dzido and E. Soczewinski, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 88.
27 W. Golkiewicz, *Chromatographia*, 14 (1981) 411.
28 A. Golkiewicz, *Chromatographia*, 14 (1981) 629.
29 T. Okumura, *J. Liq. Chromatogr.*, 4 (1981) 1035.
30 K. Hostettmann, M. Hostettmann-Kaldas and O. Sticher, *J. Chromatogr.*, 202 (1980) 154.
31 E. Stahl and J. Mueller, *Chromatographia*, 15(8) (1982) 493.
32 K. N. Bhattacharya, *Chem. Era*, 18(2) (1982) 47.
33 Y. Wen, P. Wang and X. He, *Youji Huaxue*, 5 (1981) 372.
34 C. Shufeng and J. Wang, *Huaxue Tongbao*, 10 (1982) 591.
35 I. R. Hunter and E. Heftmann, *J. Liq. Chromatogr.*, 6 (1983) 281.

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CHROMSYMP. 673

# COMPLETE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF 4-N,N-DIMETHYLAMINOAZOBENZENE-4′-THIOHYDANTOIN AND 4-DIMETHYLAMINOAZOBENZENE-4′-SULPHONYL CHLORIDE AMINO ACIDS UTILIZING THE SAME REVERSED-PHASE COLUMN AT ROOM TEMPERATURE

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## SUMMARY

Reversed-phase high-performance liquid chromatographic methods for the complete separation of all 4-dimethylaminoazobenzene-4′-sulphonyl chloride and 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin amino acids on the same Supelcosil LC-18 column at room temperature are described. The procedures are simple and reproducible, and the systems are easily interconvertible. The use of a fixed-wavelength detector at 436 nm permits amino acid analysis at levels lower than 1 pmol with a stable baseline.

## INTRODUCTION

One of the major problems in the structural analysis of red blood cell enzymes is that they are present in only small amounts. This means that only micrograms of purified protein can be obtained, as with mammalian red blood cell hexokinase (E.C. 2.7.1.1). This enzyme has been shown to exist in two or more distinct molecular forms[1]. At present, the molecular basis of hexokinase heterogeneity remains unknown, although preliminary experimental findings have suggested a post-translational modification as a possible mechanism[1]. In an attempt to characterize these modifications, we are developing methods that can be used with small amounts of pure protein. Over the last few years, many workers[2–14] have described the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for the separation of 4-dimethylaminoazobenzene-4′-sulphonyl chloride (DABS) and 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin (DABTH) amino acids. These methods are widely applied in microsequencing analysis, because their high sensitivity permits amino acid analysis at the picomole level with reliable results. However, the methods reported[2–14] for separating DABS- and DABTH-amino acids have certain drawbacks. In this paper, we describe the first complete separation of all DABS- and DABTH-amino acids and by-products on the same reversed-phase column at room temperature.

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## EXPERIMENTAL

### *Chemicals*

DABS-Cl and 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC) were purchased from Fluka (Buchs, Switzerland). Free amino acids for the preparation of DABS- and DABTH-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methanol, 2-propanol and all the other reagents for HPLC and sequencing analysis were obtained from Fluka. Analytical-reagent grade potassium dihydrogen phosphate and acetate were purchased from Merck (Darmstadt, F.R.G.). Millipore filters (0.22 $\mu$m) were obtained from Millipore (Bedford, MA, U.S.A.). The water used for the experiments was doubly distilled. All buffers were filtered through a Millipore filter (0.22 $\mu$m) before HPLC analysis.

DABS-Cl and DABITC were recrystallized according to Chang[8]. A 0.5-g amount of DABITC was dissolved in 50 ml of boiling acetone (UV spectroscopy grade) and the insoluble materials were removed by passing the solution through a sintered-glass filter funnel. When the clear solution was left at $-20$°C overnight, needle-shaped crystals precipitated. DABITC was dried under vacuum, utilizing a Savant system concentrator (Savant, Hicksville, NY, U.S.A.).

A stock solution of DABITC (1.41 mg/ml) in acetone was prepared. Aliquots (100 and 500 $\mu$l) of the stock solution were pipetted into Eppendorf tubes, dried under vacuum and stored at $-20$°C over a period of months without appreciable degradation. Fresh DABITC solution was prepared by redissolving dried DABITC stored at $-20$°C in an appropriate volume of acetone before use. The same procedure was used for DABS-Cl, giving a 4 nmol/$\mu$g (1.3 mg/ml) stock solution in acetone.

### *Preparation of standard DABS-amino acids*

Standards of mono-DABS-amino acids were prepared according to the method of Chang *et al.*[7] with slight modification. Amino acids (1 mg) were dissolved in 2 ml of 0.2 *M* sodium hydrogen carbonate buffer (pH 9.0). In this way, the large volume of buffer prevented a change in pH, as occurs with serine, threonine, aspartic acid and glutamic acid. To 100 $\mu$l of amino acid solution, 100 $\mu$l of DABS-Cl solution (2 nmol/$\mu$l in acetone) were added. The mixture was heated at 70°C for 10 min, dried under vacuum and the residue dissolved in 2 ml of 70% (v/v) ethanol. The standard DABS-amino acid solutions were stored at $-20$°C.

Bis-DABS-amino acids (lysine, histidine and tyrosine) (50 nmol) were dissolved in 100 $\mu$l of 0.2 *M* sodium hydrogen carbonate buffer (pH 9.0) and treated with 200 ml of DABS-Cl solution (4 nmol/$\mu$l in acetone). The mixture was heated at 70°C for 10 min, dried under vacuum and the residue dissolved in 500 $\mu$l of 70% (v/v) ethanol. The final concentration of the standard bis-DABS-amino acid solution was about 1 nmol per 10 $\mu$l.

### *Preparation of DABTH derivatives*

Standard DABTH-amino acids were prepared according to the method of Chang[8] with slight modifications. Amino acids (0.5 mg) were dissolved in 1 ml of triethylamineacetic acid buffer (pH 10.65). To 100 $\mu$l of this solution were added 50 $\mu$l of DABITC solution (4 nmol/$\mu$l in acetone). The mixture was heated at 54°C for 1 h, dried under vacuum and the residue dissolved in 100 $\mu$l of 50% (v/v) trifluo-

roacetic acid (TFA). The acid solution was heated at 54°C for 45 min and then dried again under vacuum. The dried DABTH-amino acid derivative was dissolved in a suitable volume of 70% ethanol and stored at −20°C.

*HPLC analysis*

The HPLC system used (Beckman, Berkeley, CA, U.S.A.) consisted of two Model 112 pumps, a Model 420 solvent programmer, a Model 210 sample injection valve and a Model 160 fixed-wavelength (436 nm) visible-range detector, equipped with a 18.5-μl flow cell. Integration of peak areas was obtained by means of an HP 3390 A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). DABS- and DABTH-amino acid analyses were performed on a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.) equipped with a stainless-steel guard column (2 cm × 4.6 mm I.D.), packed with pellicular reversed-phase material (Pellicular Packing LC-18, 40 μm) (Supelco, Bellefonte, PA, U.S.A.).The sensitivity of the detector was routinely set at 0.005 a.u.f.s. The injection volume was 20 μl. The mobile phase utilized for the separation of DABS-amino acids consisted of two eluents: 25 m*M* potassium dihydrogen phosphate (pH 6.8) (solvent A) and acetonitrile–2-propanol (80:20) (solvent B); DABTH-amino acid analysis was performed utilizing 35 m*M* sodium acetate buffer (pH 5.1) (solvent A) and acetonitrile (solvent B).

The chromatographic conditions used to obtain the chromatogram shown in Fig. 1 were as follows:1 min at 20% of solvent B, 6 min up to 26% of solvent B and held for 13 min, up to 70% of solvent B. The gradient was then returned to 20% of solvent B and the initial conditions were restored in 8 min. The flow-rate was 1.0 ml/min. The chromatographic profile reported in Fig. 2 was obtained utilizing the following gradient: 8 min at 39% of solvent B, 4 min up to 53% of solvent B and held for 28 min. The gradient was then returned to 39% of solvent B and the initial conditions were restored in 10 min. The flow-rate was 1.0 ml/min.

## RESULTS AND DISCUSSION

*Separation of DABS-amino acids*

Pre-column derivatization of amino acids with DABS-Cl followed by reversed-phase HPLC[4–7,9,12] permits the detection of DABS-amino acids at the picomole level in the visible region. Many workers [7,9,12] have discussed the advantages of this method with respect to ninhydrin[15], *o*-phthaladehyde (OPA)[16–18] or dansyl chloride[19] detection. Chang *et al.*[6,12] reported the complete separation of all DABS-amino acids at high temperatures (50°C). Recently, Winkler *et al.*[9] described a good separation of all DABS-amino acids at room temperature, except for leucine and isoleucine, which are not completely separated. Further, the pH is very critical; slight changes cause fusion of the peaks of some DABS-amino acids and a loss of resolution. In order to obtain a simple and reproducible method that allows the complete separation of all DABS-amino acids at room temperature, we tested various reversed-phase columns (Ultrasphere ODS, Micropak MCH and Supelcosil LC-18) and different buffers (acetate, phosphate), organic solvents (acetonitrile, ethanol, methanol, 2-propanol), organic modifiers (dimethylformamide, triethylamine), pH values, flow-rates and gradient forms. The best results were obtained with a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.), 25 m*M* potassium dihydrogen phosphate buffer, pH

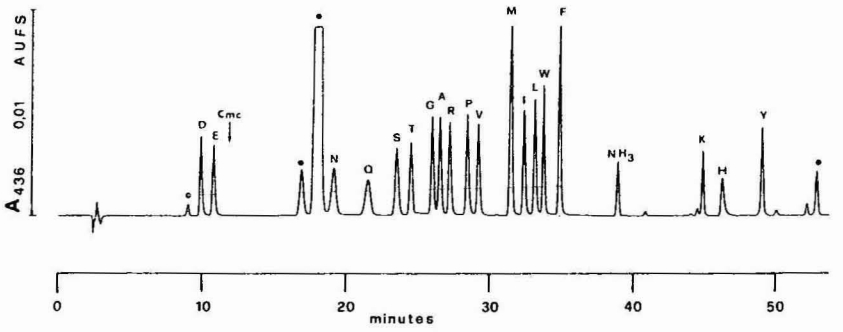


Fig. 1. Complete separation of all DABS-amino acids (*ca.* 50 pmol) by reversed-phase HPLC on a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.). Solvent A, 25 m*M* potassium dihydrogen phosphate buffer (pH 6.8); solvent B, acetonitrile–2-propanol (80:20). Chromatographic conditions as described under Experimental. Flow-rate, 1 ml/min; temperature, ambient. ●, By-products originating from the excess of reagent.

6.8 and acetonitrile–2-propanol (80:20). Fig. 1 shows the complete separation of a standard mixture of DABS-amino acids and by-products originating from the excess of reagent. For each new Supelcosil LC-18 column only a slight modification of the conditions described under Experimental are necessary in order to obtain the optimal separation. The decisive factor was the addition of 20% of 2-propanol to the acetonitrile, which permitted the complete resolution of DABS-Ser, DABS-Thr, DABS-Gly, DABS-Ala and DABS-Val. The procedure described here is the first complete separation of about 30 DABS-amino acids and by-products at room temperature.

*Separation of DABTH-amino acids*

Of great importance has been the recent development of the sensitive manual Edman micro-sequencing analysis of peptides and protein by the double coupling method, in which DABITC is followed by phenyl isothiocyanate (PITC) for complete reaction of all terminal amino groups[2,3,8,10,13]. The DABTH-amino acids obtained after each cycle can be identified by thin-layer chromatography or, more sensitively, by reversed-phase HPLC . Chang[8] reported the complete separation of DABTH derivatives on a Zorbax ODS column, except leucine and isoleucine, which were eluted together.

Recently, many workers[9–11,13,14] have proposed various RP-HPLC procedures for the separation of all DABTH-amino acids, but they have certain drawbacks. A better separation of DABTH-Ile and DABTH-Leu was obtained by Winkler *et al.*[9], who used as a decisive factor a 3-μm Ultraphere ODS column and the addition of 2% of triethylamine to the eluent. Yang and Wakil[11] described a good separation of all DABTH derivatives except DABTH-Ser and DABTH-Gln.

Similarly, the method proposed by Lu and Gracy[13] does not allow the resolution of two pairs of DABTH-amino acids, DABTH-Pro/DABTH-Phe and DABTH-Ile/DABTH-Leu. For the above reasons, we performed experiments similar to those described for DABS-amino acids in order to find a procedure that permits the complete separation of all DABTH derivatives at room temperature.

We obtained the best results by utilizing a 5-μm Supelcosil LC-18 column, as

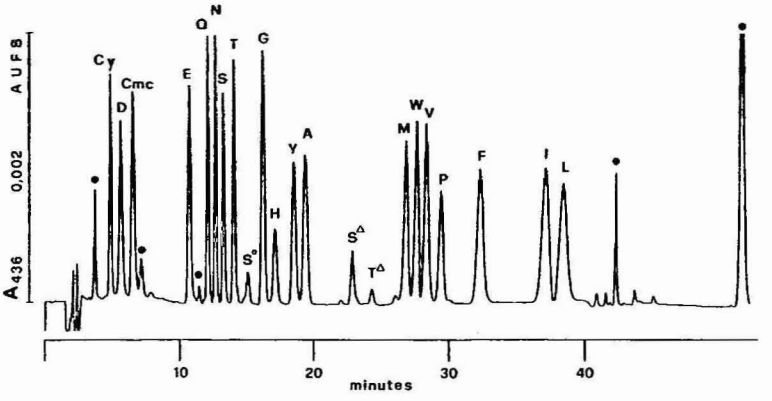


Fig. 2. Complete separation of *ca.* 2–3 pmol of standard DABTH-amino acid derivatives on a 5-$\mu$m Supelcosil LC-18 column (25 cm × 4.6 mm I.D.). Solvent A, 35 m*M* acetate buffer (pH 5.1); solvent B, acetonitrile. Chromatographic conditions as described under Experimental. DABTH peaks are identified by a single-letter code according the IUPAC-IUB Commission on Biochemical Nomenclature. Flow-rate, 1 ml/min; temperature, ambient. Cy, cysteic acid; Cmc, S-carboxymethylcysteine; S°, DABTH-Ser° (a probably hydrated DABTH-Ser with a hydroxy group at the 2-position); $T^{\Delta}$ and $S^{\Delta}$, dehydrated derivatives of threonine and serine. ●, Decomposition product of DABTH-amino acids.

shown in Fig. 2. The complete resolution of all DABTH derivatives (about 30), including cysteic acid and S-carboxymethylcysteine, can be achieved at room temperature by the use of the gradient system described under Experimental, where 35 m*M* acetic acid (pH 5.1) was solvent A and acetonitrile was solvent B.

The lower pH of 5.1 does not affect the separation of DABTH-amino acids, except DABTH-Glu, which increases its retention time towards that of DABTH-Gln. A commercial Supelcosil LC-18 column shows a very highly reproducible retention time. In our experience, only a slight modification of the gradient form is necessary for each new column. It is sufficient to modify the last part of the gradient by increasing or decreasing slightly the percentage of solvent B to obtain the complete separation of the DABTH-amino acids. The DABTH-amino acid profile reported in Fig. 2 shows the original chromatogram, obtained from the Beckman Model 160 with a fixed wavelength, set at 0.002 a.u.f.s. It can be seen that there is no appreciable rise in the baseline due to the gradient change. However, we performed all routine analyses of DABTH amino acids at 0.005 a.u.f.s. with perfect stability of the baseline, demonstrating the major advantage of the method that less than 1 pmol of DABTH can easily be measured. Because of the high sensitivity of the method, only small volumes and amounts of samples need be injected. Under these conditions, the column has a long lifetime. Over 2000 analyses have been performed up to now without observing any irreversible deterioration effects. The guard column needs to be changed after every 200–300 injections when 20-$\mu$l aliquots are analysed. The only fault we have observed after prolonged use of the same column (several months) is a slight decrease in the complete resolution of DABTH-Met and DABTH-Trp.

## CONCLUSIONS

The methods described here, based on RP-HPLC, have permitted the first

complete separation of all DABS- and DABTH-amino acids on the same column at room temperature. Under the conditions described, the procedures proposed are simple and reproducible and the systems are easily interconvertible. The use of the Beckman Model 160 fixed-wavelength detector with a sensitivity limit of 0.001 a.u.f.s. permits amino acid analyses with stable baselines at levels lower than 1 pmol.We believe that the methods will be very useful for the microsequencing analysis of pure proteins obtainable in only small amounts.

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REFERENCES

1 G. Fornaini, M. Dachà, M. Magnani and V. Stocchi, *Mol. Cell. Biochem.*, 49 (1982) 129.
2 J. Y. Chang, A. Lehman and B. Wittmann-Liebold, *Anal. Biochem.*, 102 (1980) 380.
3 J. Y. Chang, *Biochem. J.*, 199 (1981) 557.
4 J. Y. Chang, P. Martini, R. Bernasconi and D. G. Braun, *FEBS Lett.*, 132 (1981) 117.
5 J. Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 199 (1981) 547.
6 J. Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 203 (1982) 803.
7 J. Y. Chang, R. Knecht and D. G. Braun, *Methods Enzymol.*, 91 (1983) 41.
8 J. Y. Chang, *Methods Enzymol.*, 91 (1983) 455.
9 G. Winkler, F. X. Heinz and C. Kunz, *J. Chromatogr.*, 297 (1984) 63.
10 A. Foriers, M. Lauwereys and R. De Neve, *J. Chromatogr.*, 297 (1984) 75.
11 C. Y. Yang and S. J. Wakil, *Anal. Biochem.*, 137 (1984) 54.
12 J.-Y. Chang, *J. Chromatogr.*, 295 (1984) 193.
13 H. S. Lu and R. W. Gracy, *Arch. Biochem. Biophys.*, 235 (1984) 48.
14 A. Lehmann and B. Wittmann-Liebold, *FEBS Lett.*, 176 (1984) 360.
15 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 893.
16 M. J. Radjai and R. T. Hatch, *J. Chromatogr.*, 196 (1980) 319.
17 D. W. Hill, F. H. Waters, T. D. Wilson and J. D. Stuart, *Anal. Chem.*, 51 (1979) 1338.
18 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667.
19 C. de Jong, G. J. Hunghes, E. van Wieringen and K. J. Wilson, *J. Chromatogr.*, 241 (1982) 345.

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CHROMSYMP. 661

# ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ASPARTAME AND RELATED PRODUCTS

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## SUMMARY

A simple and accurate quantitative determination of aspartame (L-α-aspartyl-L-phenylalanine methyl ester), a new artificial sweetener, is described. The method, which is based on ion-pair high-performance liquid chromatography, allows the determination of aspartame in finished bulk and dosage forms, and the detection of a few related products at levels down to 0.1%.

## INTRODUCTION

Aspartame (I) is a new synthetic sweetener[1], which is now very widely used all over the world and is obtained by synthesis from two amino acids, L-phenylalanine and L-aspartic acid.

Several workers have described high-performance liquid chromatographic (HPLC) methods[2–6] for aspartame, with different procedures for the complete analysis of related products. In a previous paper[7] we described a very rapid and efficient HPLC procedure for the determination of I in the finished bulk form and of some of the more common related products (III, VI, VII, VIII and IX) (see Scheme I).

In order to extend the quantitative analysis of I to commercial formulated products, such as table-top sweeteners, and to gain further knowledge about related impurities obtained during the synthesis, we have investigated a new method, which allows the complete separation of I from nine related compounds and its determination in dosage forms. It also makes it possible to detect potential impurities in final products at levels as low as 0.1%. The method is based on ion-pair formation[8] associated with reversed-phase chromatography. As the retention time of ionized components is increased, the selectivity of the chromatographic column is enhanced and this method is useful alternative to ion-exchange chromatography.

## EXPERIMENTAL

### *Materials*

Standards of aspartame and all aspartame derivatives (III–IX) were obtained from our Chemical Development Laboratories and tested for purity by HPLC, IR, PMR and elemental analyses. They were found to be not less than 99.0% pure on

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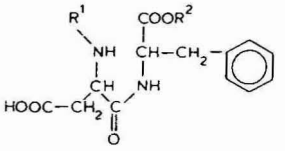
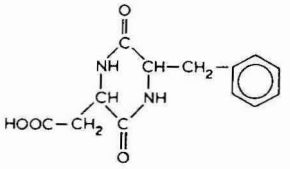


$R^1 = H; R^2 = CH_3$ (I)

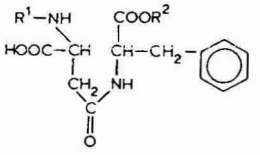
$R^1 = CHO; R^2 = H$ L-α-[(N-Formyl)aspartyl]-L-phenylalanine (IV)

$R^1 = H; R^2 = H$ L-α-Aspartyl-L-phenylalanine (VI)

$R^1 = CHO; R^2 = CH_3$ L-α-[(N-Formyl)aspartyl]-L-phenylalanine methyl ester (VIII)



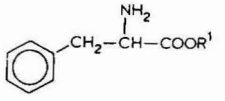
5-Benzyl-3,6-dioxo-2-piperazineacetic acid (III)



$R^1 = H; R^2 = CH_3$ L-β-Aspartyl-L-phenylalanine methyl ester (IX)

$R^1 = H; R^2 = H$ L-β-Aspartyl-L-phenylalanine (V)

$R^1 = CHO; R^2 = CH_3$ L-β-[(N-Formyl)aspartyl]-L-phenylalanine methyl ester (VII)



$R^1 = H$ L-Phenylalanine (II)

$R^1 = CH_3$ L-Phenylalanine methyl ester (X)

Scheme I.

a dry basis. L-Phenylalanine (II), L-phenylalanine methyl ester (X) and sodium hexanesulphonate were of analytical-reagent grade from Fluka (Buchs, Switzerland). Potassium monohydrogen phosphate, 85% phosphoric acid and acetonitrile (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.). Table-top sweeteners, such as Aspartina and Mini D, were Pierrel's and Vantaggio was obtained from Chiari and Forti (Treviso, Italy).

*Apparatus*

A Perkin-Elmer Series 3B high-performance liquid chromatograph, equipped with an LC-75 variable-wavelength UV detector, operating at 220 nm, a Series Σ 10 electronic calculating system and a 20-μl loop-valve automatic injector from Rheodyne (Berkeley, CA, U.S.A.) were used. The analytical column was a pre-packed RP-8, 10 μm, 250 × 4 mm I.D. column (Hibar-RP8) from Merck, kept at room temperature.

*Mobile phase*

Eluent A was prepared by dissolving in 1 l of distilled water 2.062 g of sodium hexanesulphonate monohydrate and 0.450 g of anhydrous potassium monohydrogen phosphate. The pH of the solution was then adjusted to 2.5 with dilute phosphoric acid.

Eluent B was prepared by dissolving sodium hexanesulphonate monohydrate (2.062 g) in 1 l of water–acetonitrile (2:3); the apparent pH was then adjusted to 3.0 with dilute phosphoric acid.

The eluents were filtered through a 0.45-$\mu$m membrane and degassed by applying a moderate vacuum in an ultrasonic bath.

*Chromatographic conditions*

The mobile phase was obtained mixing eluents A and B in the ratio 90:10 (v/v). After 5 min the concentration of eluent B began to increase linearly to 30% at 25 min. The flow-rate was 2 ml/min.

*Sample preparation*

*Analysis of finished bulk.* To approximately 200 mg of a weighed sample of I in a 100-ml volumetric flask 6 ml of acetonitrile are added and the powder is dissolved in a solution prepared by weighing 2.062 g of sodium hexanesulphonate into 1 l of water and adjusting the pH to 4.5 with dilute phosphoric acid.

*Analysis of table-top sweetener.* An amount of powder or tablets containing about 200 mg of I is exactly weighed and dissolved as above. If necessary, the solutions are filtered.

*Reference standard.* Aspartame (I) is weighed and dissolved as in the case of finished bulk in the range 1–3 mg/ml, and related products (II–X) are dissolved to give solutions with concentrations in the range 0.01–0.03 mg/ml.

The quantitative determination is performed by comparing the chromatographic peak areas of samples in triplicate with those of a known standard solution.

## RESULTS AND DISCUSSION

Aspartame (I) is relatively unstable in aqueous medium, giving rise to a dipeptide (VI) and mainly the corresponding diketopiperazine (III), which is devoid of sweetness[1]. The same occurs during heating of the product[9]. Because in the preparation of table-top sweeteners there is a granulation step and the drying operation involves heating of the mixture, it is necessary to monitor the levels of III and VI in the final formulations. Moreover (see Scheme I), a number of related products are formed during the synthesis, and each of them must be carefully checked in the finished bulk.

Only a few HPLC methods[3,4] separate I from III and VI and also from VIII[3], a major intermediate of the synthesis[10], in carbonated soft drinks[4] or the finished bulk form[3]. We have reported[7] a very fast HPLC procedure that also separates VII and VIII. The use of ion-pair chromatography on a reversed-phase column allows the separation of related compounds with very similar chemical structures having very close p$K_a$ values in a relatively short time.

Fig. 1 is a chromatogram showing the separation of a mixture of I–X in equal

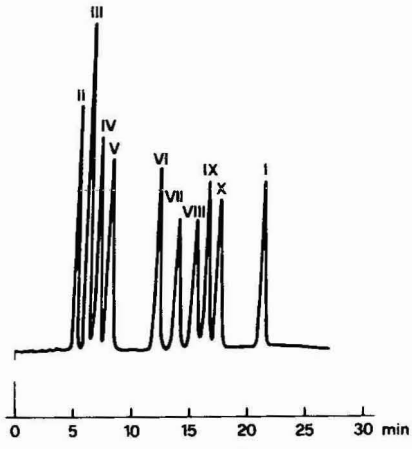
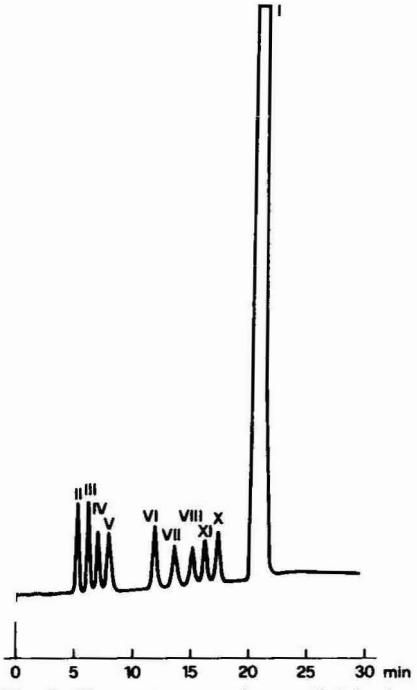



Fig. 1. Chromatogram of a mixture of I–X in equal amounts.

amounts. Baseline separation of these is accomplished in less than 25 min. Fig. 2 shows the separation of another artificial mixture, where each of the related products amount to only 1% with respect to I.

The limit of detectability of this method for the analysis of aspartame is evident from Fig. 3 and is about 0.1%.



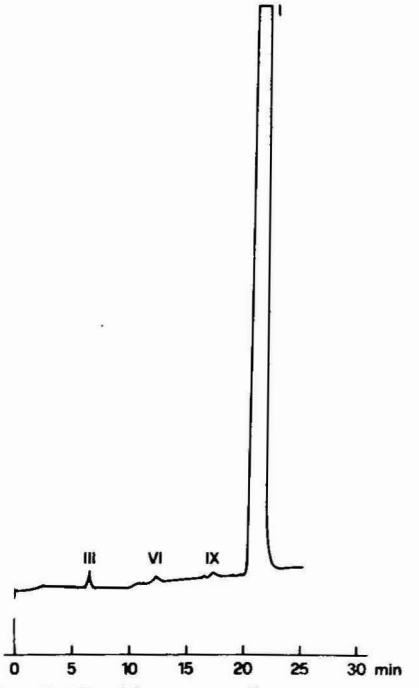




Fig. 2. Chromatogram of an artificial mixture of I and II–X, each 1% with respect to I.

Fig. 3. Chromatogram of aspartame standard, in which the level of III is 0.2%, VI is about 0.05% and IX is 0.1% with respect to aspartame.

TABLE I

VALIDATION OF ASPARTAME ANALYSIS BY PEAK-AREA MEASUREMENT

Results given are peak-area units.

| *Run No.* | *Aspartame concentration (mg/ml)* | | |
|---|---|---|---|
| | *1.0* | *2.0* | *3.0* |
| 1 | 160 982 | 321 320 | 476 494 |
| 2 | 161 781 | 320 889 | 473 476 |
| 3 | 164 431 | 323 325 | 475 696 |
| Average | 162 368 | 321 845 | 475 222 |
| Linear regression: | $A$ (area units) = $mC$ (mg/ml) + $q$, where $m$ = 156 430 and $q$ = 6960; correlation coefficient $r$ = 0.99994. | | |

TABLE II

REPRODUCIBILITY OF ASPARTAME ANALYSIS

| *Injection No.* | *Peak area* |
|---|---|
| 1 | 317 626 |
| 2 | 318 947 |
| 3 | 325 524 |
| 4 | 320 070 |
| 5 | 321 842 |
| 6 | 315 660 |
| Average | 319 945 |
| S.D. | 3449 |
| Precision ($t$ = 0.05; $n$ = 6) | ±2.77% |

TABLE III

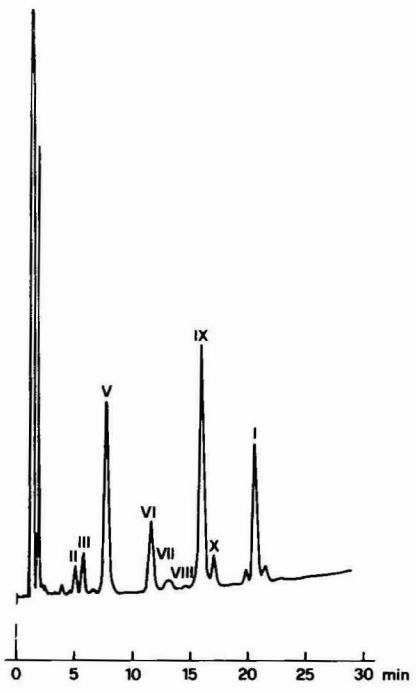
LINEAR REGRESSION FOR RELATED PRODUCTS (II–X)

$A$ (area counts) = $mC$ (mg/ml)* + $q$

| *Component* | $m$ | $q$ | $r$** |
|---|---|---|---|
| II | 160.7 | 0.085 | 0.9990 |
| III | 278.0 | −0.25 | 0.9996 |
| IV | 192.2 | −0.165 | 0.9997 |
| V | 215.4 | 0.068 | 0.9996 |
| VI | 246.5 | 0.16 | 0.9995 |
| VII | 149.2 | 0.172 | 0.9998 |
| VIII | 151.5 | 0.07 | 0.9999 |
| IX | 191.5 | −0.04 | 0.9999 |
| X | 187.15 | −0.03 | 0.9999 |

* From 0.01 to 0.03 mg/ml.

** Correlation coefficient.



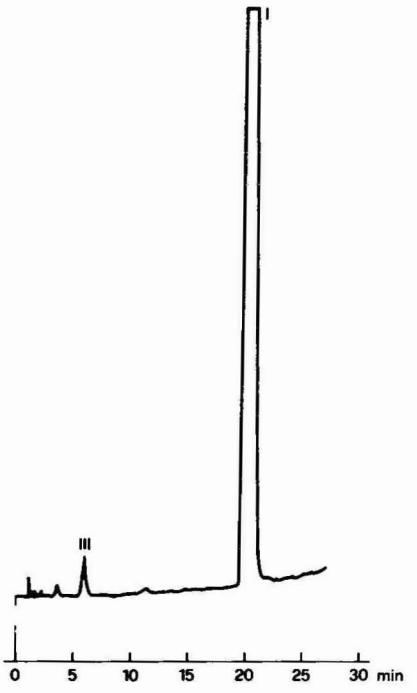




Fig. 4. Chromatogram of a mother liquor from aspartame crystallization.

Fig. 5. Chromatogram of Aspartina tablets analysis.

TABLE IV

DETERMINATION OF ASPARTAME IN COMMERCIAL DOSAGE FORMS

| *Brand name* | *Manufacturer* | *Theoretical content* | *Found** | *S.D.* | *Precision (t = 0.05)* |
|---|---|---|---|---|---|
| Mini D** | Pierrel | 3.0% | 2.99% | 0.06% | 5% |
| Vantaggio** | Chiari & Forti | 2.8% | 2.78% | 0.05% | 5% |
| Aspartina*** | Pierrel | 20 mg/tablet | 20.4 mg/tablet | 0.45 mg/tablet | 6% |

* Mean of five determinations.
** Free-flowing powder.
*** Tablet.

Table I reports data for the validation of the quantitative determination of aspartame in the range 1–3 mg/ml by peak-area measurement, based on triplicate analyses of each solution. The reproducibility of the analytical results is shown in Table II. It was determined by six replicate injections of the same solution of aspartame at a concentration of 2 mg/ml. The validity of the analysis of related compounds II–X is shown in Table III, where the linear regression and correlation coefficient are presented for each component in the range 0.01–0.03 mg/ml.

The method was applied routinely for the control of the synthesis, for purity checks of the final finished bulk and for the analysis of complex mixtures, *e.g.*, mother liquors (Fig. 4). Dosage forms (both free-flowing powder and tablets) were tested mainly for assay of the active ingredient and for the incidental increase of degradation products III and VI (Fig. 5). III, which represents the main impurity, can easily be detected at levels down to about 0.3%. Table IV gives results for the quantitative determination of aspartame obtained in the analysis of three dosage forms of different composition.

## REFERENCES

1 R. H. Mazur, J. M. Schlatter and A. H. Goldkamp, *J. Am. Chem. Soc.*, 91 (1969) 2684.
2 L. Fox, G. D. Anthony and E. P. K. Lau, *J. Assoc. Off. Anal. Chem.*, 59 (1976) 1948.
3 E. Ciranni Signoretti, A. Dell'Utri and A. De Salvo, *Boll. Chim. Farm.*, 122 (1983) 289.
4 J. Scherz, J. C. Monti and R. Jost, *Z. Lebensm.-Unters.-Forsch.*, 177 (1983) 124.
5 C. J. Argoudelis, *J. Chromatogr.*, 303 (1984) 256.
6 R. Cross and B. Cunico, *LC Liq. Chromatogr., HPLC Mag.*, 2 (1984) 678.
7 G. Verzella and A. Mangia, *J. Chromatogr.*, 346 (1985) 417.
8 W.R. Melander and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography—Advances and Perspectives*, Vol. 2, Academic Press, New York, 1980, pp. 114–303.
9 H. H. El-Shattawy, G. E. Peck and D. O. Kildsig, *Drug Dev. Ind. Pharm.*, 7 (1981) 605.
10 V. Giobbio, G. Ornato, L. Buracchi and A. Mangia, *U.S. Pat.*, 4 434 097, 1984.

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CHROMSYMP. 675

# IDENTIFICATION AND ISOLATION OF HUMAN INSULIN A AND B CHAINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A method for the isolation, identification and quantification of human insulin A and B chains by high-performance liquid chromatography (HPLC) is described. These chains were isolated from a peptide mixture produced by *E. coli* with modified genes obtained by genetic engineering. The method is based on the use of hydrophilic reagents, forming ion pairs in a reversed-phase column. Because some undesirable effects resulting from the use of phosphoric acid were observed, especially with the B chain, a new HPLC method was developed for each of the two human insulin chains. The use of trifluoroacetic acid as a counter ion for the A chain and of formic acid for the B chain led to the rapid isolation and purification of each chain by HPLC. The advantage of this method is that it provides a highly pure product, which was identified by polyacrylamide gel electrophoresis and amino acid analysis.

## INTRODUCTION

To avoid the problems encountered by patients receiving porcine or bovine insulin therapy to control diabetes[1], an interesting alternative is to administer an analogue of insulin, the structure and function of which are similar to that of human insulin. Therefore, the genes for both the A and B chains of human insulin were synthesized *in vitro*[2] and were inserted separately into two distinct strains of *E. coli*[2] in order to achieve the production of hybrid polypeptides, containing $\beta$-galactosidase, coupled to either the A or the B chain. On recovery and cleavage with cyanogen bromide[3] of the hybrid polypeptides[4], a mixture of peptides was obtained, including

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the A or B insulin chains. As this peptide mixture is insoluble in phosphoric acid[5] and as, in our hands, the use of phosphoric acid produced several undersirable effects, especially during the recovery of the B chain for lyophilization, we sought a more suitable solvent. Of the hydrophilic reagents that form ion pairs, which are used in the separation of peptides[6], formic acid was selected for the isolation of the insulin B chain because the peptide mixtures were completely soluble in it and because the B chain standard, dissolved in formic acid, also gave very good resolution. Trifluoroacetic acid (TFA) was selected for the insulin A chain. In both instances, the respective mobile phases selected gave sample $k'$ values in the correct range[7]. Lyophilization of the products yielded white solids that easily dissolved in their respective solvents, thereby permitting characterization of the products by polyacrylamide gel electrophoresis and by amino acid analysis. The products so obtained were in a form that could undergo chain recombination[4,8].

## EXPERIMENTAL

### *Apparatus*

Two different chromatographic systems, both from Spectra-Physics (Santa Clara, CA, U.S.A., and Darmstadt, F.R.G.) were used. The first was an SP8750 modular analytical system with a Rheodyne (Berkeley, CA, U.S.A.) injector loop (10 $\mu$l), an SP8700 solvent delivery system, an SP8400 variable-wavelength detector and an SP4100 computing integrator. The second was an SP8000 semi-preparative system with a 100-$\mu$l injector loop. In both systems a Waters Radial-Pak $\mu$Bondapak $C_{18}$ cartridge (10 cm × 8 mm I.D. 10 $\mu$m), contained in a Z-Module Radial Compression Separation System (Waters Assoc., Milford, MA, U.S.A.), was used. Samples and solvents were clarified by filtration through porous membranes (0.22 $\mu$m) from Millipore (Milford, MA, U.S.A.).

### *Reagents*

HPLC-grade water was obtained from triply distilled water by using a Norganic Filter Apparatus (Millipore XX1500-710). Acetonitrile (LiChrosolv grade) was obtained from Merck (Darmstadt, F.R.G.), and trifluoroacetic acid (Sequanal grade) from Pierce (Rockford, IL, U.S.A.). Formic acid (J. T. Baker, Phillipsburgh, NJ, U.S.A.) was redistilled under vacuum (20 mmHg). The A and B insulin chains (Sigma, St. Louis, MO, U.S.A.) used as standards were from porcine pancreas (I-3505) and from bovine pancreas (I-5500). Acrylamide was obtrained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

### *Procedure*

Both eluent A [0.0125% TFA (pH 3.2) in ultra-pure water for A chain and 0.5% formic acid (pH 2.7) in ultra-pure water for B chain] and eluent B (acetonitrile) were filtered through a porous membrane (0.22 $\mu$m) and degassed with helium. The A or B chains used as standards, dissolved in eluent A, were filtered through a porous membrane (0.22 $\mu$m) and were chromatographed individually to determine their retention times (Figs. 1 and 2). The external standard procedure was used to quantify the products. Various amounts of the A chain (2.7–18.3 $\mu$g) and B chain (5–40 $\mu$g) were used individually to determine the linearity of the response. To calculate the

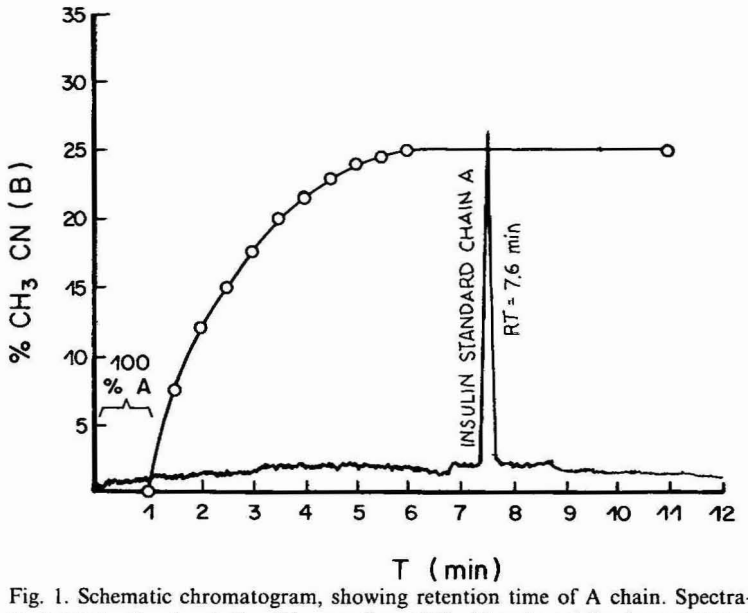


Fig. 1. Schematic chromatogram, showing retention time of A chain. Spectra-Physics system: column, Radial-Pak μBondapak $C_{18}$ (10 cm × 8 mm I.D., 10 μm); mobile phase, 100% eluent A (1 min), followed by a convex gradient from 0 to 25% acetonitrile (eluent B) in 0.0125% TFA (pH 3.2) (eluent A); flow-rate, 2.5 ml/min; UV detection (280 nm).

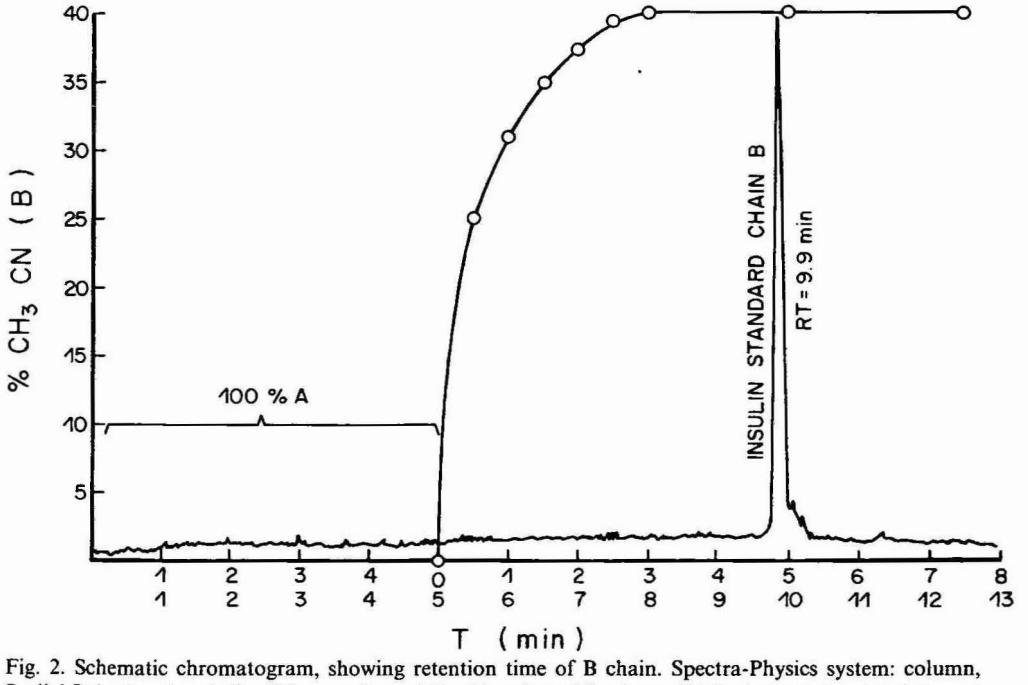


Fig. 2. Schematic chromatogram, showing retention time of B chain. Spectra-Physics system: column, Radial-Pak μBondapak $C_{18}$ (10 cm × 8 mm I.D., 10 μm); mobile phase, 100% eluent A (5 min), followed by a convex gradient from 0 to 40% acetonitrile (eluent B) in 0.5% formic acid (pH 2.7) (eluent A); flow-rate, 2.5 ml/min; UV detection (280 nm).

TABLE I

STATISTICAL EVALUATION OF RESULTS FROM HPLC OF INSULIN A AND B CHAIN STANDARDS

Both standards were run five times at each concentration with the same chromatographic conditions, as in Figs. 1 and 2.

| *Chain* | *Sample (μg)* | *Standard deviation (μg)* | *Confidence limit (μg)* |
|---|---|---|---|
| A | 2.5– 5 | 0.03994 | ± 0.0351 |
| | 10.0–15 | 0.259 | ± 0.0227 |
| | 15.0–20 | 0.08064 | ± 0.07924 |
| B | 5.0–10 | 0.1179 | ± 0.189 |
| | 10.0–15 | 0.08809 | ± 0.09998 |
| | 20.0–40 | 0.160076 | ± 0.18167 |

confidence limits of the method[9], both standards were run five times at each concentration (Table I).

*Chromatographic conditions*

The Spectra-Physics system was programmed to provide a stepwise convex gradient elution to separate the A or B chains of human insulin from the mixture of peptides. To increase the differences between the $k'$ values of the peaks of the peptide

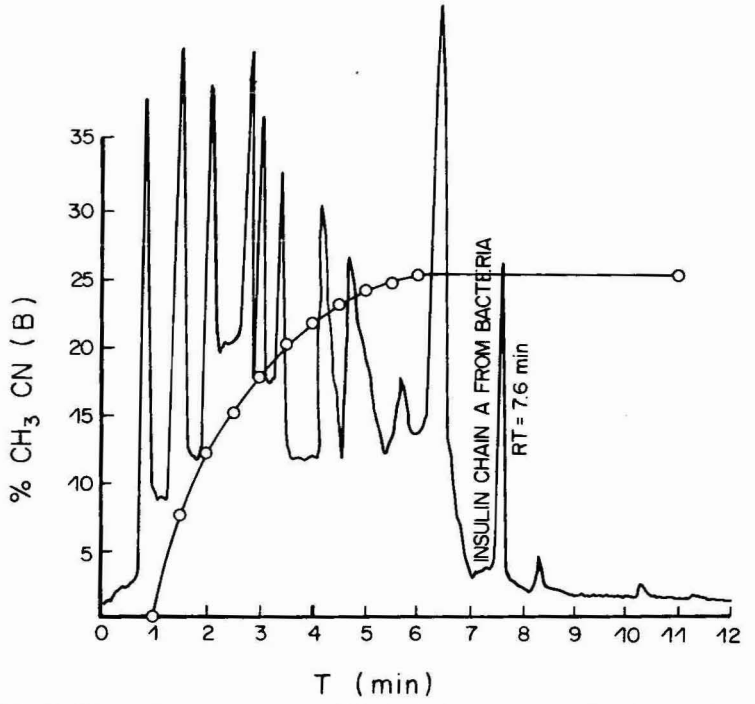


Fig. 3. Schematic chromatogram of A chain, isolated from the mixture of peptides. Chromatographic conditions as in Fig. 1.

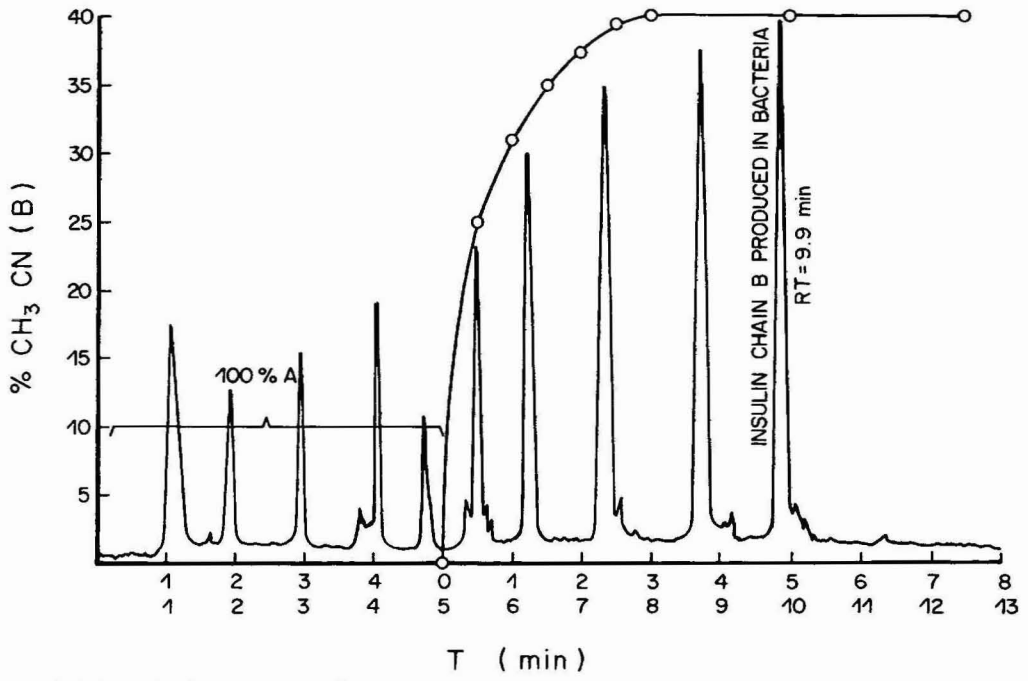


Fig. 4. Schematic chromatogram of B chain, isolated from the mixture of peptides. Chromatographic conditions as in Fig. 2.

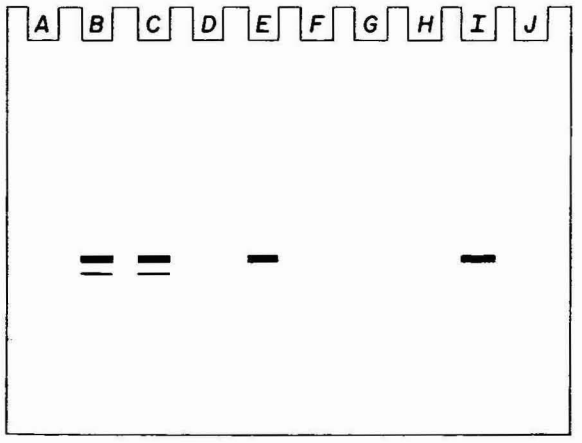


Fig. 5. Schematic electropherogram: polyacrylamide gel electrophoresis of insulin A chain produced in bacteria and purified by HPLC was carried out in a continuous gel, 16% acrylamide, Tris–$H_3BO_3$. Lanes B and C: 3 and 5 $\mu$g, respectively, of insulin A chain, purified by HPLC, showing one major band that migrates with the standard. lanes E and I: 5 $\mu$g of standard insulin A chain. Lanes A, D, F, G, H and J: without samples.

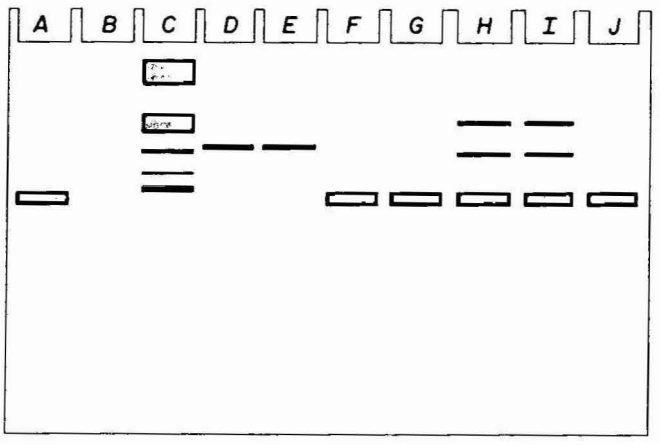


Fig. 6. Schematic electropherogram: polyacrylamide gel electrophoresis of insulin B chain produced in bacteria and purified by HPLC was carried out in a continuous gel: stacking gel, 18% acrylamide, Tris–HCl, 2.12 *M*, pH 9.18. Separation gel, 18% acrylamide, Tris–$H_2SO_4$, 0.0267 *M*, pH 6.1. Samples were dissolved in 7 *M* urea, Tris–$H_3BO_3$, 0.205 *M*, pH 8.6, diluted with water (1:5). Lanes A, G and J: 6 μg of bovine chain B used as standard and chromatographed by HPLC. Lane C: unknown peptide mixture separated from chain B by HPLC. Lane D and E: other peptides separated from chain B by HPLC. Lane F: *ca.* 7 μg of B chain, purified by HPLC, showing a single band that migrates with the standard. Lane H and I: 5 and 10 μg, respectively, of the peptide mixture before HPLC.

TABLE II

AMINO ACID ANALYSIS OF INSULIN A CHAIN, PRODUCED IN BACTERIA, AND OF THE A CHAIN STANDARD

Approximately 20 μg of porcine A chain and 20 μg of *E. coli* A chain purified twice by HPLC were hydrolysed and analysed in parallel.

| *Amino acid* | *A chain from bacteria* | | *A chain used as standard* | |
|---|---|---|---|---|
| | *Found* | *Predicted* | *Found* | *Predicted* |
| Aspartic acid | 1.85 | 2.0 | 1.75 | 2.0 |
| Threonine | 0.93 | 1.0 | 1.11 | 1.0 |
| Serine | 2.14 | 2.0 | 2.09 | 2.0 |
| Glutamic acid | 3.83 | 4.0 | 3.45 | 4.0 |
| Glycine | 1.12 | 1.0 | 1.31 | 1.0 |
| Valine | 0.38 | 1.0 | 0.49 | 1.0 |
| Isoleucine | 1.43 | 2.0 | 1.12 | 2.0 |
| Leucine | 1.96 | 2.0 | 1.47 | 2.0 |
| Tyrosine | 2.02 | 2.0 | 1.30 | 2.0 |
| Cysteine | N.D.* | 4.0 | N.D.* | 4.0 |

* N.D. = not determined.

mixture and the peaks of human insulin A or B chain, pure eluent A was used for a certain period of time: for the A chain 1 min (Fig. 1) and for the B chain 5 min (Fig. 2); after this time, a convex-shaped gradient of a mixture of eluent A and eluent B was started. For the A chain the limiting buffer was TFA (pH 3.2) in 25% acetonitrile (see legend to Fig. 1), and for the B chain formic acid (pH 2.7) in 40% acetonitrile (see legend to Fig. 2). The mobile phase was pumped at a flow-rate of 2.5 ml/min and the column effluent was monitored at 280 nm.

Chromatography was performed at room temperature using a μBondapak $C_{18}$ cartridge. The injection volume of the sample was 10 μl for the analytical determinations and 100 μl for the isolation and purification of human insulin A and B chains.

## RESULTS AND CONCLUSIONS

That both the A and B chains were separated from the peptide mixture (Figs. 3 and 4) by use of this HPLC method is demonstrated by the well resolved peaks corresponding to the standards. The recovered materials were re-chromatographed individually in the same systems and yielded peaks containing the purified A and B chains, respectively.

The very high purity of the products was demonstrated by polyacrylamide gel electrophoresis of the samples, in which a major band, migrating with the appropriate standard, was observed in each instance (Figs. 5 and 6). The results of the amino acid analysis of the samples were in agreement with the expected composition (Tables II and III)[10].

TABLE III

AMINO ACID ANALYSIS OF INSULIN B CHAIN, PRODUCED IN BACTERIA AND OF THE BOVINE B CHAIN STANDARD

Approximately 20 μg of bovine B chain and 20 μg of *E. coli* B chain purified twice by HPLC were hydrolysed and analysed in parallel.

| *Amino acid* | *B chain from bacteria* | | *Bovine B chain used as standard* | |
|---|---|---|---|---|
| | *Found* | *Predicted* | *Found* | *Predicted* |
| Aspartic acid | 1.33 | 1 | 1.0 | 1 |
| Threonine | 1.99 | 2 | 1.03 | 1 |
| Serine | 1.25 | 1 | 1.04 | 1 |
| Glutamic acid | 2.92 | 3 | 2.84 | 3 |
| Proline | 0.91 | 1 | 1.01 | 1 |
| Glycine | 3.01 | 3 | 3.32 | 3 |
| Alanine | 1.03 | 1 | 2.01 | 2 |
| Cisteine | N.D.* | 2 | N.D.* | 2 |
| Valine | 2.85 | 3 | 2.81 | 3 |
| Leucine | 3.86 | 4 | 3.44 | 4 |
| Tyrosine | 1.78 | 2 | 1.83 | 2 |
| Phenylalanine | 2.64 | 3 | 2.86 | 3 |
| Histidine | 1.86 | 2 | 1.8 | 2 |
| Lysine | 0.91 | 1 | 0.82 | 1 |
| Arginine | 1.05 | 1 | 1.19 | 1 |

* N.D. = not determined

REFERENCES

1 Y. Aharonowitz and G. Cohen, *Sci. Am.*, 245 (1981) 66.
2 D. V. Goeddel, D. G. Kleid, F. Bolívar and H. L. Hyneker, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 106–110.
3 E. Gross, *Methods Enzymol.*, 11 (1967) 238–255.
4 X. Alvarado, O. Ladrón de Guevara, G. Estrada, L.Guereca, S. Antonio, N. Cruz and F. Bolívar, unpublished results.
5 T. W. Hearn and W. A. Hancock, *Biochem. Sci.*, 3 (1979) 58–62.
6 K. J. Wilson, A. Honegger and G. J. Hughes, *Biochem. J.*, 199 (1981) 43–51.
7 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, 2nd ed., pp. 260–261.
8 G. Estrada, O. Ladrón de Guevara, X. Alvarado, S. Antonio, N. Cruz, L. Guereca and F. Bolívar, unpublished results.
9 W. J. Dixon and F. J. Massey, *Introduction to Statistical Analysis*, McGraw-Hill, New York, 1957.
10 A. L. Lehninger, *Biochemistry*, 2nd, ed., 1975, p. 109.

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## Note

# Separation of phenylthiohydantoin-amino acids by high-performance liquid chromatography and some applications in dansyl Edman sequence analysis

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Reversed-phase high-performance liquid chromatography (HPLC) has, in recent years, revolutionized the techniques for both the isolation of the fragments and the analytical procedures necessary for determination of the primary structure of peptides and proteins. In particular, numerous HPLC methods have been proposed for the analysis of amino acid phenylthiohydantoin (PTH) derivatives formed during any of the various versions of the classical direct Edman degradation, including the more recent automated micro-sequencing procedures[1–6]. Most of these methods prescribe the inclusion of non-volatile buffers of low ionic strength and pH value in the range 3.5–5.5 in the formula of at least one of the solvents, and chromatography is often performed at relatively high temperatures. Thus some problems may occur, since slight alterations in these parameters will produce significant shifts in the retentions of several PTH-amino acids (PTH-AA). Moreover, adverse effects of salts and temperature on the maintenance of hydraulic components of the instrument and on lifetime of columns, respectively, must be taken into consideration. We report here an alternative procedure for the separation of PTH-AA by HPLC, which in part obviates these disadvantages. This procedure was developed as part of an attempt to overcome the major difficulty with the dansyl Edman technique of sequence analysis, namely differentiation between acids and amides, and to improve identification of histidine, tryptophan and arginine. Typical results obtained are presented.

## MATERIALS AND METHODS

Analyses were performed on a chromatographic unit consisting of two Beckman Model 112 pumps, a Beckman Model 420 controler a Beckman Model 210 sample injection valve, a Beckman Model 160 UV detector, set at 254 nm, and a Perkin-Elmer Model 561 dual-pen chart recorder. The column was a Du Pont Zorbax ODS (5 $\mu$m, 250 × 4.6 mm I.D.). For the rapid separation of PTH derivatives of dicarboxylic and basic amino acids, the column was a Spheri-5 RP-18 (Brownlee, guard column, 30 × 4.6 mm I.D., 5 $\mu$m).

Standard PTH-AA (Pierce) were dissolved in methanol and stored at −20°C. Dansyl Edman degradation was performed according to Gray[7]. The ethyl acetate extracts that contain the anilino-thiazolinone derivatives usually discarded were evaporated to dryness, and then treated with 1 *N* hydrochloric acid at 80°C for 5 min.

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Most PTH-AA were extracted with ethyl acetate and the solution was dried. PTH derivatives of basic amino acids remaining in the aqueous phase were lyophilized. The dried PTH-AA were then redissolved in 0.2% methanolic trifluoroacetic acid, and sample volumes equal to or less than 25 $\mu$l were injected into the column.

Solid-phase sequencing was performed using the LKB 4020 Sequencer. Coupling of peptides was carried out with aminopropyl-glass, activated either by reaction with *p*-phenylene diisothiocyanate or through the C-terminal homoserine lactone, according to the procedures recommended in the LKB Slid-Phase Sequencing Handbook.

Acetonitrile, methanol and 2-propanol were HPLC-grade solvents, obtained from Carlo Erba; ethanol and trifluoroacetic acid were purchased from Merck. Deionized water was first glass-distilled and then passed through a Sep-pak $C_{18}$ cartridge (Waters Assoc.).

## RESULTS AND DISCUSSION

A typical elution profile of a mixture of nineteen standard PTH-AA is shown in Fig. 1; methionine was eluted between valine and proline, and lysine was eluted 1 min before tryptophan. The eluents were (A) 0.2% aqueous trifluoroacetic acid and (B) acetonitrile–2-propanol–ethanol (39.2:9.8:1, v/v/v). The column was equilibrated

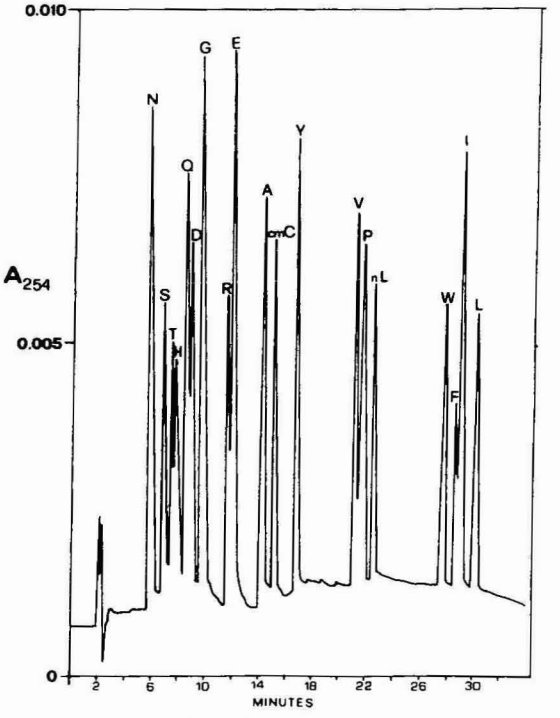


Fig. 1. Separation of standard PTH-amino acids by HPLC on a Zorbax ODS column. The chromatographic system is described in the text. Sample size: 50 pmol of each derivative. The peaks are labelled by one-letter abbreviations for the amino acids, plus nL = norleucine and cmC = carboxymethylcysteine.

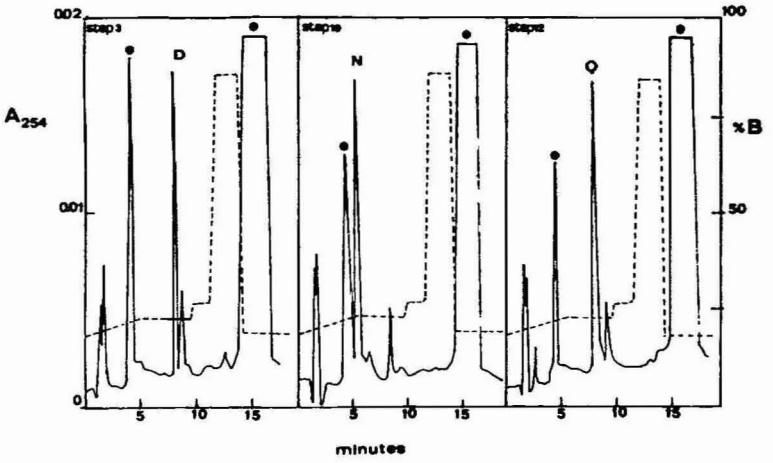


Fig. 2. Selected steps from manual dansyl Edman degradation of 25 nmol of a peptide from serine hydroxymethyltransferase. After the conversion step, an amount from 5 to 20% of the sample was injected onto the column. HPLC conditions as in the text (shorter elution program). Peaks marked with dots are the by-products of the Edman degradation, present in the samples recovered from the dansyl Edman technique. The dashed line shows the theoretical gradient.

with A–B (80:20) at a flow-rate of 1.1 ml/min, at room temperature; the separation was obtained by using the gradient programme reported in Table I; the pressure was 2500 p.s.i. It is noteworthy that immediately after elution of PTH-leucine the system is ready for a new analysis, since the column recycling is completed. Thus, the method can keep pace with the requirements of modern automated sequencers.

One advantage of this method is the simplicity of solvent preparation, which does not require distillation or delicate pH adjustment. In addition, the solvent systems and low temperature used ensure long column life and minimize instrumental

TABLE I

HPLC GRADIENT

| *Time (min after injection)* | *Function* | *Value* | *Duration* (min)* |
|---|---|---|---|
| 0.0 | Flow-rate | 1.1 ml/min | – |
| 0.0 | Amount B | 20% | – |
| 0.05 | Amount B | 23% | 6.0 |
| 4.5 | Flow-rate | 0.7 ml/min | 0.2 |
| 9.1 | Flow-rate | 0.8 ml/min | 0.1 |
| 9.2 | Amount B | 27% | 0.2 |
| 11.0 | Amount B | 33% | 0.5 |
| 11.0 | Flow-rate | 0.8 ml/min | 0.1 |
| 15.5 | Flow-rate | 1.1 ml/min | 0.1 |
| 15.5 | Amount B | 36% | 9.5 |
| 22.0 | Flow-rate | 0.7 ml/min | 0.2 |
| 23.0 | Flow-rate | 1.1 ml/min | 6.5 |
| 25.5 | Amount B | 20% | 1.5 |
| 31.0 | Start | | |

* Time required to reach the programmed value of the indicated function.

problems. Flow-programming during the course of chromatography was of chief importance in controlling the resolution and analysis time. In particular, a decrease in the flow-rate was essential for resolution of the polar derivatives, whereas the two subsequent increases by step from 0.7 to 1.1 ml/min allowed resolution of PTH-AA from Ala to nor-Leu in reasonable time. Finally, decreasing the flow-rate to 0.7 ml/min could split PTH-Ile from PTH-Phe. Moreover, the adverse effects of column ageing on the separation can be compensated for by decreasing the flow-rate by *ca.* 10% at steps 4, 5 and 11. When peak broadening increases with column use, a regeneration treatment, consisting of a washing with 100 ml of benzene–2-propanol (30:70, v/v), restored column performance. These precautions ensured satisfactory reproducibility of the retention times of the peaks over more than 6 months of continuous use (more than 1000 injections) of a single column with standard PTH-AA and with derivatives generated during the operation of a solid-phase sequencer; essentially similar results were obtained with a substitute Zorbax column.

The method has been applied to the solution of some problems with the dansyl Edman method of sequence analysis. This procedure is still very popular for the structural analysis of peptides at the nanomolar level because of its economy and accessibility to non-specialized laboratories, yet it has certain drawbacks. For instance, it does not allow identification of the amidation state of dicarboxylic amino acid residues, and identification of some other residues, such as those of Cm-cys, Trp, Arg and His, is often difficult. The PTH derivatives of these amino acids may be recovered from a side-fraction, usually discarded in the course of classical dansyl Edman procedure (see Materials and methods). In fact, in our laboratory they were successfully identified recently by routine application of the above-described HPLC

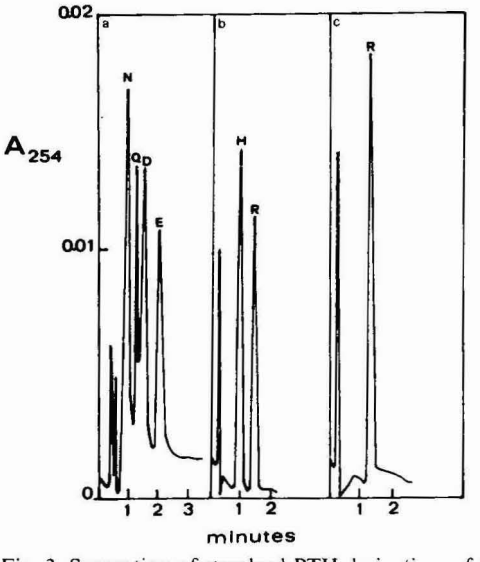


Fig. 3. Separation of standard PTH derivatives of (a) dicarboxylic amino acids and their amides and (b) basic amino acids, on a mini Spheri-5 RP-18 column. The chromatographic system is described in the text. Isocratic elution was accomplished at room temperature by 20% of solvent B at a flow-rate of 0.9 ml/min (a) or 1.1 ml/min (b). Identification of step 8 from manual dansyl Edman degradation of 25 nmol of a peptide from serine hydroxymethyltransferase is reported in (c).

method. The by-products of the Edman degradation, which in the dansyl Edman version are present in the ethyl acetate extracts, together with the anilino-thiazolinone derivatives of the amino acids, do not interfere with the analysis of polar PTH-AA. A shorter elution protocol may be chosen for their identification, with recycling starting after 11 min and analysis being completed in 18 min. The chromatograms in Fig. 2 illustrate the identification by the latter procedure of some PTH-AA recoverd in the course of the dansyl Edman degradation of the peptide GLDPQCWGVNVQPYSGSPANFAVYTALVEPH from cytosolic serine hydroxymethyltransferase, an enzyme from rabbit liver, the sequence of which is under investigation in our laboratory.

In addition, we have set up conditions for the faster and more economical solution of specific problems individually encountered in the course of dansyl Edman degradation, by following a minicolumn approach, similar to that reported by Bhown and Bennett[8]. Fig. 3a shows the separation of the PTH derivatives of Glu, Gln, Asp and Asn, obtained isocratically in 3 min on a Spheri-5 RP-18 guard column. Under similar conditions (Fig. 3b), it is possible to separate PTH-Arg and PTH-His, present in the aqueous phase after conversion of the anilino-thiazolinones. An obvious advantage of these identification procedures is the dramatic reduction of expenditure for the column (the cost of a Guard column is one-sixth that of a Zorbax ODS). An example of the application of this method in the course of sequence determination of peptide IFYRRGVRSVDPKTGKE from serine hydroxymethyltransferase is presented in Fig. 3c.

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REFERENCES

1 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569–573.
2 N. D. Johnson, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 100 (1979) 335–338.
3 G. E. Tarr, *Anal. Biochem.*, 111 (1981) 27–32.
4 D. Hawke, P.-M. Yan and J. E. Shively, *Anal. Biochem.*, 120 (1982) 302–311.
5 M. W. Hunkapiller and L. E. Hood, *Methods Enzymol.*, 91 (1983) 493.
6 P. Pucci, G. Sannia and G. Marino, *J. Chromatogr.*, 270 (1983) 371–377.
7 W. R. Gray, *Methods Enzymol.*, 25 (1972) 121–138.
8 A. S. Bhown and J. C. Bennett, *Anal. Biochem.*, 137 (1984) 256–260.

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CHROMSYMP. 651

# COUPLING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPH WITH A LIQUID SCINTILLATION DETECTOR: EXAMPLE IN THE FIELD OF PYRETHROIDS

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## SUMMARY

The resolving power of high-performance liquid chromatography (HPLC) and the high specificity and sensitivity of in-line scintillation detection (SD) were used to study the transformation of tralomethrin, a new synthetic pyrethroid. It was believed that the insecticidal activity of tralomethrin resulted from its transformation to the active deltamethrin. Radiolabelled tralomethrin was first incubated with two different types of excitable membranes. The samples were then analysed by HPLC–SD and shown to contain intact tralomethrin. These data and electrophysiological experiments were used to rule out deltamethrin as the cause of insecticidal activity in tralomethrin.

## INTRODUCTION

Radiolabelled molecules are useful tools for investigating the disposition of pharmaceuticals in animal organisms. However, although the radioactivity permits the detection of minute amounts of a compound, it is not suitable for specifying whether the compound is transformed or not. Indeed, the fate of the compound depends on its possible biotransformation. After such a transformation, the analysis of the reaction mixture is effected by means of a chromatographic procedure that allows the separation and, possibly, the identification of the transformed products. Thin-layer chromatography, followed by either autoradiography or liquid scintillation counting, may be used in such instances[1–3]. A few examples[4,5] highlight the advantages of coupling high-performance liquid chromatography (HPLC) with a radioactivity detector able to monitoring continuously the eluate from the chromatographic column,

Tralomethrin {(*S*)-α-cyano-3-phenoxybenzyl-(1*R*,3*S*)-2,2-dimethyl-3-[(*R*,*S*)-1,2,2,2-tetrabromoethyl]cyclopropane carboxylate} (RU 25474), a new pyrethroid insecticide, consists of two isomers, RU 24784 and RU 24785. Three of its four asymmetric carbon atoms have a well defined configuration, 1*R*,3*S*,α*S* (the same as in deltamethrin[6]), whereas the fourth may exist in either the *R* (RU 24784) or the *S* (RU 24785) configuration (Fig. 1).

The insecticidal activity of tralomethrin was suspected to result from its pos-

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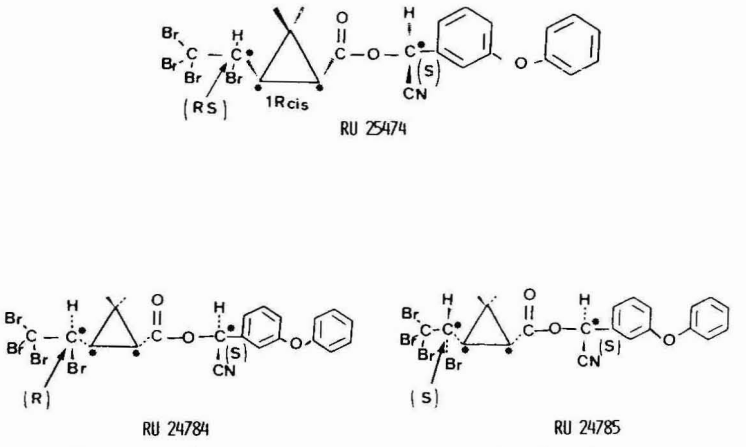


Fig. 1. Structures of the two epimeric components of tralomethrin.

sible transformation to deltamethrin[1,2,7]. In this study, radiolabelled tralomethrin, bound to the abdominal nerve cords of the American cockroach (*Periplaneta americana*), was analysed by means of HPLC coupled with liquid scintillation detection (SD). It was first observed that tralomethrin intoxication behaves differently from that by deltamethrin[8]. Here, the lack of biotransformation of tralomethrin is confirmed. Indeed, the neurotoxic activity of compounds, *e.g.*, pyrethroids, and in this instance tralomethrin, has been studied *in vitro* with two different excitable membranes: insect axonal membrane and mammalian neuroblastoma cells in culture[8]. For a precise knowledge concerning the fate of tralomethrin, we have used the radiolabelled compound. Analysis of these preparations by HPLC–SD allowed us to characterize and identify this compound unequivocally.

## EXPERIMENTAL

### *Materials*

Tralomethrin (RU 25474) was obtained by chemical synthesis[9]. The $^{14}C$ compound was labelled at the two *gem*-methyl groups of the cyclopropane ring (59.3 mCi/mmole).

[$^{14}C$]Deltamethrin was also labelled at the two *gem*-methyl group of the cyclopropane ring (60 mCi/mmole).

The internal standard was commercially available [$^{14}C$]$\beta$-naphthol (CMM 218) (Commissariat à l'Énergie Atomique, Saclay, France).

### *Apparatus and procedures*

The HPLC apparatus consisted of an M380 Chromatem pump (Touzart et Matignon, Berkeley, CA, U.S.A.), a Rheodyne 7125 injector with a loop of 20 $\mu$l and a LiChrosorb Si 60, 5-$\mu$m column (25 cm × 0.25 in. I.D.) (Merck, Darmstadt, F.R.G.). The Flo-one/DR (Radiomatic Instrument & Chemical Co., FL, U.S.A.) liquid scintillation detector was connected to the column outlet; keyboard operation permitted the selection of pump flow-rate and scale multiplier. The flow-rate of the pump was adjustable between 0.1 and 10 ml/min in order to provide the optimal flow. A microprocessor processed the data from scintillation counting and provided the results by printing the number of counts and drawing a curve on a chart recorder.

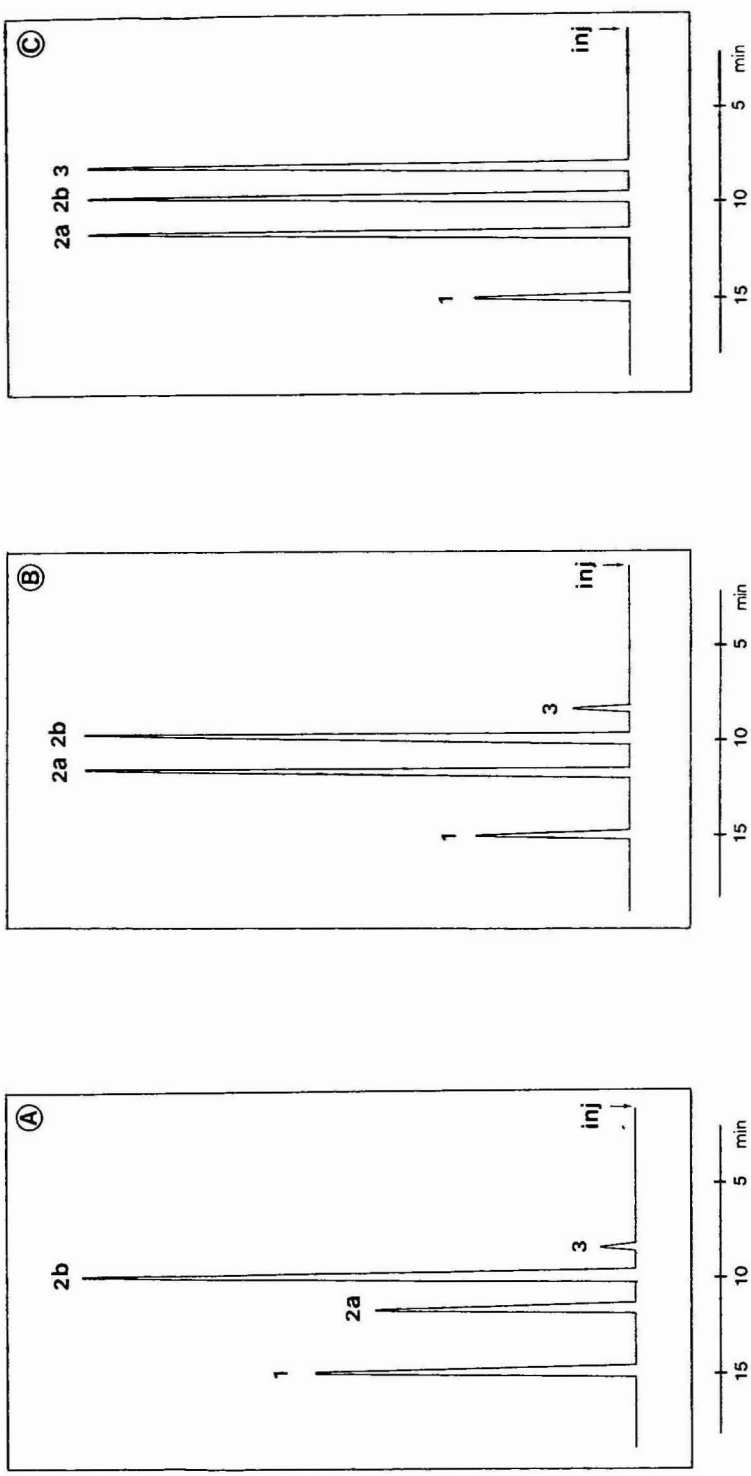


Fig. 2. Separation of tralomethrin (two epimers) by HPLC coupled with a liquid scintillation detector. Peaks: 1 = internal standard, [$^{14}$C]$\beta$-naphthol; 2a and 2b = pure [$^{14}$C]tralomethrin epimers; 3 = [$^{14}$C]deltamethrin (pre-existing impurity). (A) Chromatographic profile of the medium before incubation with nerve cord cells. 1, 6 $\mu$Ci; 2, 9.9 $\mu$Ci; 3, 0.4 $\mu$Ci. (B) Chromatographic profile of the extract obtained 1 after incubation with the medium analysed in A. 1, 0.2 $\mu$Ci; 2, 2.1 $\mu$Ci; 3, 0.1 $\mu$Ci. (C) Chromatographic profile of the nerve cord extract (B), showing an increase in the [$^{14}$C]deltamethrin peak after addition of this compound.

*Analytical conditions*

The eluent (spectrographic grade materials from Fluka, Buchs, Switzerland) consisted of 1700 ml of hexane, 140 ml of pentane, 25 ml of acetonitrile, 45 ml of dioxane, 15 ml of 2-propanol and Lumaflow I fluid scintillator (Kontron, Zurich, Switzerland) at a flow-rate of 1 ml/min. The flow-rate of the Chromatem pump was 0.6 ml/min.

Under these conditions, the sensitivity of this method was 20 pCi/mmole, corresponding to 15 pmole of tralomethrin applied to the column.

## RESULTS

Analysis of [$^{14}$C]tralomethrin (RU 25474), extracted from the incubation medium prior to its application to the cockroach nerve cells, showed the two diastereoisomeric constituents (RU 24784 and RU 24785, 2a and 2b) (Fig. 2A). It also revealed a low content (about 4%) of an impurity, 3, with a retention time identical with that of deltamethrin. This impurity is probably the result of the preparation method of tralomethrin.

After incubation with cockroach nerve cords for 1 h and extraction, the chromatographic profile of RU 25474 (Fig. 2B) was not altered, and the impurity content (about 4.5%) was not significantly modified. As the addition of [$^{14}$C]deltamethrin results in an increase in the impurity peak (Fig. 2C), it is assumed to be due to that compound.

## CONCLUSIONS

From these results, it can be deduced that no significant biotransformation of tralomethrin molecules occurs when the latter are applied to cockroach nerve cords for 1 h. As it has been demonstrated that tralomethrin acts in less than 1 h and as the electrophysiological response to tralomethrin intoxication differs noticeably from that of deltamethrin, it can be concluded that the low amount of deltamethrin present in tralomethrin as an impurity cannot be held responsible for the insecticidal activity of tralomethrin.

This study exemplifies the ability of HPLC, coupled with liquid scintillation detection, to differentiate between diastereoisomers with regard to their retention times and to quantify them accurately on the basis of their radioactivity.

## REFERENCES

1 L. O. Ruzo and J. E. Casida, *J. Agric. Food Chem.*, 29 (1981) 702.
2 L. M. Cole, L. O. Ruzo, E. J. Wood and J. E. Casida, *J. Agric. Food Chem.*, 30 (1982) 631.
3 L. M. Cole, J. E. Casida and L. O. Ruzo, *J. Agric. Food Chem.*, 30 (1982) 916.
4 L. N. Mackey, P. A. Rodriguez and F. B. Schroeder, *J. Chromatogr.*, 208 (1981) 1.
5 S. Baba, M. Horie and K. Watanabe, *J. Chromatogr.*, 244 (1982) 57.
6 M. Elliot, A. W. Farnham, N. F. Janes, P. M. Needham and D. A. Pulman, *Nature (London)*, 248 (1974) 710.
7 S. N. Irving and T. E. M. Fraser, *J. Agric. Food Chem.*, 32 (1984) 111.
8 M. Roche, C. Frelin, P. Bruneau and C. Meinard, *Pestic. Biochem. Physiol.*, 24 (1985) in press.
9 Roussel Uclaf, *French Pat.*, 2 364 884, 1978.

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CHROMSYMP. 663

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPH COUPLED WITH TWO DETECTORS: A UV SPECTROMETER AND A POLARIMETER

## EXAMPLE IN THE FIELD OF PYRETHROIDS: IDENTIFICATION OF ENANTIOMERS

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SUMMARY

Identification of enantiomers is possible by using two detectors in high-performance liquid chromatography: a UV spectrometer and a polarimeter. In a mixture of enantiomers and diastereoisomers, such as those resulting from a chemical structure with several asymmetric carbons, the UV spectrometer allows the detection of the diastereoisomers, whereas the polarimeter permits the identification of the enantiomers.

Deltamethrin, an optically pure synthetic pyrethroid widely used as an agricultural insecticide, can undergo partial isomerization, especially through the action of light. Every photoisomer in the resulting mixture is a diastereoisomer of deltamethrin with two possible enantiomeric forms. The above method was applied to determine the spatial configurations of the isomers formed, and conclusions were drawn about the mechanism of photoisomerization.

## INTRODUCTION

High-performance liquidchromatography (HPLC), coupled with UV spectrometric detection, is widely used for the separation, identification and quantification of diastereoisomers, such as those encountered in the pyrethroid series[1]. However, this method is not suitable for determining the absolute configurations when enantiomeric forms are present, the separation of the latter requiring the use of a chiral chromatographic sorbent[2,3].

An easier way of identifying enantiomers is to use a combination of a UV detector with a polarimetric detector, both coupled with HPLC. The recorder integrator simultaneously indicates the UV absorption and the rotation as a function of retention time. Use of these data permits the analysis of the mixture, provided that a sample of at least one optically pure enantiomer of each diastereoisomer is available[4–6].

Deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl-(1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate] (RU 22974) is a synthetic pyrethroid widely

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used as an agricultural insecticide[7]. It is an optically active compound that contains three asymmetric carbon atoms with the configurations 1*R*,3*R*,α*S* (Fig. 1)[8,9]. Through the action of light, deltamethrin undergoes isomerization. Isomerization of all three asymmetric centres could lead to eight isomers (four diastereoisomers, each having two enantiomeric forms). Previous studies[10,11] have indicated the opening and recyclization of the cyclopropane ring (involving the asymmetric centres 1 and 3) and the possible isomerization of the benzylic carbon atom (involving the centre α). Coupling HPLC with both UV spectrometry and polarimetry, and having some optically pure samples at our disposal, thus enabled us to determine the absolute configuration of the photoisomers of deltamethrin. Additional information about the mechanism of photoisomerization was consequently deduced; we can assume that the benzylic carbon atom (α) has not undergone isomerization.

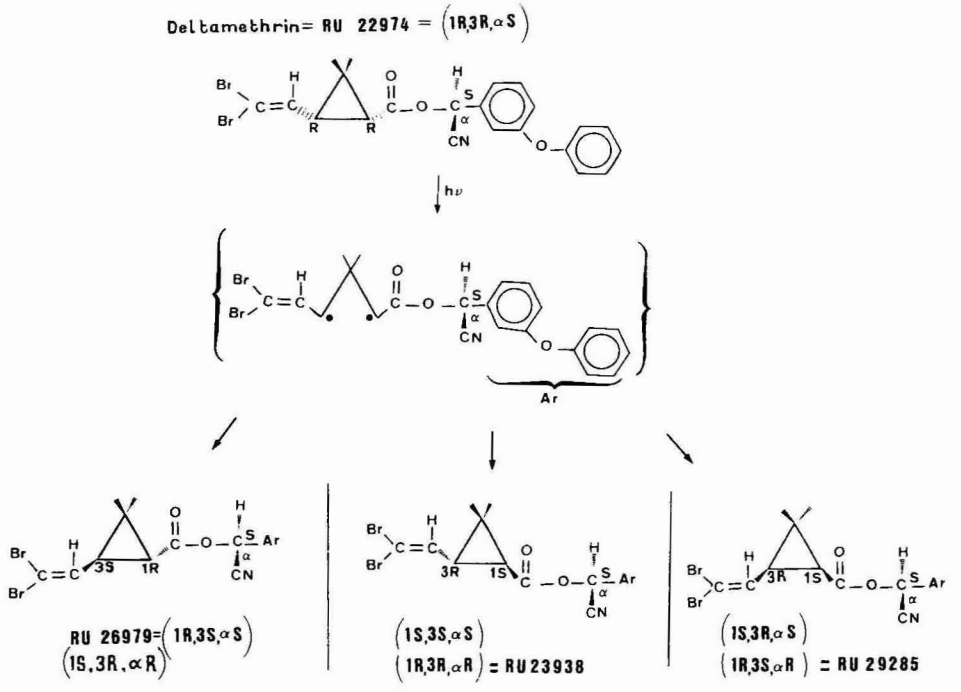


Fig. 1. Photoisomerization of deltamethrin.

## EXPERIMENTAL

### *Materials*

Deltamethrin (RU 22974) was obtained by chemical synthesis and is now commercially available*. Three of its diastereoisomers, all having the *R* configuration at C-1 1*R*,3*R*,α*R* (RU 23938), 1*R*,3*S*,α*S* (RU 26979) and 1*R*,3*S*,α*R* (RU 29285), were previously prepared by an unequivocal procedure[12]. They are combinations of 1*R*,3*R* (*cis*) or 1*R*,3*S* (*trans*) acidic moieties with either *R* or *S* cyanobenzylic alcohols.

* Trade name, DECIS (Roussel Uclaf); previous trivial name, decamethrin.

*Apparatus and procedures*

Deltamethrin, as a thin, crystalline layer (0.1 mg/cm$^2$), was submitted to irradiation in a SUNTEST apparatus, equipped with a 150 klux xenon vapour lamp (both from Heraeus, Hanau, F.R.G.), which has an emission spectrum approximating that of the sun. After irradiation for 60 h, the resulting photoisomerization mixture was analysed.

The HPLC system consisted of a LiChrosorb Si 60, 5-$\mu$m column (250 mm $\times$ 4 mm I.D.) (Merck, Darmstadt, F.R.G.). an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.) and a Rheodyne 7125 injection valve (Rheodyne Cotati, CA, U.S.A.) with a 20-$\mu$l loop.

The detection systems consisted of a Varichrom UV 50 variable-wavelength UV detector (Varian, Palo Alto, CA, U.S.A.) and a Perkin-Elmer 241 LC polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a 0.65-mm I.D. microflow cell, having a 10-cm optical pathway. The latter was used at 302 nm* and the resulting recording range was $\pm 0.5°$ full-scale. These two detectors were connected by a stainless-steel capillary tube.

The UV absorption and optical rotation data were fed into an SP 4200 data system (Spectra-Physdics, San Jose, CA, U.S.A.), which traced the corresponding curves and calculated the areas under the peaks.

*Analytical conditions and reagents*

The analytical flow-rate was 1.3 ml/min. The mobile phase consisted of 1900 ml of hexane, 45 ml of acetonitrile, 100 ml of pentane, 10 ml of dioxane and 1.5 ml of 2-propanol (spectrographic grade, Fluka, Buchs, Switzerland). This eluent was suitable for separating all the apolar components of the photoisomerization mixture. The polar photodegradation products were not investigated. The UV detector was used at 230 nm. Dimethyl isophthalate (Merck), having a UV absorption maximum at 226 nm, was used as an internal standard.

## RESULTS AND DISCUSSION

*Principle of the determination*

The area, $s$, under each peak of the polarimetric curve measures the amplitude of the optical rotation (OR) and is therefore proportional to the amount, $m$, of the diastereoisomer and to its rotary power. When the compound is optically pure (single enantiomer) with a rotatory power $\alpha_0$, then

$$s = k_1 m \alpha_0 \text{ (OR)}$$

The area, $S$, under each corresponding UV absorption (UV) peak depends only on the amount, $m$, of the compound:

$$S = k_2 m \text{ (UV)}$$

* The 302 nm wavelength was selected from those provided by a mercury vapour lamp. The maximum of the optical rotatory dispersion for this kind of derivatives is located at 223–230 nm, and these wavelengths would have ensured greater accuracy.

In any case, the ratio $s/S$ (OR/UV) is independent of the amount of the compound and, for a determined pure enantiomer, this ratio is a constant, which depends on the value of its $\alpha_0$ rotatory power:

$$r = \frac{s}{S} = \frac{k_1 m \alpha_0}{k_2 m} = \frac{k_1}{k_2} \cdot \alpha_0 = k_3 \alpha_0$$

If the diastereoisomer detected by chromatography were a blend of its two enentiomers [in the proportions of $1-x$ for the enantiomer of rotary power $\alpha$ to $x$ for the antipode $(-\alpha)$], then the UV absorption would be identical, $S = k_2 m$, whereas the optical rotation would have been affected:

$$\begin{aligned} s' &= k_1(1 - x)m\alpha_0 + xm(-\alpha_0) \\ &= k_1 m(1 - 2x)\alpha_0 \end{aligned}$$

Therefore, the ratio $s'/S$ would also have been altered:

$$r' = \frac{k_1 m(1 - 2x)\alpha_0}{k_2 m} = k_3(1 - 2x)\alpha_0$$

$$r' = r(1 - 2x); \quad r - r' = 2rx$$

The difference between the values of the ratios $r$ and $r'$ is directly proportional to the content $x$ of the antipodal isomer. If it is equal to zero, then the diastereoisomer is optically pure, and is identical with the standard sample. If the $r'$ ratio has the same absolute value, but is reversed ($r' = -r$), then $x = 1$. The diastereoisomer is optically pure, but is antipodal to the standard sample. If there was no optical rotation, $r' = 0$, then $x = 0.5$, and the diastereoisomer would be the racemic mixture of the enantiomers.

*Accuracy of the method*

The inaccuracy concerniong the value of the ratio $r$ is a result of experimental errors in determining the values of the areas $s$ and $S$. Optical rotation determinations are less precise than the UV absorption measurements, and as the value of $s$ is directly proportional to the rotary power $\alpha_0$ of the compound, the lower the rotatory power, the greater is the inaccuracy. For this reason, amplification of the OR signal has been advised[6]. A statistical study (ten analyses) of a mixture of optically pure samples indicated that the error in the ratio r may be in the range $\pm 5$ to $\pm 10\%$, depending on the compound.

*Results*

The first practical experiment was the analysis of the artificial mixture of deltamethrin and some of its optically pure diastereoisomers[12] in equal amounts (except RU 26979, for which the amount was tripled because of its low rotatory power). These compounds [RU 22974 (deltamethrin), RU 23938, RU 26979 and RU 29285] were thought to be present in the photoisomerization mixture. On one hand, their retention times could be determined on the basis of the UV absorption curve, and

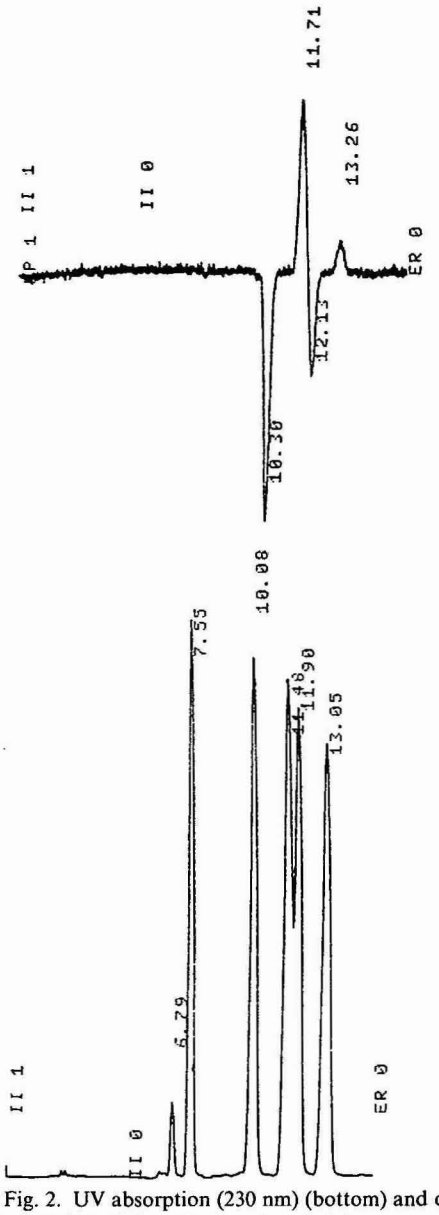


Fig. 2. UV absorption (230 nm) (bottom) and optical rotation (302 nm) (top) of the standard samples as a function of the retention time (min).

| *Compound* | *Retention time (min)* | |
|---|---|---|
| | *UV* | *OR* |
| Unknown impurity | 6.79 | |
| Internal standard | 7.55 | |
| RU 23938 | 10.08 | 10.30 |
| RU 22974 | 11.48 | 11.71 |
| RU 29285 | 11.90 | 12.13 |
| RU 26979 | 13.05 | 13.26 |

TABLE I

RATIOS $r$ (OR/UV) OF DELTAMETHRIN PRODUCTS

| *Compound* | *Configuration* | *Retention time (min)* | *Ratio r** | *Error (%)* |
|---|---|---|---|---|
| Internal standard | | 7.6 | | |
| RU 22974 | 1*R*,3*R*,α*S* | 11.5 | +0.32 | + 6.5 |
| RU 23938 | 1*R*,3*R*,α*R* | 10.1 | −0.42 | + 5 |
| RU 26979 | 1*R*,3*S*,α*S* | 13.0 | +0.08 | +10 |
| RU 29285 | 1*R*,3*S*,α*R* | 11.9 | −0.16 | + 8.5 |

* Mean values of ten determinations.

on the other, the polarimetric recorder revealed that two of the samples (RU 22974)and RU 26979) are dextrorotatory, whereas the other two (RU 23938 and RU 29285) are laevorotatory (Fig. 2).

The ratio $r$ (OR/UV) was calculated for each compound and the data are listed in Table I.

The second experiment, carried out under identical conditions, concerned the mixture resulting from the photoisomerization of deltamethrin. The analysis indicated the presence of four main components, A, B, C and D (Fig. 3), and revealed that their retention times correspond to those of the samples investigated in the first experiment.

The UV absorption curve gives an idea of their relative amount. The addition of an internal standard in both the first and the second experiments permits the quantitative determination of the amounts of each detected diastereoisomer. Their total equals 51.3% of the initial amount of deltamethrin. The calculated relative amounts are listed in Table II.

The simultaneously recorded optical rotation indicates that every component of the mixture is dextrorotatory (Fig. 3). The ratios $r'$ (OR/UV) were calculated for the compounds A, B, C and D (Table II). Their absolute values are not significantly different from those corresponding to the standard samples. However, two of them are opposite in sign.

The close parallel between these and the previous data allows us to assume that, within the limits of experimental error, the retention times are equivalent and so are the absolute values of the ratio $r$ (OR/UV).

TABLE II

RATIOS $r'$ (OR/UV) OF DELTAMETHRIN PRODUCTS)

| *Compound* | *Retention time (min)* | *Ratio r'** | *Relative amount (%)* |
|---|---|---|---|
| Internal standard | 7.8 | | |
| A | 11.3 | +0.33 | 20.7 |
| B | 9.9 | +0.42 | 6.2 |
| C | 13.0 | +0.07 | 56.7 |
| D | 11.8 | +0.16 | 16.5 |

* Mean values of three determinations.

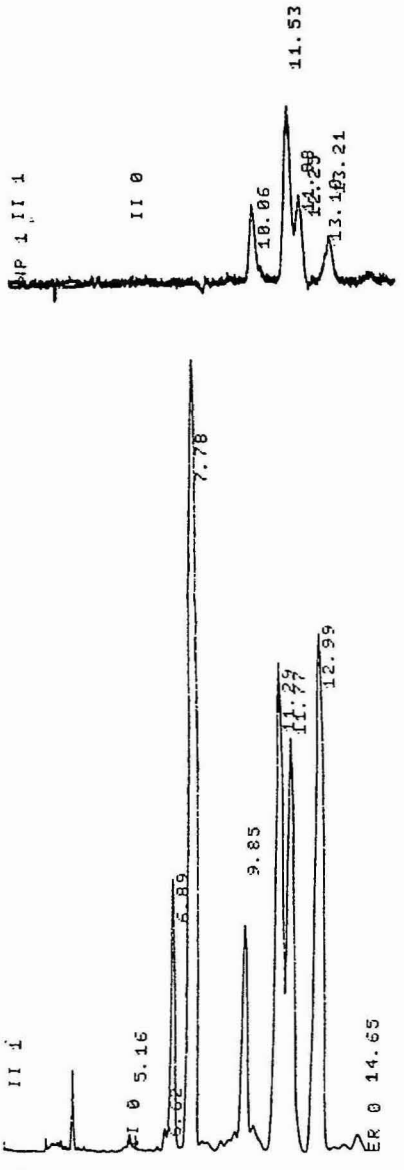


Fig. 3. UV absorption (230 nm) (bottom) and optical rotation (302 nm) (top) of the photoisomerization products as a function of the retention time (min).

| *Compound* | *Retention time (min)* | |
|---|---|---|
| | *UV* | *OR* |
| Unknown impurity | 6.89 | |
| Internal standard | 7.78 | |
| B | 9.85 | 10.06 |
| A | 11.29 | 11.53 |
| C | 11.77 | 11.98 |
| D | 12.99 | 13.10 |

TABLE III

ABSOLUTE COMFIGURATIONS OF DELTAMETHRIN PRODUCTS

| *Component* | *Absolute configuration* |
|---|---|
| A | 1*R*,3*R*,α*S* |
| B | 1*S*,3*S*,α*S* |
| C | 1*R*,3*S*,α*S* |
| D | 1*S*,3*R*,α*S* |

## CONCLUSIONS

Taking into account the sign of the ratio $r'$, it can be deduced that two components of the photoisomerization mixture are likely to be identical with the standards: A corresponds to RU 22974 (deltamethrin) and C to RU 26979. The two other components are likely to be the antipode of the other two standards: B corresponds to the antipode of RU 23938 and D to the antipode of RU 29285. The absolute configurations of the four components are then likely to be those indicated in Table III.

In each of them the *S* configuration of the benzylic carbon atom is maintained, while the two asymmetric centres, 1 and 3, may exist in both the *R* and *S* configurations. This observation leads to the following conclusions concerning the photochemical pathways of isomerization. Racemization of the asymmetric centres 1 and 3 occurs and probably results from the opening and re-cyclization of the cyclopropane ring, as suggested previously[10,11].

The lack of photoisomers with the *R* configuration at the benzylic carbon atom negates the question of possible racemization of the alcohol group. This question could not have been answered whilst the detection method was not suitable for characterizing enantiomers. HPLC, coupled with UV spectrometry and polarimetry, is suitable for resolving this kind of problem. Some technical improvements, especially with regard to the light source of the polarimeter, should improve the accuracy of the method.

## REFERENCES

1 C. Meinard, J. C. Suglia and P. Bruneau, *J. Chromatogr.*, 176 (1979) 140.
2 W. H. Pirkle, J. M. Finn, B. C. Hamper, J. Chreiner and J. R. Pribish, *ACS Symp. Ser.*, 185 (1982) 245.
3 A. A. Kurganov, A. B. Tevlin and V. A. Davankov, *J. Chromatogr.*, 261 (1963) 223.
4 W. Bohme, *Chromatogr. Newl.*, 28 (1980) 709.
5 W. Bohme, G. Wagner, U. Oheme and U. Priesnitz, *Anal. Chem.*, 54 (1982) 709.
6 B. S. Scott and D. L. Dunn, *J. Chromatogr.*, 319 (1985) 419.
7 *Deltamethrin Monograph*, Roussel Uclaf, Paris, 1982.
8 M. Elliott, A. W. Farnham, N. F. Janes, P. H. Janes, P. H. Needham and D. A. Pulman, *ACS Symp. Ser.*, 2 (1974) 80; *Nature (London)*, 248 (1974) 710; *Pestic. Sci.*, 6 (1975) 537.
9 J. D. Owen, *J. Chem. Soc., Perkin Trans. 1*, (1975) 1865.
10 L. O. Ruzo, R. L. Holmstead and J. E. Casida, *J. Agric. Food Chem.*, 25 (1977) 1385.
11 L. O. Ruzo, in D. H. Hutson and E. R. Roberts (Editors), *Progress in Pesticide Biochemistry*, Wiley, New York, 1982, Vol. 2, Ch. 1.
12 J. Tessier and J. P. Demoute, unpublished results.

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# REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF A NOVEL PROLINE-RICH TRYPTOPHYLLIN

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## SUMMARY

A tryptophan-containing tridecapeptide of amphibian origin (tryptophyllin-13, TPH-13), having the sequence <Glu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH, was found to give peak splitting in isocratic and gradient reversed-phase high-performance liquid chromatography. The influence of many experimental conditions (column temperature, type of bonded phase, buffer pH, type of organic solvent, flow-rate, on-column incubation of the peptide, presence of a denaturant in the mobile phase, oxidation state of the peptide) on the chromatographic pattern of TPH-13 under selected gradient conditions, was studied. The raising of column temperature to 45°C was always found to suppress peak splitting, whereas the other modifications affected the resolution and/or relative intensity of the two peaks of the doublet. Using a mobile phase buffer with a pH near neutrality, the two peaks could be partially separated by preparative high-performance liquid chromatography, and were shown to be in equilibrium with each other. A pronounced conformational isomerism of the peptide chain, due to the presence of five proline residues, three of which are consecutively linked, is suggested to give rise to two major conformers of comparable stability and different hydrophobicity, detectable as a doublet by reversed-phase high-performance liquid chromatography.

## INTRODUCTION

During the isolation of dermorphins[1] from skin extracts of *Phyllomedusa rohdei*, a South American frog, another set of new peptides was identified, characterized by the presence of a tryptophan residue in all their sequences. This common feature, together with their amphibian origin, suggested the collective name "tryptophyllins" (TPHs) for these peptides[2]. TPHs with different chain lengths have been isolated, ranging from some tetrapeptides to a tridecapeptide, and their primary structures have been determined[3–5]. They have been replicated by conventional synthetic methods together with some analogues, and investigated in several biological systems[6–8].

The heaviest tryptophyllin characterized so far, TPH-13, has the following amino acid sequence:
<Glu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH

0021-9673/85/$03.30 

An interesting feature of its structure is the abundance of proline. Five residues are present in the molecule, and three of them are consecutively linked.

The natural peptide and its synthetic replicate were checked for purity and identity by different techniques, including reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis. The products were initially analysed on a cyanopropylsilyl column under isocratic conditions (60% acetonitrile in 0.1 *M* formic acid)[5], yielding a single sharp peak both individually and also when injected together. Nevertheless, only methionine sulphoxide was released as a C-terminal amino acid from the analysed sample of natural TPH-13 by carboxypeptidase Y (CP-Y). However, methionine, instead of methionine sulphoxide, appeared when the synthetic replicate was subjected to digestion with CP-Y[2]. This suggested that the natural product had been oxidized *via* the purification procedure, as already found for other methionine-containing peptides[9–11]. We have consequently developed a gradient elution method for separating the reduced peptide from its oxidized counterpart, and we have indeed confirmed that the preparation of natural TPH-13 examined contained methionine sulphoxide. While testing different RP-HPLC conditions, we also observed a seemingly anomalous retention behaviour of the natural and synthetic sample, namely, splitting of the chromatographic peak into an approximately symmetric doublet.

There have been various reports on single protein species yielding multiple and/or irregularly shaped peaks using RP-HPLC[12–16], as well as in high-performance size-exclusion[17] and ion-exchange chromatography[18,19]. A variety of properties of macromolecular substances can potentially play a role in this behaviour (*e.g.* conformational changes in protein structure) and the above-cited observations have in general been attributed to changes in the tridimensional structure of the biopolymer, which are caused by denaturation.

The finding that pure TPH-13 yields double peaks under some RP-HPLC conditions prompted us to investigate its chromatographic behaviour more thoroughly, with the goal of ascertaining whether peak splitting of this medium-sized peptide could also be related to conformational isomerism. As already pointed out, this tryptophyllin spans a proline-rich sequence, which might give rise to a series of conformers of comparable stability, due to the small difference in energy content between *cis*- and *trans*-conformations of proline residues[20]. The full characterization of this proline-rich peptide by RP-HPLC, described in the present paper, provides indications that two major conformational isomers of TPH-13 may be responsible for peak splitting.

## EXPERIMENTAL

### *Chromatography*

All chromatographic experiments were performed on a Hewlett-Packard 1084 B apparatus, equipped with an HP 1040 diode array detector, controlled through an HP 85 computer.

A μBondapak $C_{18}$ and a μBondapak CN column (300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) were used throughout the investigation. A flow-rate of 1 ml/min was always employed, except when the effects of flow-rate changes on the chromatographic pattern were studied. The temperature of the column was kept

at 0°C by immersing it in an ice-water bath, or controlled through the 1084 B thermostated oven when temperatures above ambient (*ca.* 25°C) were desired. The detection wavelength was fixed at 280 nm.

*Materials*

Natural TPH-13 and its tryptic fragments were obtained as already described[5]; the synthetic replicate was synthesized by conventional methods[8]. All other materials were of analytical grade.

The samples for chromatography were made up in 50% ethanol (*ca.* 1 mg/ml), unless otherwise indicated. Solutions and buffers containing urea were freshly prepared daily.

*Chemical methods*

Synthetic TPH-13 was oxidized to [Met(O)$^{13}$]TPH-13 by treatment with 0.18% hydrogen peroxide in 0.05 *M* acetic acid (peptide concentration, *ca.* 0.5 mg/ml) for 15 min at room temperature. Reduction of extractive TPH-13 was achieved with 2-mercaptoethanol according to the method described by Floor and Leeman for the reduction of substance P sulphoxide[9].

## RESULTS

*Preliminary investigation*

Synthetic and natural TPH-13 were found to be unresolved by isocratic RP-HPLC on a cyanopropyl(CN)-bonded phase[5]. They were still eluted together in a sharp peak when the mobile phase (60% acetonitrile in 0.1 *M* formic acid) previously used on the CN column was employed on an octadecyl($C_{18}$)-bonded phase. Eluents containing the same buffer and lower percentages (from 50 to 30%) of acetonitrile were also tested on both columns, but the two peptides were not resolved. Moreover, poorly shaped or anomalous peaks were obtained as the acetonitrile content of the mobile phase was reduced. In particular, a clear doublet with a low retention time was observed when the samples were analysed on the $C_{18}$ column with 40% acetonitrile in 0.1 *M* formic acid as eluent (see Fig. 1), both individually and in a mixture. The split peak coalesced reversibly as column temperature was raised to 45°C.

Conditions were then changed from isocratic to gradient elution. The amount of acetonitrile, always in the same aqueous buffer, was linearly increased from 16 to 64% in 15 min from injection. The elution profile of a mixture from synthetic and natural TPH-13 is shown in Fig. 2. Two comparable doublets are observed, each with clearly different retention times. After mild oxidation of the mixture with peracetic acid, only the early-eluted doublet was still present, whereas reduction with 2-mercaptoethanol[9] left only the more retained doublet. This confirmed the occurrence of methionine as the sulphoxide in the natural peptide analysed. Synthetic TPH-13 was always employed in the subsequent chromatographic characterization.

*Studies at different temperatures*

The above-described gradient method was used in the investigation of the influence of temperature on the elution pattern of TPH-13. As already pointed out for isocratic elution with 40% acetonitrile from the $C_{18}$ column, the increase of temper-

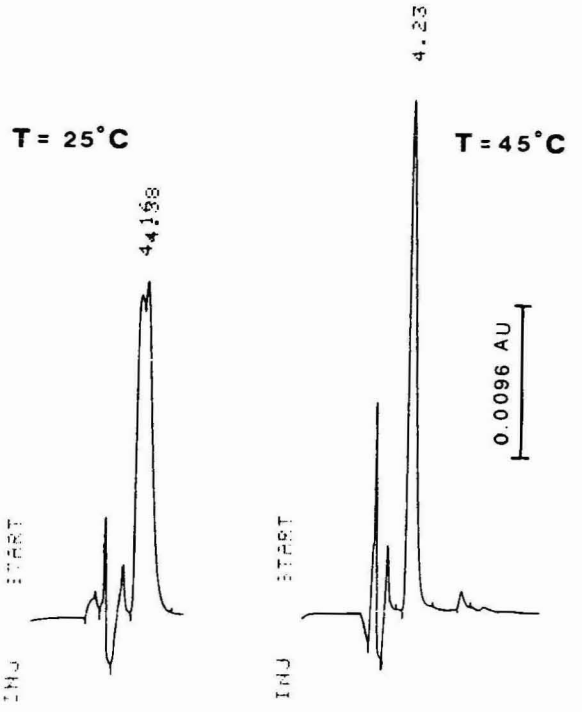


Fig. 1. Elution profile of TPH-13 under isocratic RP-HPLC conditions as a function of column temperature. Conditions: column, μBondapak $C_{18}$; eluent, 40% acetonitrile in 0.1 *M* formic acid; sample size, *ca.* 10 μg.

ature to 45°C caused coalescence of the double peak into a single peak with the retention time of the earlier-eluted component of the doublet (see Fig. 3). On the other hand, at 0°C the resolution of the split peak was better than at room temperature. Furthermore, the more or less 50:50 ratio between the two "horns" of the doublet was not dramatically changed by lowering the temperature. In addition, this ratio was roughly the same as that observed with isocratic elution (see Fig. 1).

TPH-13 behaved similarly when analysed on the CN column. In this case, a slightly slower gradient (from 16 to 64% acetonitrile in 20 min) than that employed on the $C_{18}$ column was used; otherwise, a strongly asymmetrical single peak was observed at room temperature, instead of a doublet. The ratio between the two components was still about 50:50.

*pH studies*

These experiments, as well as those described further below, were performed on the $C_{18}$ column, always with a linear gradient from 16 to 64% acetonitrile in 15 min. The behaviour of TPH-13 with a 0.05 *M* ammonium acetate buffer at two pH values (buffer A, pH 6.7; and buffer B, pH 7.8 with ammonia) was examined. The elution profiles of TPH-13 obtained with buffers A and B were quite similar: both showed a very clear, approximately symmetric doublet, much better resolved than at acidic pH. The retention time of TPH-13 decreased as the pH value of the buffer

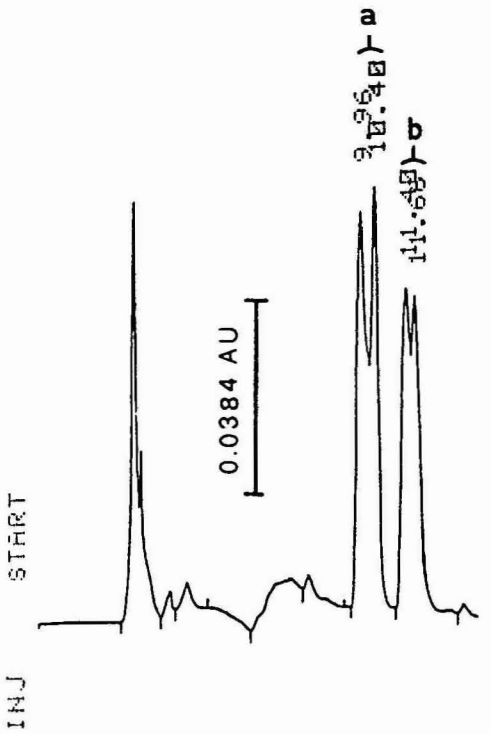


Fig. 2. Elution profile of a mixture of synthetic and natural TPH-13 under gradient RP-HPLC conditions at room temperature. Conditions: column, μBondapak $C_{18}$; eluent A, 0.1 *M* formic acid; eluent B, acetonitrile; gradient, from 16 to 64% B in 15 min; sample size, *ca.* 35 μg of synthetic peptide and *ca.* 45 μg of natural peptide. a = natural TPH-13, containing methionine in the sulphoxide form; b = synthetic [$Met^{13}$] analogue.

increased, in agreement with the acidic isoelectric point of the peptide (p*I* = 4.6)[2]. The variations of column temperature had similar effects on the chromatographic pattern to those already observed at acidic pH: in both cases, the doublet coalesced at 45°C, whereas at 0°C, peak splitting was still clearer than at room temperature. In particular, when buffer B was used, the two components of the doublet were resolved virtually at the baseline on the refrigerated column (see Fig. 4).

*Interconversion inside the doublet*

Preliminary experiments with 0.1 *M* formic acid as the aqueous buffer were performed, in order to investigate the equilibration between the two peaks of the doublet once they had been separated by preparative HPLC. These tests gave negative results, since the same elution pattern was always observed, even upon immediate reinjection of the pools corresponding to each of the two peaks. This finding might also suggest that peak splitting of TPH-13 was an artifact.

A pH value close to neutrality for the buffer seemed more suitable for this separation: both acidic and basic catalyses are negligible, and the interconversion

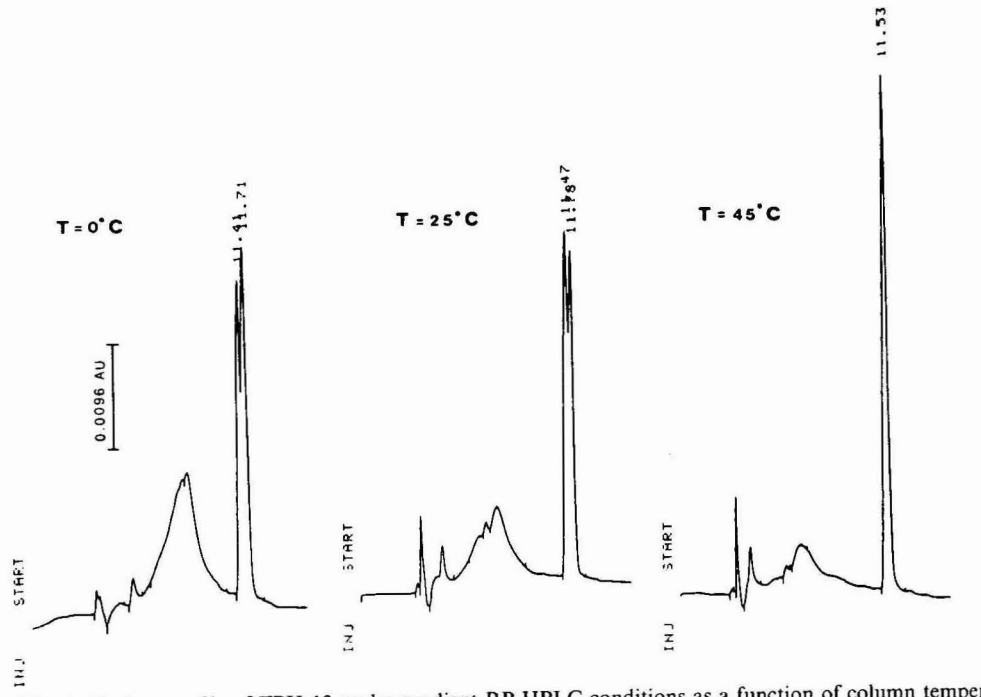


Fig. 3. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions are as in Fig. 2, except for sample size (*ca.* 10 μg).

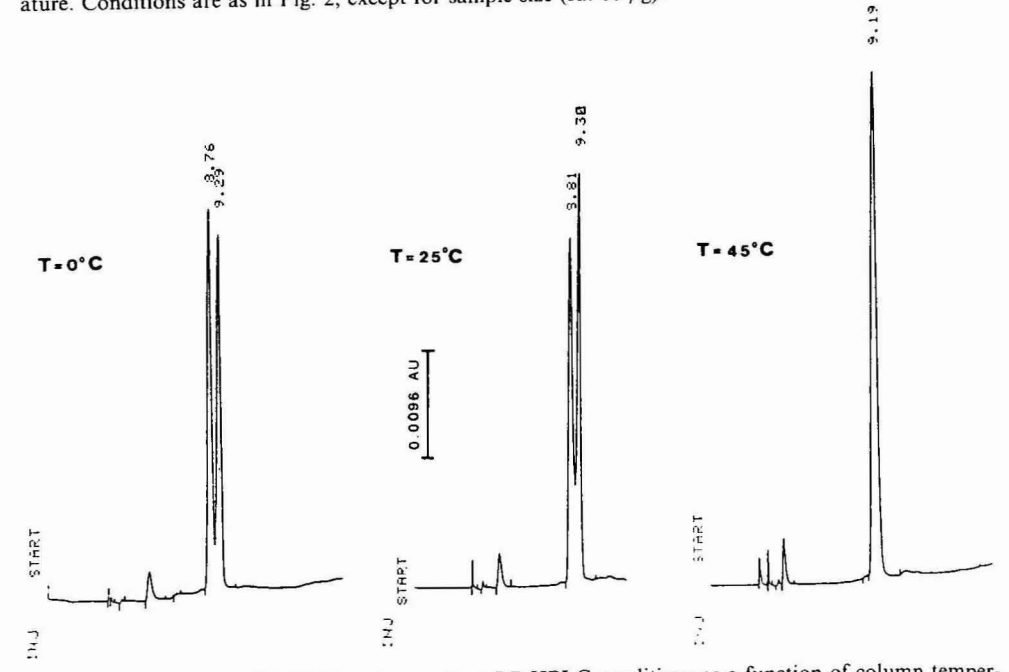


Fig. 4. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions: eluent A, 0.05 *M* ammonium acetate (pH 7.8 with ammonia); sample size, *ca.* 10 μg; other conditions as in Fig. 2.

between different conformations of the peptide chain should consequently be slow. Buffer A was then used. Two pools (pools 1 and 2) were collected after injection of TPH-13 into the refrigerated column; their elution patterns are shown in Fig. 5. Upon immediate reinjection (fig. 5a and c), each pool appeared largely enriched in the expected component, although contaminated with the other. The two pools were left at room temperature for 24 h while equilibrating. They were then reinjected, and yielded two superimposable chromatograms (see Fig. 5b and d). It was consequently confirmed that the two horns of the doublet are actually different species, whose interconversion rate depends on the pH of the buffer in the mobile phase.

*Influence of the organic modifier*

Acetonitrile is the most frequently used organic solvent in RP-HPLC of peptides and proteins[21]. Nevertheless, in many cases it was indicated as being responsible for denaturation of these biopolymers during chromatography, since high concentrations of this component are often required to obtain elution. The use of a more hydrophobic solvent, such as 2-propanol, results in the elution of hydrophobic peptides at lower concentrations of organic solvent[22]. The modifications induced in the chromatographic behaviour of TPH-13 by the replacement of acetonitrile with 2-propanol were consequently investigated.

The elution pattern obtained with 0.1 *M* formic acid as the aqueous buffer was not substantially modified, compared to the profile resulting from the use of acetonitrile (see Fig. 3): a virtually symmetric doublet was still present at room temperature, coalescing into a single peak at 45°C. The lower temperature was not investigated, because the high viscosity of 2-propanol led to excessive column pressure. The use of this solvent actually reduced the retention time of TPH-13, and it also significantly improved separation between the two peaks of the doublet.

*Effects of contact time*

The interaction of peptides and proteins with the hydrophobic surface of the column matrix in RP-HPLC certainly modifies the tridimensional structure of the polymer, and it was found to induce reversible or irreversible denaturation[12,14–16,23]. The unfolding kinetics of some proteins in RP-HPLC were recently studied by Benedek *et al.*[15]. From their work, it appears that the contact time between protein and chromatographic matrix exerts a great influence on the extent of denaturation.

The flow-rate affects the retention time of a product and, consequently, the time during which it remains in contact with the column. Therefore, the behaviour of TPH-13 in gradient elution at acidic pH was examined with both lower (0.7 ml/min) and higher (2 ml/min) flow-rates than are otherwise used (1 ml/min). Doubling the flow-rate did not significantly change the elution pattern: a doublet was observed at room temperature (better resolved than with a flow-rate of 1 ml/min), and a sharp singlet at 45°C. The lower temperature could not be investigated, because the operating pressure limit of the system was exceeded. With a flow-rate of 0.7 ml/min, the chromatographic behaviour of TPH-13 was not remarkably modified at room temperature or at 45°C, whereas at 0°C the amount of the later-eluted component of the doublet was apparently increased.

In order to increase the contact time without changing the flow-rate and the slope of the gradient, an on-column incubation period was added to the elution time.

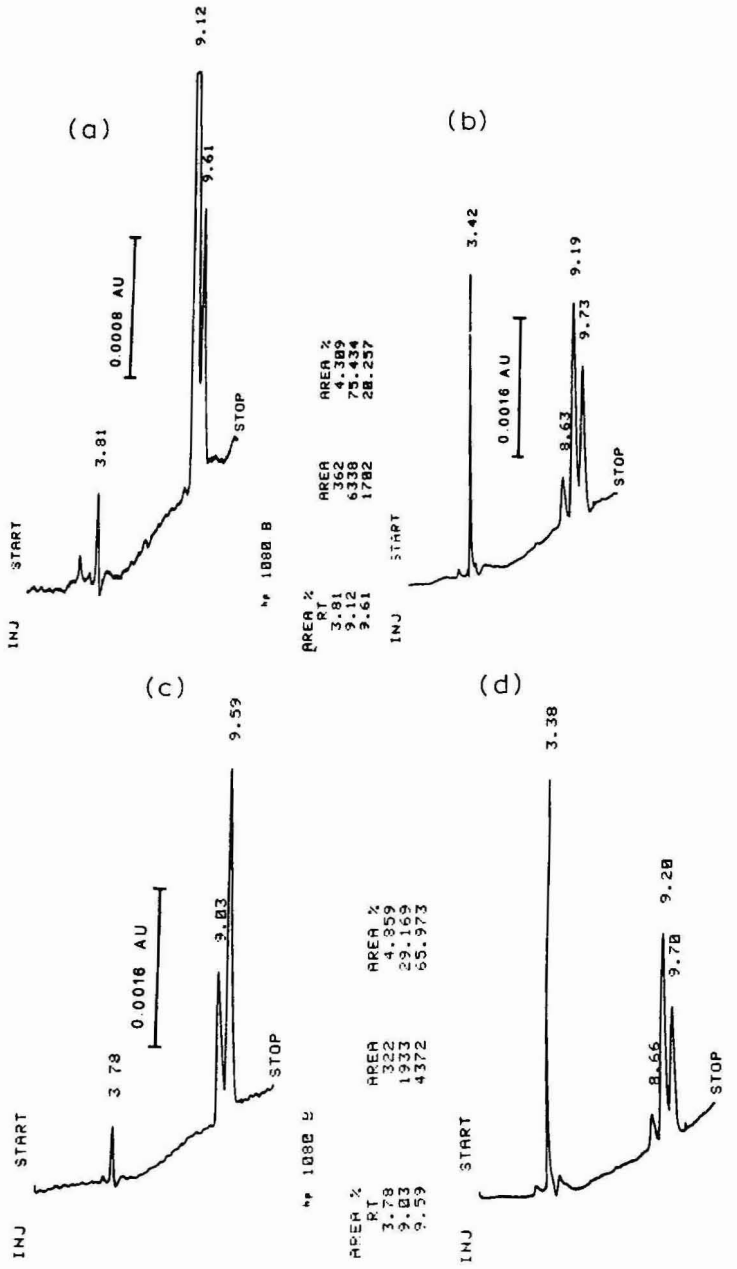


Fig. 5. Elution profiles of the two pools collected after injection of TPH-13 (*ca.* 50 μg) into the refrigerated column. Conditions are as in Fig. 2, except for eluent A (0.05 *M* ammonium acetate, pH 6.7). Pools 1 and 2 correspond to the early- and late-eluted peak of the TPH-13 doublet, respectively: pool 1 reinjected (50 μl) immediately (a) and after 24 h (b); pool 2 reinjected (50 μl) immediately (c) and after 24 h (d). The small peak with a retention time of *ca.* 8.6 min was, in both cases, due to the oxidized peptide. Reinjection conditions as above.

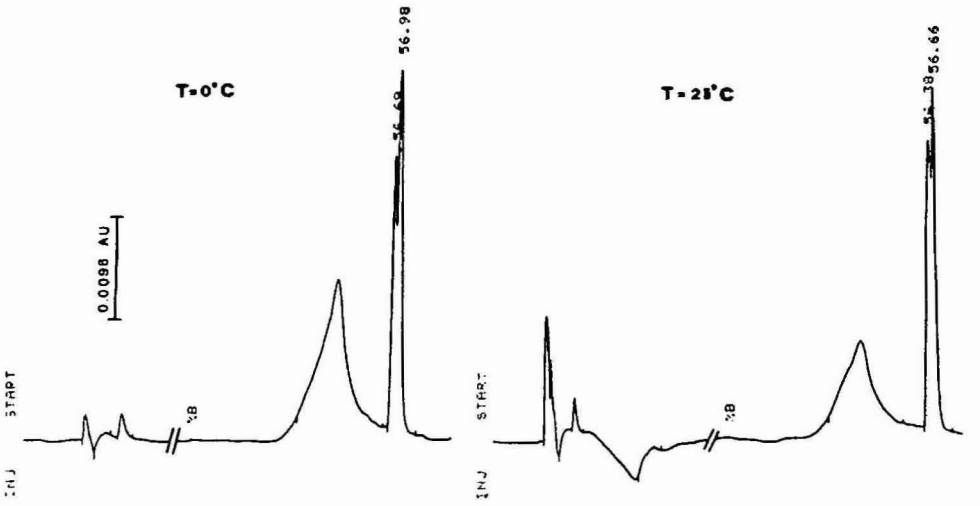


Fig. 6. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. The injected peptide was incubated on the column by keeping the mobile phase isocratic (16% B) for 45 min before the start of the gradient, which is marked by "% B" on the chromatogram. Conditions are as in Fig. 2, except for sample size (*ca.* 10 μg).

In this case, the mobile phase was kept isocratic for 45 min before the start of the gradient. The resulting elution profiles at room temperature and 0°C are shown in Fig. 6: the typical doublet of TPH-13 appears asymmetric, as the more retained horn prevails, and asymmetry is very remarkable in the chromatogram obtained at the lower temperature.

*Effects of a denaturing agent*

The behaviour of TPH-13 in the presence of a commonly used denaturing agent, such as urea, was also investigated. The acidic buffer employed so far (0.1 *M* formic acid) was used, plus 6 *M* urea, and the peptide sample was prepared in the starting mobile phase (*ca.* 1 mg/ml). These modifications strongly affected the elution pattern of TPH-13 at room temperature, as shown in Fig. 7. Again, a clearly asymmetric doublet was observed, but, in contrast to what was found when the contact time between peptide and column was increased, the earlier-eluted component prevailed. The double peak still coalesced as the column temperature was raised to 45°C. The lower temperature could not be considered because of the high viscosity of the mobile phase.

Since the presence of urea in the buffer also resulted in a higher pH value (3.5) than that of the unmodified buffer (2.5), a control analysis with 0.1 *M* formic acid, adjusted to pH 3.5 with sodium hydroxide, was performed. The elution pattern under these conditions was similar to that obtained with the unmodified buffer, also when the peptide sample was dissolved in the starting mobile phase of the previous experiment. The retention time at pH 3.5 was slightly lower than that at pH 2.5, in agreement with the observed trend toward decreased retention as pH was increased (see above). In contrast, when the urea-containing buffer at pH 3.5 was used, a stronger

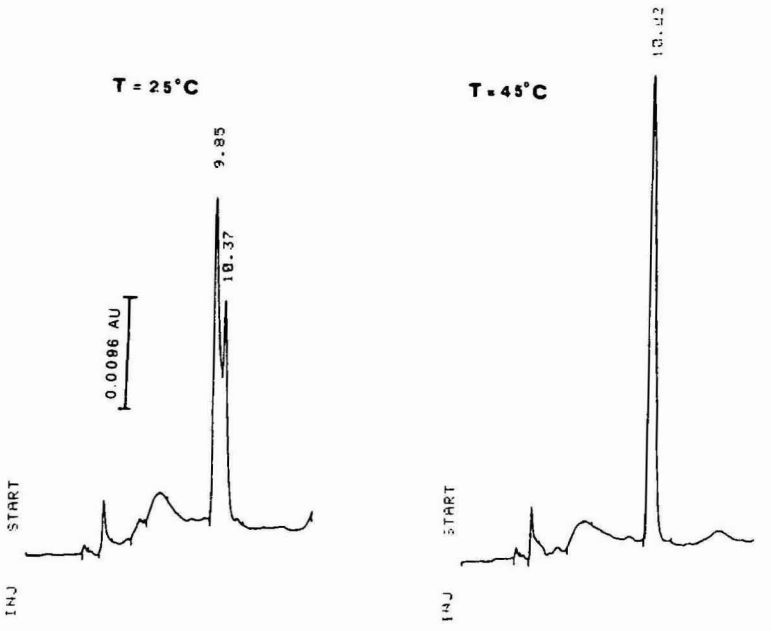


Fig. 7. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions: eluent A, 0.1 *M* formic acid–6 *M* urea (pH 3.5); sample size, *ca.* 10 μmol; other conditions as in Fig. 2.

reduction of the retention time of TPH-13 was observed (see Fig. 7), suggesting a specific effect of the denaturing agent.

*Chromatographic behaviour of the proline-rich fragment of TPH-13*

Results quite similar to those previously illustrated, concerning the influence of column temperature and pH of the mobile phase on the elution pattern of TPH-13, as well as interconversion inside its typical doublet, were obtained with the (4–13) tryptic fragment (H-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH). In agreement with its enhanced hydrophobic character, the decapeptide always had an elution volume slightly larger than that of TPH-13.

The results obtained in the investigation on the chromatographic behaviour of TPH-13 have been described qualitatively so far. They could also be expressed in a semiquantitative manner by measurement of resolution ($R_s$) and intensity ratio ($A_2/A_1$) between the two peaks of the TPH-13 doublet under various experimental conditions. The variations of $R_s$ and ($A_2/A_1$) were then calculated with respect to the characteristic values under reference conditions, *i.e.*: column temperature, 0 and 25°C; bonded phase, $C_{18}$; buffer pH, 2.5 (0.1 *M* formic acid); organic solvent, acetonitrile; flow-rate, 1 ml/min; on-column incubation time, none; urea concentration in the buffer, none; TPH-13, in the reduced state. The results obtained are shown in Table I.

TABLE I

EFFECT OF THE MODIFICATION OF EXPERIMENTAL CHROMATOGRAPHIC CONDITIONS ON $R_s$ AND $A_2/A_1$ IN RP-HPLC OF TPH-13

$R_s$, resolution of the two peaks of the TPH-13 doublet; $A_2/A_1$, ratio of the areas of the late- and early-eluted peak, respectively.

$$\Delta R_s\,(\%) = \frac{R_s - R_{s,ref}}{R_{s,ref}} \times 100 \qquad \Delta(A_2/A_1)\,(\%) = \frac{(A_2/A_1) - (A_2/A_1)_{ref}}{(A_2/A_1)_{ref}} \times 100$$

$R_{s,ref}$ and $(A_2/A_1)_{ref}$, values of $R_s$ and $A_2/A_1$, respectively, measured under reference conditions (see text). The effects of the experimental condition modifications have been evaluated according to the following ranking factors: for $\Delta R_s$ (%): $1 = \Delta R_s \leqslant 20\%$, $2 = 20\% < \Delta R_s \leqslant 70\%$, $3 = \Delta R_s > 70\%$, $0 = \Delta R_s = 0\%$; and for $\Delta(A_2/A_1)$ (%): $1 = \Delta(A_2/A_1) \leqslant 10\%$, $2 = 10\% < \Delta(A_2/A_1) < 25\%$, $3 = \Delta(A_2/A_1) \geqslant 25\%$. + and − signs after the factors mean positive and negative variations, respectively.

| *Modified experimental conditions* | | *T = 25°C* | | *T = 0°C* | |
|---|---|---|---|---|---|
| | | *$\Delta R_s$ (%)* | *$\Delta(A_2/A_1)$ (%)* | *$\Delta R_s$ (%)* | *$\Delta(A_2/A_1)$ (%)* |
| Column temperature | 0°C | 1 + | 3 + | − | − |
| Bonded phase* | CN | − | 3 −** | 1 + | 3 − |
| Buffer pH | 3.5 | 1 + | 1 + | − | − |
| | 6.7 | 2 + | 1 − | 3 + | 3 − |
| | 7.8 | 3 + | 1 + | 3 + | 3 − |
| Organic solvent | 2-propanol | 2 + | 2 + | − | − |
| Flow-rate | 2 ml/min | 2 + | 1 − | − | − |
| | 0.7 ml/min | 1 − | 2 + | 1 − | 3 + |
| Incubation time | 45 min | 0 | 2 + | 0 | 3 + |
| Urea concentration (in the buffer) | 6 mol/l | 3 + | 3 − | − | − |
| Oxidation state of TPH-13 | [Met(O)$^{13}$]TPH-13 | 2 + | 2 − | 3 + | 2 − |

* In addition to the bonded phase, the gradient time was also changed, from 15 min (reference condition) to 20 min.

** The variation of $(h_2/h_1)$ (ratio of the heights of the late- and early-eluted peak of the doublet, respectively) with respect to the reference condition was calculated instead of the variation of $(A_2/A_1)$, because the poor resolution obtained at room temperature did not allow the two peaks to be separately integrated (see Fig. 5).

## DISCUSSION AND CONCLUSIONS

Analysis of the chromatographic pattern of TPH-13 under different RP-HPLC conditions (see also Table I) suggests the following generalizations:

(1) Raising the column temperature to 45°C had the most drastic effect on the elution profile, since it changed the pattern from a doublet to a single peak under all the conditions investigated. In particular, under conditions in which the elution volumes were hardly affected by temperature, the resulting peak had a retention time close to that of the horn that decreased in intensity as column temperature was lowered from 25 to 0°C (see pH studies), or that was already prevalent at room temperature (see analysis in the presence of 6 *M* urea).

(2) The contact with the more hydrophobic bonded phase ($C_{18}$ compared to CN) favoured the shifting of equilibrium inside the doublet towards the later-eluted

peak. This effect cannot simply be due to a "denaturation" of the peptide, caused by its interaction with the stationary phase, since the addition of a known denaturant like urea to the mobile phase had just the opposite effect. On the other hand, the replacement of acetonitrile by 2-propanol as the organic solvent also increased the relative intensity of the later-eluted peak.

(3) The presence of methionine sulphoxide in the peptide significantly improved separation between the two peaks. Thus, the bulky, polar sulphoxide function seems to accentuate the difference in hydrophobicity between the species yielding the two horns and/or seems to reduce their interconversion rate; moreover, it also remarkably affects the position of equilibrium between them. The increase of buffer pH to 6.7 and 7.8 had similar effects on resolution, but it showed a strong influence on the relative intensity of the two peaks only at low temperature.

In addition to the parameters whose influence on the chromatographic pattern of TPH-13 was purposely studied, other factors might contribute to peak splitting: (a) formation of dimeric (or polymeric) complexes due to ionic or hydrogen bonds and (b) dissociation of the ionizable groups in the side chains, occurring at pH values higher than that of the reference buffer.

The formation of non-monomeric structures was suggested by Lundanes and Greibrokk[24] to be responsible for double peaks observed in the chromatograms of pure peptides. The contribution of this phenomenon was already minimized in the case of TPH-13 by the use of sufficiently concentrated buffers, and it could be completely excluded by addition of urea to the buffer, which affected the relative intensity of the two peaks of the doublet but did not suppress it. With respect to the second factor, Horváth *et al.*[25] showed that ampholytic solutes may be eluted with different retention times, depending on the distribution of charge. Although the equilibrium forms of ampholytes are not likely to be separated by chromatography, the hydrochlorides of basic amino acids were found to give one early-eluted peak, containing chloride, and one retained peak without chloride[24]. A significant contribution of dissociation to the peak splitting of TPH-13 was actually excluded because of the close resemblance between the chromatographic behaviour of TPH-13 and that of its (4-13) fragment, which has no ionizable group in the side chains.

On the other hand, the investigation of interconversion inside the doublets characteristic of TPH-13 and of the tryptic decapeptide showed that, with a mobile phase containing a virtually neutral buffer, the two species yielding the double peak both have a slow rate of equilibration. This finding, together with the influence of different parameters on resolution and the relative intensity between the two peaks of the doublet, suggests that these horns might be attributable to conformational isomers, although each of them is not likely to correspond to exactly the same isomeric species under all the conditions investigated, which differed widely from each other. Moreover, changing the pH of the buffer might also have reversed the elution order of the supposed conformers.

With respect to the actual nature of these isomers, the proline-rich sequence that characterizes both intact TPH-13 and its tryptic fragment might give rise to two major conformers, with comparable stability and different hydrophobicity, whose relative intensity and rate of isomerization would be affected by the chromatographic conditions used to resolve them. In this connection, the triprolyl sequence appears to be a very interesting feature; in fact, the onset of a helical (polyproline-II-like)

structure* was shown to occur in oligopeptides containing three or more consecutively linked proline residues[27].

On the other hand, even simple proline-containing dipeptides were found, by Melander *et al.*[28], to yield peak splitting in RP-HPLC when proline was not at the N-terminus, and this phenomenon was ascribed to the slow kinetics of isomerization of the X–Pro imidic bond, which is on the same time-scale as the chromatographic separation of these peptides. The dynamic effect of such secondary equilibria in RP-HPLC was recently studied by the same authors[29], who also applied their theoretical work to the kinetic study of *cis–trans* proline isomerization in some dipeptides by RP-HPLC[30]. Also, the isolated proline residues present in the TPH-13 sequence at positions 4 and 12 might consequently be responsible for the peak splitting of this peptide. Engaging of the proline imino group in a peptide bond is needed for an appreciable *cis–trans* equilibrium, therefore a significant contribution of the [Pro$^4$] residue can be readily excluded, since the (4–13) fragment, which has this proline as an N-terminus, still yields double peaks under the same HPLC conditions as the intact molecule. On the contrary, the strong improvement of chromatographic resolution observed following methionine oxidation as well as carboxyl dissociation (see Table I), which both affect the C-terminal moiety of TPH-13, suggests that the [Pro$^{12}$] residue might be a determinant for peak splitting. In this regard, the heptapeptide dermorphin[1], which has a C-terminal sequence (Tyr-Pro-Ser-$NH_2$) similar to that of TPH-13 (Tyr-Pro-Met-OH), was also found to give double peaks under some RP-HPLC conditions, while its analogue, [Gly$^6$]-dermorphin, yielded a single sharp peak[31]. Moreover, in the case of dermorphin, the relative intensity of the two resulting peaks agrees well with NMR data concerning the presence of *cis*-proline in the peptide chain[32].

Since the RP-HPLC technique cannot give definitive information on the conformational isomerism hypothesized for TPH-13, studies by other techniques (NMR and circular dichroism) are in progress to confirm the indications provided by this work. In addition, isomer specific proteolysis (ISP) with proline-specific endopeptidase and other proteolytic enzymes, suggested as an indirect method for distinguishing *cis*- and *trans*-proline residues[33,34], is now being tested on TPH-13 and its proline-rich fragment in order to provide further information on the conformational equilibrium of these peptides.

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REFERENCES

1 P. C. Montecucchi, R. de Castiglione and V. Erspamer, *Int. J. Pept. Protein Res.*, 17 (1981) 316.
2 P. C. Montecucchi, *Peptides*, in press.
3 P. C. Montecucchi, L. Gozzini, V. Erspamer and P. Melchiorri, *Int. J. Pept. Protein Res.*, 23 (1984) 276.

* Polyproline is known to exist either as a compact right-handed helix (designated form I) that contains *cis*-peptide bonds and has close intramolecular contacts, or as a left-handed, highly extended helix (form II) with *trans*-peptide bonds[26].

4 L. Gozzini, P. C. Montecucchi, V. Erspamer and P. Melchiorri, *Int. J. Pept. Protein Res.*, 25 (1985) 323.
5 P. C. Montecucchi, L. Gozzini and V. Erspamer, *Int. J. Pept. Protein Res.*, in press.
6 G. Perseo and R. de Castiglione, *Int. J. Pept. Protein Res.*, 24 (1984) 155.
7 G. Perseo, M. Gigli and R. de Castiglione, *Int. J. Pept. Protein Res.*, 25 (1985) 316.
8 R. de Castiglione, V. Erspamer, M. Gigli, L. Gozzini, P. C. Montecucchi and G. Perseo, *Abstract Book, IX American Peptide Symposium, Toronto, Canada, June 23–28, 1985*, p. 90.
9 E. Floor and S. E. Leeman, *Anal. Biochem.*, 101 (1980) 498.
10 W. Vale, J. Spiess, C. Rivier and J. Rivier, *Science*, 213 (1981) 1394.
11 L. Rusconi and P. C. Montecucchi, *J. Chromatogr.*, 346 (1985) 390.
12 A. R. Kerlavage, C. J. Weitzmann, T. Hasan and B. S. Cooperman, *J. Chromatogr.*, 266 (1983) 225.
13 R. M. Moore and R. R. Walters, *J. Chromatogr.*, 317 (1984) 119.
14 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
15 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
16 S. A. Cohen, K. Benedek, Y. Tapuhi, J. C. Ford and B. L. Karger, *Anal. Biochem.*, 144 (1985) 275.
17 R. J. T. Corbett and R. S. Roche, *Biochemistry*, 23 (1984) 1888.
18 E. S. Parente and D. B. Wetlaufer, *J. Chromatogr.*, 288 (1984) 389.
19 E. S. Parente and D. B. Wetlaufer, *J. Chromatogr.*, 314 (1984) 337.
20 G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.*, 23 (1968) 284.
21 Several references in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, CRC Press, Boca Raton, FL, 1984.
22 D. H. Coy, in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, pp. 197–203.
23 F. E. Regnier, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 350.
24 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
25 Cs. Horváth, W. Melander and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
26 W. L. Mattice and L. Mandelkern, *Biochemistry*, 9 (1970) 1049.
27 N. Helbecque and M. H. Loucheux-Lefebvre, *Int. J. Pept. Protein Res.*, 19 (1982) 94.
28 W. R. Melander, J. Jacobson and Cs. Horváth, *J. Chromatogr.*, 234 (1982) 269.
29 W. R. Melander, H. J. Lin, J. Jacobson and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4527.
30 J. Jacobson, W. R. Melander, G. Vaisnys and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4536.
31 L. Rusconi, unpublished results.
32 E. Arlandini, M. Ballabio, R. de Castiglione, B. Gioia, M. L. Malnati, G. Perseo and V. Rizzo, *Int. J. Pept. Protein Res.*, 25 (1985) 33.
33 L. N. Lin and J. F. Brandts, *Biochemistry*, 18 (1979) 43.
34 L. N. Lin and J. F. Brandts, *Biochemistry*, 23 (1984) 5713.

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# α- AND β-CYCLODEXTRINS AS SELECTIVE AGENTS FOR THE SEPARATION OF ISOMERS BY REVERSED-PHASE HIGH-PERFORMANCE THIN-LAYER AND COLUMN LIQUID CHROMATOGRAPHY

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## SUMMARY

Comparative studies on the application of cyclodextrins as sorbents for the separation of various isomers by two techniques, reversed-phase thin-layer chromatography and reversed-phase high-performance liquid chromatography were performed in parallel. The effects of α- or β-cyclodextrin concentration in the mobile phase solution on the $k'$ and $R_F$ values of *o*-, *m*- and *p*-cresols, -nitrophenols, -nitroanilines, and 1- and 2-methylnaphthalenes and -nitronaphthalenes were investigated.

With both techniques, substantial selectivity towards positional isomers (*o*-, *m*- and *p*-substituted benzenes and 1- and 2-substituted naphthalenes) was observed, which resulted from inclusion in β-cyclodextrin cavities.

Reversed-phase thin-layer chromatography was found to be a very useful, simple and fast procedure for preliminary studies of inclusion processes in the mobile phase solution and of their influence on chromatographic separation.

## INTRODUCTION

α- and β-cyclodextrins (CD) are torus-shaped cyclic oligosaccharides, made up of six and seven α-1,4-linked D-glucopyranose units, respectively. The inside of the CD cavities is relatively hydrophobic; it is formed by a circular configuration of hydrogen atoms and glucoside oxygen atoms while all the hydroxyl groups are on the outside of the molecule. This structure gives rise to the remarkable ability of CD to form inclusion compounds with various molecules and ions[1]. The fit of the entire or at least part of the guest molecules in the CD (host) cavity determines the stability of the inclusion compounds and the selectivity of the complexation process.

This property of CD has been used to advantage in many separation tech-

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niques, including classical liquid chromatography[2,3]. In high-performance liquid chromatography (HPLC) two approaches have recently been devised for separating various compounds through CD complexation: the use of chemically bonded α- or β-CD silica stationary phases[4–8]; and the use of CD as mobile phase components in reversed-phase systems[9–13].

Our previous studies taking the latter approach have dealt with the separation of *o*-, *m*- and *p*-nitrobenzoic acids[9], *cis*- and *trans*-*o*-, *m*- and *p*-nitrocinnamic acids[10], and the resolution of mandelic acid[11] and some of its derivatives into enantiomers[12,13]. CD, dissolved in the mobile phase, have also been used by Hinze and Armstrong[14] and Burkert *et al.*[15] in thin-layer chromatography (TLC) on a polyamide stationary phase for the resolution of *o*-, *m*- and *p*-substituted benzoic acids, phenols and naphthols.

The present paper reports further comparative studies on the application of CD as mobile phase components for the separation of various isomers, performed in parallel by two chromatographic techniques: reversed-phase HPLC and reversed-phase TLC. The model compounds were: *o*-, *m*- and *p*-cresols (C), -nitrophenols (NP), -nitroanilines (NA) and -chloromandelic acids (CMA); 1- and 2-methylnaphthalenes (MN) and -nitronaphthalenes (NN); and mandelic acid (MA).

The aim of this study was to establish a simple and fast procedure for preliminary optimization of separation conditions in reversed-phase systems containing CD in mobile phase solutions. In these experiments, we used precoated RP-18 $F_{254s}$ TLC plates, which have only recently become available. In contrast to those used previously[16,17], they are easily wettable by aqueous mobile phase solutions in prevailing concentrations of water used to dissolve CD for reversed-phase HPLC.

## EXPERIMENTAL

α-CD was supplied by Janssen Chimical (Beerse, Belgium) and β-CD by Chinoin (Budapest, Hungary). All other materials were of analytical or reagent grade and were used without further purification.

Chromatographic experiments were performed with a HPLC unit constructed at the Institute of Physical Chemistry, Polish Academy of Sciences (Warsaw, Poland) and equipped with a UV detector (254 nm). For HPLC, use was made of stainless-steel columns (250 × 4.5 mm I.D.), packed with 10 μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.). For TLC, the TLC precoated plates RP-18 $F_{254s}$ of Merck (product no. 15683) were used. All were 5 cm in length with the exception of the plates for the resolution of cresols, which were *ca.* 17 cm long.

The spots were usually *ca.* 2 mm in diameter. Portions of 0.5–1 μl of 0.1–1% methanolic solutions of samples were applied 8 mm from the lower edge of the plates. Ascending TLC was performed in a glass jar. In all experiments with CD the plates were pretreated as follows: ascending development was carried out for 2 h with the same eluent that would be used for "normal" development. After the samples had been dried and spotted, the plates were developed in a second jar with the same eluent as in the pretreatment. Development times varied from 10 min to 30 min, depending on the percentage of ethanol and the concentration of CD in the eluents. The spots were located under UV light by quenching of fluorescence at 254 nm. The mobile phases (the same for HPLC and TLC experiments) consisted of aqueous solutions

containing various concentration of α- or β-CD, appropriate amounts of ethanol (20 vol. % and 50 vol. %), and in some cases suitable phosphate buffer components. HPLC and TLC chromatograms were carried out in parallel at room temperature (*ca.* 22°C).

## RESULTS AND DISCUSSION

The $k'$ and $R_F$ values of the model compounds, determined on reversed phases by HPLC and TLC with mobile phase solutions containing various concentration of α- or β-CD are collected in Tables I–III.

TABLE I

$k'$ AND $R_F$ VALUES OF 1- AND 2-METHYLNAPHTHALENES (MN), 1- AND 2-NITRONAPHTHALENES (NN), *o*-, *m*- AND *p*-CHLOROMANDELIC ACIDS (CMA) AND MANDELIC ACID (MA) ON RP-18 HPLC COLUMNS AND TLC PLATES WITH MOBILE PHASES CONTAINING VARIOUS CONTENTRATIONS OF β-CD IN ETHANOL–PHOSPHATE BUFFER (pH 6.7) (50:50,v/v)

| *Compounds* | *[β-CD] ($10^{-3}$ M)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *0.0* | | *5* | | *10* | | *20* | |
| | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ |
| 1-MN | 29.8 | 0.03 | >100 | 0.00 | >100 | 0.00 | >100 | 0.00 |
| 2-MN | 31.5 | 0.02 | 33.6 | 0.04 | 33.1 | 0.03 | 22.7 | 0.04 |
| 1-NN | 7.85 | 0.08 | 9.02 | 0.07 | >100 | 0.00 | >100 | 0.00 |
| 2-NN | 9.98 | 0.08 | 11.35 | 0.06 | 10.67 | 0.07 | 8.5 | 0.08 |
| *o*-CMA | 7.48 | 0.08 | 7.88 | 0.06 | 8.3 | 0.09 | >100 | 0.00 |
| *m*-CMA | 7.85 | 0.08 | 9.1 | 0.06 | 28.4 | 0.02 | >100 | 0.00 |
| *p*-CMA | 0.34 | 0.19 | 0.28 | 0.16 | 0.32 | 0.24 | 0.30 | 0.29 |
| MA | 0.15 | 0.72 | 0.13 | 0.72 | 0.14 | 0.76 | 0.13 | 0.75 |

Examination of these data leads to the conclusion that similar effects due to CD complexation are observed by both techniques. The elution sequences are the same. The values of $k'$ determined from HPLC and TLC data are approximately the same (or at least of the same order of magnitude). The results obtained appear to be consistent, considering the fact that we have sometimes observed similar discrepancies using CD solutions in columns packed with reversed-phase sorbents from different batches. Thus TLC on precoated reversed-phase plates can be very useful as a pilot procedure for studying CD complexation processes in the mobile phase solution and their influence on chromatographic separations.

As suggested earlier[10], β-CD imparts a substantial selectivity towards positional isomers on reversed-phase systems. The effects arising from α-CD comlexation, observed by both techniques, are slighter and not general for all disubstituted benzene derivatives.

TABLE II

$k'$ AND $R_F$ VALUES OF *o*-, *m*- AND *p*-CRESOLS (C), -NITROANILINES (NA) AND -NITROPHENOLS (NP) ON RP-18 HPLC COLUMNS AND TLC PLATES WITH MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS OF β-CD IN ETHANOL–WATER (20:80, v/v)

| *Compound* | *[β-CD] ($10^{-3}$ M)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *0.0* | | *5* | | *10* | | *20* | |
| | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ |
| *o*-C | 17.0 | 0.05 | 13.07 | 0.06 | 11.05 | 0.08 | 8.87 | 0.09 |
| *m*-C | 16.16 | 0.06 | 11.71 | 0.07 | 9.64 | 0.08 | 7.48 | 0.11 |
| *p*-C | 16.42 | 0.06 | 10.37 | 0.07 | 7.97 | 0.11 | 5.68 | 0.13 |
| *o*-NP | 15.5 | 0.04 | 13.4 | 0.05 | 10.7 | 0.05 | 8.5 | 0.08 |
| *m*-NP | 12.7 | 0.06 | 9.8 | 0.07 | 8.2 | 0.13 | 6.9 | 0.16 |
| *p*-NP | 7.9 | 0.07 | 6.3 | 0.11 | 4.4 | 0.17 | 3.1 | 0.21 |
| *o*-NA | 14.8 | 0.04 | 11.07 | 0.05 | 9.13 | 0.06 | 7.27 | 0.08 |
| *m*-NA | 7.72 | 0.07 | 5.87 | 0.09 | 4.93 | 0.08 | 4.00 | 0.14 |
| *p*-NA | 5.82 | 0.09 | 2.65 | 0.19 | 1.78 | 0.22 | 1.12 | 0.38 |

The $R_F$ values listed in Tables I and II show that in some cases the separation of positional isomers by TLC is easily obtained (*e.g.*, *o*-, *m*- and *p*-nitroanilines), whereas in other cases particular conditions are required (*e.g.*, *o*-, *m*- and *p*-cresols, known to be difficult to separate by LC). However, improved separation by TLC may be obtained by multiple development or by increasing the migration distance. Under these conditions, clear-cut separation of the three isomers was obtained. A comparison of HPLC and TLC separations with β-CD solutions is shown in Fig. 1.

TABLE III

$k'$ AND $R_F$ VALUES OF *o*-, *m*-, AND *p*-CRESOLS (C), -NITROANILINES (NA) AND -NITROPHENOLS (NP) ON RP-18 HPLC COLUMN AND TLC PLATES WITH MOBILE PHASES CONTAINING VARIOUS CONCENTRATIONS OF α-CD IN ETHANOL–WATER (20:80, v/v)

| *Compound* | *[α-CD] ($10^{-3}$ M)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *0.0* | | *5* | | *10* | | *20* | |
| | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ | $k'$ | $R_F$ |
| *o*-C | 17.0 | 0.05 | 15.40 | 0.05 | 14.84 | 0.05 | 13.22 | 0.05 |
| *m*-C | 16.16 | 0.06 | 14.55 | 0.06 | 13.96 | 0.06 | 12.66 | 0.06 |
| *p*-C | 16.42 | 0.06 | 14.73 | 0.06 | 14.02 | 0.06 | 12.56 | 0.06 |
| *o*-NP | 15.5 | 0.04 | 13.11 | 0.04 | 11.72 | 0.04 | 10.84 | 0.05 |
| *m*-NP | 12.7 | 0.06 | 12.14 | 0.07 | 9.84 | 0.07 | 7.98 | 0.09 |
| *p*-NP | 7.9 | 0.07 | 6.52 | 0.08 | 4.21 | 0.09 | 3.40 | 0.13 |
| *o*-NA | 14.80 | 0.04 | 13.10 | 0.04 | 11.60 | 0.04 | 9.12 | 0.06 |
| *m*-NA | 7.72 | 0.07 | 6.74 | 0.08 | 5.76 | 0.09 | 4.60 | 0.11 |
| *p*-MA | 5.82 | 0.09 | 4.58 | 0.12 | 3.22 | 0.19 | 2.24 | 0.25 |

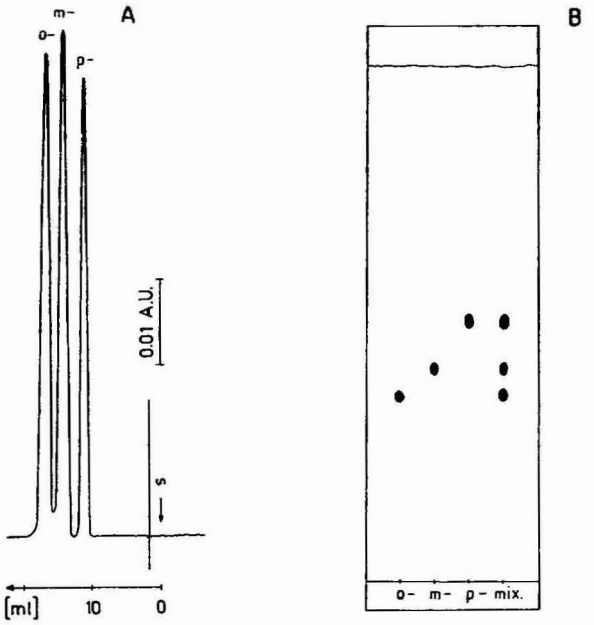


Fig. 1. Separation of *o*-, *m*- and *p*-cresols by (A) HPLC and (B) TLC with a mobile phase containing 0.02 *M* β-CD in ethanol–water (20:80, v/v). (A) HPLC column (250 × 4.5 mm I.D. RP-18), flow-rate 1.8 ml/min; (B) TLC (RP-18) chromatogram after six developments of a plate, 17 × 5 cm.

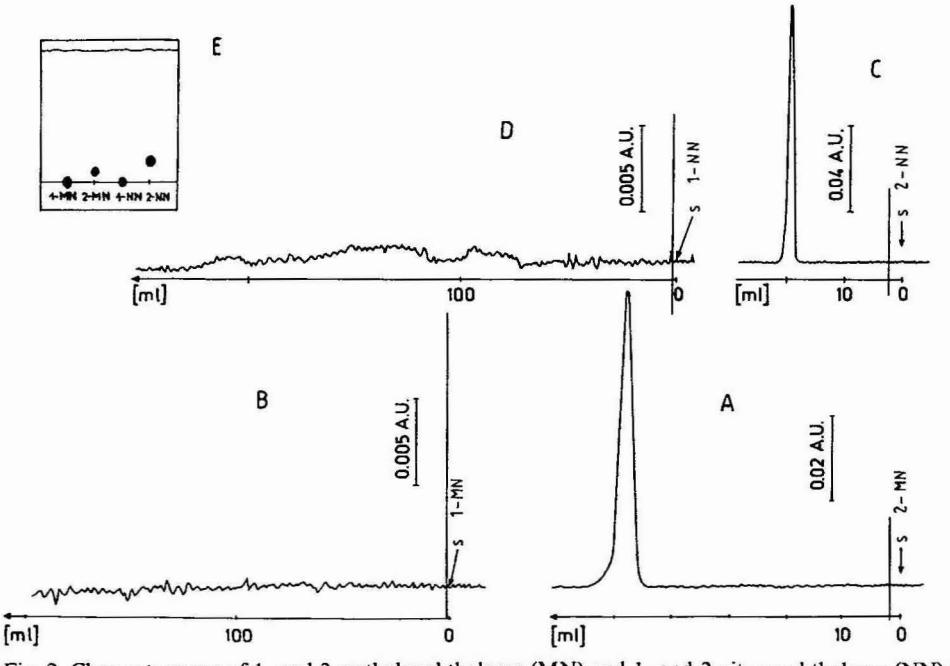


Fig. 2. Chromatograms of 1- and 2-methylnaphthalenes (MN) and 1- and 2-nitronaphthalenes (NN) by HPLC and TLC with a mobile phase containing 0.02 *M* β-CD in ethanol–phosphate buffer (pH 6.7) (50:50, v/v). (A) 2-MN; flow-rate, 1.8 ml/min; (B) 1-MM; flow-rate, 2.4 ml/min (no peak eluted); (C) 2-NN; flow-rate, 1.8 ml/min; (D) 1-NN; flow-rate, 2.4 ml/min (no peak eluted); (E) TLC of 1-MN, 2-MN, 1-NN, and 2-NN: chromatogram after two developments of a plate 5 × 4 cm.

It was observed that both 1-MN and 1-NN are irreversibly adsorbed on the RP-18 phase from β-CD solutions. Fig. 2 demonstrates the behaviour of 1- and 2-MN and 1- and 2-NN in aqueous ethanol solution (50 vol. %), containing β-CD, observed by reversed-phase HPLC and TLC techniques.

The separation on reversed-phase HPLC columns is too specific for the determination of two isomers in a mixture: only one peak is eluted (that of naphthalene, substituted on position 2) whereas 1-NN and 1-MN remain adsorbed on the column. In such a case, only TLC may be used for evaluating the composition of mixtures of 1- and 2-MN or 1- and 2-MN. Two spots are detected by TLC, the first corresponding to 1-substituted naphthalenes at the start (initial position) and the second corresponding to 2-substituted naphthalenes. It should be noted that the resolution of two isomeric monosubstituted naphthalenes is very poor without β-CD (see Table I). The unusual irreversible adsorption of (1-MN · β-CD) and (1-NN · β-CD) complexes on the RP-18 phase is under further investigation.

The attempts to perform a direct comparison of the results described above with those of Hinze and Armstrong[14] and Burkert *et al.*[15] seem to be unreasonable because they concern two different sorbents (RP-18 and polyamide phases) and perhaps different mechanisms.

## REFERENCES

1 J. Szejtli, *Cyclodextrins and their Inclusion Complexes*, Akadémiai Kiadó, Budapest, 1982.
2 W. L. Hinze, *Sep. Purif. Methods*, 10 (1981) 159–237.
3 E. Smolkova-Keulemansová, *J. Chromatogr.*, 251 (1982) 17.
4 K. Fujimura, T. Ueda and T. Ando, *Anal. Chem.*, 55 (1983) 446.
5 Y. Kawaguchi, M. Tanaka, M. Nakae, K. Funazo and T. Schono, *Anal. Chem.*, 55 (1983) 1852.
6 D. W. Armstrong and W. De Mond, *J. Chromatogr. Sci.*, 22 (1984) 411.
7 D. W. Armstrong, W. De Mond, A. Alak, W. L. Hinze, T. E. Riehl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234.
8 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. De Mond and T. Ward, *Anal. Chem.*, 57 (1985) 237.
9 D. Sybilska, J. Lipkowski and J. Wójcikowski, *J. Chromatogr.*, 253 (1982) 95.
10 D. Sybilska, J. Dębowski, J. Jurczak and J. Zukowski, *J. Chromatogr.*, 286 (1984) 163.
11 J. Dębowski, D. Sybilska and J. Jurczak, *J. Chromatogr.*, 237 (1982) 303.
12 J. Dębowski, D. Sybilska and J. Jurczak, *Chromatographia*, 16 (1982) 198.
13 J. Dębowski, J. Jurczak and D. Syubilska, *J. Chromatogr.*, 282 (1983) 83.
14 W. L. Hinze and D. W. Armstrong, *Anal. Lett.*, 13 (A12) (1980) 1093.
15 W. G. Burkert, Ch. N. Ownesby and W. L. Hinze, *J. Liq. Chromatogr.*, 4 (1981) 1065.
16 G. Grassini-Strazza and M. Cristalli, *J. Chromatogr.*, 214 (1981) 209.
17 U. A. Th. Brinkman and G. de Vries, *J. Chromatogr.*, 258 (1983) 43.

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CHROMSYMP. 676

# DETERMINATION OF TRACE AMOUNTS OF LANTHANIDES IN ROCKS AND MINERALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Rare-earth elements have been determined by a high-performance liquid chromatographic procedure. The hydrochloric acid solution containing the rare-earth elements coming from the main separation of the whole group, contained in a rock sample and separated by a classical ion-exchange procedure, was eluted through a stainless-steel column packed with microparticulate silica, with bonded cation-exchange groups. Complete separation of individual lanthanides was achieved. A variable-wavelength detector was used following post-column complex formation with pyridylazoresorcinol. Results obtained on test solutions and on internal rock reference samples show good reproducibility and precision.

## INTRODUCTION

The rare-earth elements (REE) may be considered the best representatives of trace elements with very similar physico-chemical properties, and are the most useful indicators of geochemical processes, because all have essentially the same chemical properties and display small, regular differences in mass and also in ionic radius (as a result of the lanthanide contraction). Since the compositional trend of the REE (or lanthanides) has been used by several authors[1–4] to investigate the evolution of igneous rocks and since the impact of REE abundance on the environment seems to become more and more important[5,6], an accurate determination of these elements is a very important step in analytical chemistry related to geochemical work.

Several techniques can be used in the analytical approach to such a problem. The majority of data in international standards on rocks and minerals, relating to REE content, have been obtained by radiometric techniques, such as instrumental and radiochemical neutron activation analysis[7–9]. However, these techniques are expensive and not readily available. In order to devise an alternative and widely applicable technique, we have studied atomic absorption spectrophotometry with electrothermal atomisation (ETA–AAS) of the lanthanides[10–13], but ETA–AAS allows an accurate and sensitive determination for only the heavy REE. Recent research has stressed increased interest in high-performance liquid chromatography (HPLC), par-

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ticularly as applied to inorganic analytical problems. One of the most interesting groups of metals investigated is the lanthanides, owing to the well-known difficulties in their analysis.

We have carried out a systematic study of lanthanide separation in order to apply HPLC procedures to their determination in natural or industrial samples. An improved application of HPLC to REE and transition-element (d-block) analyses has been carried out by Cassidy and Elchuk[14,15] and Hwang *et al.*[16]. By a similar procedure we have previously[17,18], determined trace amounts of terbium and cerium in terbium, lanthanum-, and yttrium-doped oxide–sulphide phosphors.

The method is based on colorimetric detection of the lanthanide-PAR [4-(2-pyridylazo)resorcinol] monosodium salt, by formation of the dye after HPLC separation in a cation-exchange column and elution with DL-2-hydroxyisobutyric acid, buffered at pH 4.6 in a gradient elution mode. The whole group of lanthanides was previously separated on classical cation-exchange columns filled with strongly acidic sulphonated resin (Dowex 50W-X8) after acid solubilization of the rock samples[19].

## EXPERIMENTAL

### *Reagents*

The following reagents were used: 40% hydrofluoric acid; 70% perchloric acid; 36% hydrochloric acid; 3 *M* hydrochloric acid in 25% ethanol solution; 4 *M* hydrochloric acid; borosilicate glass tube (180 m × 20 mm I.D.), packed with Dowex 50W X8 (acid form, 200–400 mesh) ion-exchange resin; 4-(2-pyridylazo)resorcinol monosodium salt, 0.050 mg/l, in 2 *M* ammonium hydroxide and 1 *M* ammonium acetate aqueous solution; DL-2-hydroxyisobutyric acid (HIBA), 0.03 and 0.07 *M* aqueous solutions buffered at pH 4.6 with sodium hydroxide; Partisil PXS 10/25 SCX (Whatman) cation-exchange stainless-steel HPLC column; REE standard solutions, obtained by dissolving pure oxides in mineral acids.

### *Apparatus*

The following equipment was used: Perkin-Elmer 2 liquid chromatograph, equipped with a linear gradient programmer; Rheodyne M 7125 load injection valve (175-$\mu$l loop); constant-flow peristaltic pump, Gilson Minipuls 2, used to transfer the PAR complexing solution; Perkin-Elmer LC 5 variable-wavelength spectrophotometric detector, equipped with Perkin-Elmer LC Autocontrol; Kipp & Zonen B5 strip-chart recorder; LDC 10 calculating integrator.

### *Procedure*

Weigh out 1000.0 mg of finely powdered rock sample (120 mesh) in a PTFE dish. Moisten the powder with water to avoid spattering. Add 10 ml of 40% hydrofluoric acid and 10 ml of 70% perchloric acid, mixing with a platinum or PTFE rod. Allow to stand overnight in the fume-cupboard and then evaporate to dryness. Add 10 ml of each the two acids and evaporate to dryness again, then dilute to *ca.* 100 ml to give 0.6 *M* hydrochloric acid concentration. Pass this solution through a borosilicate glass tube (180 × 20 mm I.D.), filled with Dowex 50W X8 (acid form, 200–400 mesh) ion-exchange resin. Elute the matrix major and minor elements, which may interfere in the subsequent colorimetric PAR detection (sodium, potassium, calcium,

magnesium, iron, aluminium, titanium and some strontium), with 300 ml of 3 *M* hydrochloric acid in 25% ethanol solution. Elute the REE group (plus barium, scandium, yttrium, and the rest of the strontium) with 200–250 ml of 4 *M* hydrochloric acid. If necessary, evaporate this eluate to less than 50 ml in a PTFE beaker; then dilute to 50 ml in a standard flask. This last solution is injected into the HPLC system via a 175-μl loop. A linear concentration gradient is started, from 0.03 *M* HIBA to 0.07 *M* HIBA (both buffered at pH 4.6 with sodium hydroxide) at 3%/min to the total solvents, at a constant the total flow-rate of 1.2 ml/min. The sample solutions are chromatographed on a stainless-steel cation-exchange column (Partisil PXS 12/25 SCX). The eluted metal ions are measured with a variable-wavelength detector (520 nm) after a post-column complexing reaction with PAR in a "T" cell by mixing the HIBA–REE eluate with PAR solution (from the peristaltic pump). The REE contents are calculated by the well-known calibration curve or standard addition method.

TABLE I

OCCURRENCE OF SOME MAJOR, MINOR, AND TRACE ELEMENTS IN SOME INTERNATIONAL REFERENCE ROCKS AND IN CALIBRATING SOLUTIONS

Values are given as percentages for the elements listed as their oxides, and in parts per million for the remainder.

| *Element* | *GS-N granite* | *NIM-L lujavrite* | *Calibrating solution* |
|---|---|---|---|
| $Al_2O_3$ | 14.69 | 13.64 | 15.00 |
| $Fe_2O_3$ | 3.73 | 9.96 | 5.00 |
| MnO | 0.06 | 0.77 | 0.50 |
| MgO | 2.32 | 0.28 | 1.00 |
| CaO | 2.54 | 3.22 | 2.00 |
| $Na_2O$ | 3.76 | 8.57 | 5.00 |
| $K_2O$ | 4.40 | 5.51 | 5.00 |
| $TiO_2$ | 0.69 | 0.48 | 0.50 |
| $P_2O_5$ | 0.27 | 0.06 | 0.10 |
| Ba | 1400 | 450 | 1000 |
| Ce | 140 | 240 | 100 |
| Cr | 55 | 10 | 50 |
| Dy | 1.5 | 3 | 5 |
| Er | 1.5 | 2 | 5 |
| Eu | 1.7 | 1.2 | 5 |
| Gd | 5 | 3 | 5 |
| Ho | 0.5 | 0.5 | 5 |
| La | 75 | 250 | 100 |
| Lu | 0.2 | 0.2 | 5 |
| Nd | 50 | 48 | 50 |
| Ni | 34 | 11 | 50 |
| Pr | 19 | 20 | 50 |
| Sc | 7 | 0.3 | 5 |
| Sm | 8.2 | 5 | 10 |
| Sr | 570 | 4600 | 1000 |
| Tb | 0.6 | 0.7 | 5 |
| Tm | 0.3 | 0.3 | 5 |
| Y | 19 | 22 | 50 |
| Yb | 1.7 | 3 | 5 |

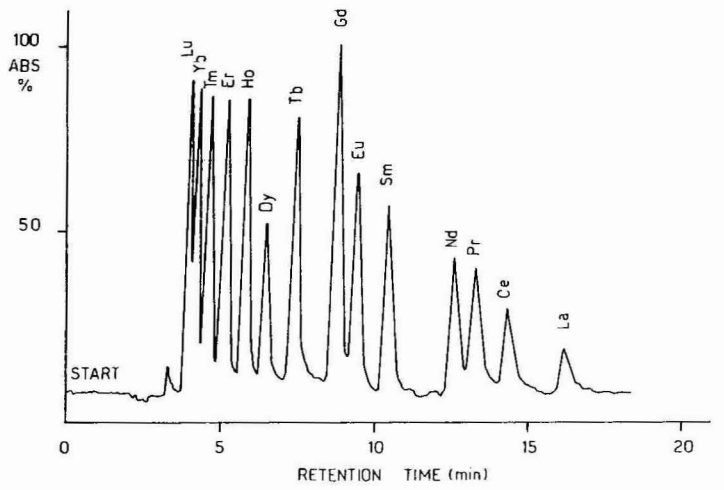


Fig. 1. HPLC chromatogram of the whole group of rare-earth elements in the standard solution, after ion-exchange separation.

## RESULTS AND DISCUSSION

To evaluate the separations (both whole REE group and individual lanthanides) a calibration has been carried out by employing a "matrix" solution, containing major (sodium, potassium, calcium, magnesium, iron and aluminium), minor (titanium, barium, scandium, yttrium and strontium) and trace (REE) elements at the same ratio (between matrix and analysed element) occurring in natural samples[20,21] (Table I). An aliquot of this solution was passed through the entire separation–

TABLE II

REE SENSITIVITY AND RECOVERY DATA FOR THE CALIBRATING SOLUTION

| *Element* | *Conc. taken (ppm)* | *Conc. found (ppm)* | *Recovery (%)* | *Sensitivity limit* | |
|---|---|---|---|---|---|
| | | | | *ppm* | *ng* |
| Ce | 50 | 51 | 102 | 1.0 | 20 |
| Dy | 5 | 5 | 100 | 0.3 | 6 |
| Er | 5 | 4.8 | 96 | 0.3 | 6 |
| Eu | 5 | 4.9 | 98 | 0.4 | 8 |
| Gd | 5 | 5.1 | 102 | 0.3 | 6 |
| Ho | 5 | 4.7 | 94 | 0.3 | 6 |
| La | 100 | 99 | 99 | 1.0 | 20 |
| Lu | 5 | 4.7 | 94 | 0.5 | 10 |
| Nd | 50 | 50.5 | 101 | 0.5 | 10 |
| Pr | 50 | 50 | 100 | 1.0 | 20 |
| Sm | 10 | 9.9 | 99 | 0.3 | 6 |
| Tb | 5 | 5 | 100 | 0.15 | 3 |
| Tm | 5 | 4.8 | 96 | 0.2 | 4 |
| Y | 50 | 49.5 | 99 | 0.3 | 6 |
| Yb | 5 | 4.9 | 98 | 0.1 | 2 |

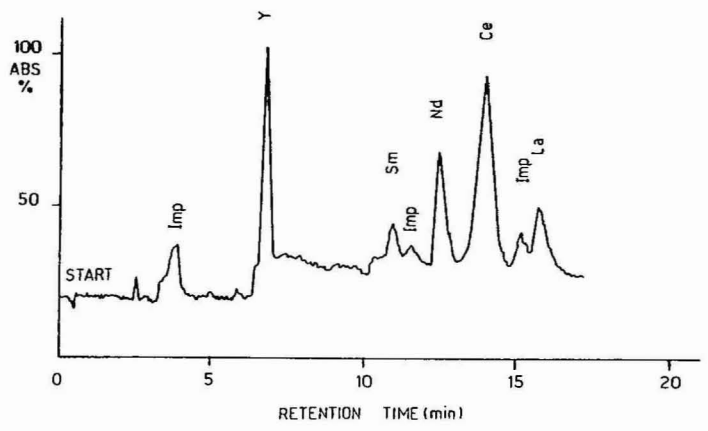


Fig. 2. HPLC chromatogram of the REE in rock NIM-L after acid treatment and ion-exchange chromatography.

HPLC–colorimetric procedure in order to test the proposed method. The chromatogram of the whole REE group is shown in Fig. 1.

Tests to evaluate the sensitivity and recovery of the proposed method have also been performed (Table II).

The suggested procedure has also been applied to the characterization of two international reference rock samples: NIM-L (lujavrite) of the National Institute of Metallurgy, Pretoria, South Africa, and STM-1 (syenite) of the U.S. Geological Survey, Reston, VA, U.S.A. The chromatogram obtained by our procedure is shown in Fig. 2. The results obtained are compared with literature values in Table III.

## CONCLUSION

We believe that the proposed procedure shows adequate sensitivity and reproducibility for the determination of REE in most commonly studied silicate rocks. Moreover, such a determination appears to be faster and less expensive than those performed by other instrumental methods.

TABLE III

HPLC RESULTS OF SOME REE DETERMINATIONS IN STM-1 AND NIM-L INTERNATIONAL ROCK REFERENCE SAMPLES

| *Element* | *STM-1 (syenite)* | | *NIM-L (lujavrite)* | |
|---|---|---|---|---|
| | *Found* | *Certified* | *Found* | *Certified* |
| Ce | 160.0 ± 10.7 | 146 ± 10 | 134 ± 3.4 | 232 ± 40 |
| La | 132 ± 20 | 146 ± 10 | 138 ± 4 | 228 ± 24 |
| Nd | 79 ± 8 | 78 ± 15 | 49 ± 20 | 48 ± 15 |
| Pr | – | – | 17 ± 8 | 21 |
| Y | 32 ± 2 | 45 | 13 ± 5 | 33 ± 21 |
| Sm | 42 ± 12 | 13 ± 2 | – | – |

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REFERENCES

1 C. C. Schnetzler and J. A. Philpotts, in L. H. Ahrens (Editor), *Origin Distribution of the Elements*, Pergamon, London, 1968.
2 L. A. Haskin, F. A. Frey, R. A. Schmitt, R. M. Smith, in L. H. Ahrens F. Press, S. K. Runcorn and H. C. Urey (Editors), *Physics and Chemistry of the Earth*, Vol. VII, Pergamon, London, 1966.
3 J. Felsche, A. G. Herrmann, in K. H. Wederpohl, C. W. Correns, D. M. Shaw, K. K. Turekian, J. Zemann (Editors), *Handbook of Geochemistry*, Springer-Verlag, Berlin, 1970.
4 L. A. Haskin, in L. H. Ahrens and G. Protas (Editors), *Proceedings of the Second Symposium on the Origin and Distribution of the Elements*, Pergamon, Oxford, 1979.
5 M. E. Switzer, *Sci. Prog. (Oxford)*, 65 (1978) 19.
6 C. H. Evans, *TIBS*, 12 (1983) 445.
7 T. W. Steele, A. Wilson, R. Goudis, P. J. Ellis and A. J. Radford, *Geostandards Newslett.*, 2 (1978) 71.
8 K. Govindaraju and H. De La Roche, *Geostandards Newslett.*, 1 (1977) 67.
9 F. J. Flanagan, *U.S. Geol. Surv., Prof. Paper*, (1976) 840.
10 A. Mazzucotelli, *Rend. Soc. It. Mineral. Petrol.*, 35 (1979) 599.
11 A. Mazzucotelli and R. Frache, *Analyst*, 105 (1980) 497.
12 A. Mazzucotelli and R. Frache, *Mikrochim. Acta*, 2 (1981) 323.
13 A. Mazzucotelli, M. Galli and R. Frache, *Analyst*, 107 (1982) 104.
14 R. H. Cassidy and S. Elchuck, *J. Chromatogr. Sci.*, 18 (1980) 217.
15 R. H. Cassidy and S. Elchuck, *J. Liq. Chromatogr.*, 4 (1981) 379.
16 J. H. Hwang, J. S. Shih, Y. C. Yen and S. C. Wu, *Analyst (London)*, 106 (1981) 869.
17 A. Mazzucotelli, A. Dadone, R. Frache and F. Baffi, *Chromatographia*, 15 (1982) 697.
18 A. Dadone, A. Mazzucotelli, R. Franche and F. Baffi, *Ann. Chim. (Rome)*, 74 (1984) 289.
19 R. Franche and A. Mazzucotelli, *Talanta*, 23 (1976) 415.
20 K. Govindaraju, *Geostandards Newslett.*, 8 (1984) 3.
21 R. G. Hansen and E. J. Ring, *MINTEK (South Africa)*, Rep. No. M134 (1984).

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CHROMSYMP. 668

# CHIRAL RECOGNITION MECHANISMS IN THE ENANTIOSELECTIVITY OF CHIRAL HYDROGEN BONDING ADDITIVES IN LIQUID–SOLID CHROMATOGRAPHY

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## SUMMARY

The addition of a chiral hydrogen bonding additive, derived from N-acetyl-L-valine, to the non-aqueous mobile phase in silica gel liquid chromatography permits the chiral recognition of D- and L-amino acid derivatives as N-acetyl-O-*tert.*-butyl esters. Chiral N-acetyl-L-valine-*tert.*-butylamide is firmly adsorbed on the silica gel surface and forms the chiral hydrogen-bonded phase. The recognition process by which enantioselectivity is attained occurs predominantly in this *de facto* stationary phase. The main driving force for the recognition may be attributed to steric effects, exerted by the silica gel surface in adsorbing the solute–additive associates, rather than to the stability difference between the diastereomeric hydrogen-bonded associates as observable in solution.

In contrast, the recognition process of chiral N-acetyl-L-valine-*tert.*-butyl ester occurs predominantly in the bulk of the mobile phase. This additive, which shows retention on the silica gel similar to that of the solutes to be resolved, has little effect on recognition in the stationary phase process. The enantioselectivity should therefore be ascribed to stability differences between the diastereomeric hydrogen-bonded associates in the mobile phase.

## INTRODUCTION

This study is a continuation of our earlier work which showed that the addition of chiral hydrogen bonding additives (CHBA) to the non-aqueous mobile phase in liquid–solid chromatography permits the recognition of molecular chiralities. This is a unique recognition technique in which CHBA are regarded as "hydrogen-bonding solvents", which induce transient diastereomeric hydrogen-bond solvation with solute enantiomers and serve as a stronger solvent component to aid their separate elution. Up to now, versatility in chiral recognition with the use of CHBA has been known only in part for two CHBA: chiral N-acetyl-L-valine-*tert.*-butylamide (CHBA 1)[1–3] and (*R,R*)-diisopropyl tartramide[4,5]. The former is capable of resolving amino acid enantiomers as N-acetyl-O-*tert.*-butyl ester derivatives in silica gel liquid chromatography[1,2]. On the basis of its excellent selectivity, the scope of this method was extended to the highly sensitive resolution of complex mixtures of amino acid deriv-

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atives, such as N-(4-nitrobenzoyl)-O-isopropyl esters[3]. The explanation of the origin of resolution of the enantiomers has yet to be solved.

To assist in the solution of this problem, the following examinations were carried out: (1) determination of the amount of CHBA 1 adsorbed on a silica gel surface, (2) immobilization of the CHBA 1 structure on the surface of the gel and (3) resolution by use of an alternative L-valine additive, subjected to the same derivatization of the N-acetyl-O-*tert*.-butyl ester as that of the amino acid enantiomers to be resolved. This paper discusses the chiral recognition mechanisms responsible for the enantioselectivity of CHBA on the basis of the separation of D- and L-amino acid derivatives.

## EXPERIMENTAL

Isocratic chromatography was performed with two liquid chromatographic systems. One was a liquid chromatograph with a micro-bore column, as used previously[3] but without a sample injector; a Rheodyne Model 7413 injector with a 0.5-$\mu$l loop was used instead. The other was also a laboratory-built system, equipped with a Merck Hibar pre-packed column (25 × 0.4 cm I.D.)[2]. This column contained 2.0 g of LiChrosorb Si 60 (5 $\mu$m) silica gel. For detection, UV absorption at either 265 or 254 nm was used: at 265 nm for the chromatographic experiments with CHBA 1 owing to transparency of the additive, and at 254 nm with N-acetyl-L-valine *tert*.-butyl ester (CHBA 2). The chromatographic columns were maintained at a constant temperature of 20°C.

Two dimensions of micro-bore columns were used. One column, slurry packed with silica gel [Spherosil XOA-600 (5 $\mu$m); Prolabo, Paris], was 25 × 0.1 cm I.D. and the other, packed with aminopropyl silica [Nucleosil-5$NH_2$ (5 $\mu$m); Macherey, Nagel & Co., Düren, F.R.G.], was 50 × 0.1 cm I.D. The amino packing contained 0.84 mmole of amino groups per gram, as calculated from the mean nitrogen contents obtained by three elemental analyses of this material (found: C, 3.81; H, 1.04; N, 1.17%).

The columns were packed by a high-pressure slurry technique according to the following procedure. About 600 mg of the packing, dried over $P_2O_5$ under reduced pressure, was suspended in 9 ml of methanol–2-propanol–carbon tetrachloride (2:3:11, v/v) with sonication. The homogenized mixture was poured into the slurry reservoir (1.0 cm I.D., inner volume 10 ml) and forced from the reservoir into the column using chloroform at 10 000 p.s.i. The column was then equilibrated with the chromatographic solvent, consisting of ethyl acetate–*n*-hexane (20:80, v/v), to determine the number of theoretical plates of the column. The silica column had 7700 plates per 25 cm and the aminopropyl silica column 12 000 plates per 50 cm. These values were obtained using di-*n*-butyl phthalate as the solute at a solvent flow-rate of 60 $\mu$l/min.

All solvents used for chromatography were distilled prior to use.

### *Measurement of the amount of CHBA 1 adsorbed on the silica gel surface*

The Merck Hibar column, with its bottom connected to a variable-wavelength UV detector (Jasco UVIDEC-100-II; Japan Spectroscopic, Tokyo, Japan), was immersed in a constant-temperature bath (Yamato Model BL-21) at 20°C. The column

was washed successively with 200 ml of ethyl acetate and chloroform, then equilibrated in chloroform–*n*-hexane (40:60, v/v). The hold-up volume of the column, attached to the liquid chromatograph, was determined by injecting benzene into the column. The above mixture, containing 15 m*M* of CHBA 1, was made to pass through the column, and the eluent from the column was collected until the silica gel column attained equilibrium with the mobile phase solvent. Equilibrium was confirmed by constant UV absorption by CHBA 1, monitored at 230 nm, and constant retention of the solute enantiomers of the racemic N-acetyl-$N^{ind}$-*tert.*-butyltryptophan *tert.*-butyl ester after three injections. After the volume of the combined eluents had been precisely measured, the solvent was removed under reduced pressure and the residual additive was weighed. The amount of CHBA 1 adsorbed on the silica gel surface was calculated as the loss of the additive in the mobile phase solvent corresponding to the particular volume of eluent from which the hold-up volume, determined prior to equilibration, had been removed. By this procedure, 196.06 mg of the CHBA 1 were found to be held on 2.0 g of the silica gel in the column.

The amount of CHBA 1 in the mobile phase bulk was considered to correspond to that in the alternative hold-up volume, as also determined by using benzene, following attainment of equilibrium of the additive. The observed amount was 9.64 mg of CHBA 1 in 3 ml of the hold-up volume.

*Preparation of chiral acids and procedure for in situ modification of packed amino columns*

The chiral graft glutaryl-L-valine-*tert.*-butylamide monocarboxylic acid was prepared by the following procedure. 4-Benzyloxycarbonylbutanoyl N-4-hydroxysuccinimide ester, obtained by dicyclohexylcarbodiimide coupling between glutaric acid monobenzyl ester and N-hydroxysuccinimide, and L-valine-*tert.*-butylamide hydrochloride [subliming at *ca.* 148°C; $[\alpha]_D^{23} = +45.6°$ ($c$ = 1.01, methanol)] were condensed in the presence of triethylamine. The benzyl ester obtained was hydrogenated to afford the desired material. Recrystallization from ethyl acetate gave pure material of m.p. 143–147°C; $^1$H NMR ($C^2HCl_3$), $\delta$ 0.93 (d, 3H, $J$ = 6.6 Hz), 0.94 (d, 3H, $J$ = 6.6 Hz), 1.35 (s, 9H), 1.79–2.09 (m, 3H), 2.23–2.54 (m, 4H), 4.01–4.10 (dd, 1H), 5.99 (brs, 1H), 7.87 ppm (brd, 1H, $J$ = 9.1 Hz); IR (KBr), 3300, 3100, 2980, 2950, 2890, 2600 (br), 1720, 1640, 1560, 1460, 1395, 1370, 1340, 1260, 1225, 1160, 1050, 930 $cm^{-1}$; mass spectrometry ($m/z$), calculated for $C_{14}H_{26}O_4N_2$ 286, found 287 $(M + H)^+$; $[\alpha]_D^{22} = -38.7°$ ($c$ = 1.00, ethanol).

The chiral acid, glutaryl-L-valine *tert.*-butyl ester monocarboxylic acid, was prepared in a manner similar to that above. Coupling of the 4-benzyloxycarbonylbutanoyl N-4-hydroxysuccinimide ester and L-valine *tert.*-butyl ester hydrochloride [m.p. 144–146°C; $[\alpha]_D^{20} = +23.2°$ ($c$ = 0.41, ethanol); lit[6]. m.p. 147–149°C], followed by hydrogenation gave the desired material: $^1$H NMR ($C^2HCl_3$), $\delta$ 0.91 (d, 3H, $J$ = 6.8 Hz), 0.94 (d, 3H, $J$ = 6.8 Hz), 1.48 (s, 9H), 1.77–2.24 (m, 3H), 2.24–2.64 (m, 4H), 4.42–4.57 (dd, 1H), 6.48 (brd, 1H, $J$ = 9.4 Hz), 7.62 ppm (brs, 1H); IR ($CHCl_3$; 0.07 *M*), 3480, 3400, 3280, 3140 (br), 2940, 2900, 2840, 1710, 1660, 1500, 1445, 1385, 1362, 1330, 1305, 1195, 1140, 1030, 835, 700 $cm^{-1}$; mass spectrometry ($m/z$), calculated for $C_{14}H_{28}O_5N$ 287, found, 288 $(M + H)^+$; $[\alpha]_D^{21} = -24.6°$ ($c$ = 1.00, ethanol).

These chiral acids were ionically bonded in a 50 × 0.1 cm I.D. aminopropylsilica column by an *in situ* modification procedure described by Pirkle and Finn[7].

*Preparation of CHBA and solutes*

The chiral additive N-acetyl-L-valine *tert.*-butyl ester was prepared by treating the L-valine *tert.*-butyl ester hydrochloride with a mixture of acetic anhydride and triethylamine. Recrystallization from ethyl acetate gave a pure material of m.p. 106–106.5°C. Analysis: calculated for $C_{11}H_{21}O_3N$, C 61.37, H 9.83, N 6.51; found, C 61.45, H 9.99, N 6.49%; $[\alpha]_D^{18} = -29.9°$ ($c$ = 1.03, ethanol). The preparation and characterization of the chiral additive N-acetyl-L-valine-*tert.*-butylamide have already been reported[2].

The N-acetylamino acid *tert.*-butyl esters used as solutes are the same as those described previously[2] except for the tyrosine derivative. The L-enantiomer and its racemate were prepared from the corresponding O-benzyltyrosine (Kokusan Chemical, Tokyo, Japan) according to the procedure described previously[2]. The tyrosine derivative was characterized as follows: N-acetyl-O-benzyl-DL-tyrosine *tert.*-butyl ester: m.p. 98–100°C [L-enantiomer, m.p. 93–96°C; $[\alpha]_D^{18} = +16.5°$ ($c$ = 1.02, ethanol)]; $^1$H NMR ($C^2HCl_3$), $\delta$ 1.40 (s, 9H), 1.96 (s, 3H), 3.02 (d, 2H, $J$ = 5.9 Hz), 4.55–4.83 (m, 1H), 5.04 (s, 2H), 5.89 (brd, 1H), 6.83–7.13 (m, 4H), 7.39 ppm (s, 5H); IR (KBr), 3380, 3000, 2950, 1720, 1675, 1615, 1590, 1535, 1520, 1470, 1460, 1440, 1390, 1370, 1325, 1285, 1250, 1180, 1135, 1125, 1000, 970, 860, 815, 760, 715, 700 $cm^{-1}$; mass spectrometry ($m/z$), calculated for $C_{22}H_{27}O_4N$ 369, found 369 $(M)^+$.

## RESULTS AND DISCUSSION

*Outline of optical resolutions with a CHBA*

Consider the solute enantiomers $S_l$ and $S_d$, present at high dilution, in a silica gel column containing a CHBA. Chiral recognition depends on two categories of equilibrium relationships: a mobile and a stationary phase, in which the CHBA associates with each enantiomer. Assuming that diastereomeric hydrogen-bond associations consist in the formation of binary associated complexes, as has been shown in many instances, the following equilibrium expressions are given:

$$S_l + CHBA \overset{K_l}{\rightleftharpoons} C^*S_l$$

$$S_d + CHBA \overset{K_d}{\rightleftharpoons} C^*S_d$$

where $C^*S_l$ and $C^*S_d$ are complexes. These expressions allow us to recognize easily two possible origins of the observed resolution of enantiomers: a difference in stability between diastereomeric associations (*i.e.*, relative magnitudes of the equilibrium constants $K_l$ and $K_d$) and a difference in chromatographic mobility of intact associated complexes overriding any stability difference.

If complex formation occurs predominantly in the mobile phase, the observed resolution may be ascribed to different association constants. That is, the more strongly complexed solute enantiomer is less strongly adsorbed on a stationary phase and is the first to be eluted. Complex formation by which enantioselectivity is attained may also occur from interaction between the solute and CHBA adsorbed on the silica gel surface. This selectivity, if any, may differ from that obtained in the mobile phase

bulk because of the surface responsible for adsorption of the molecules. Should the association stereoselectivity not be affected by the surface, then the more strongly complexed solute enantiomer is the last to be eluted.

The above mobile and stationary phase processes are superimposed on a scheme of competition between the CHBA in the mobile phase bulk and that adsorbed on the silica gel surface for the solute enantiomer. Assuming the competitive hydrogen-bond association to be present in the presently discussed chromatography, secondary partitioning of intact diastereomeric complexed solutes must be subordinated to the two-phase processes, as the complexed solute–CHBA interaction on the stationary surface is considered eventually to be resolved into the competitive association between the two CHBA for the solute. Therefore, in this paper, we discuss the chiral recognition mechanisms on the basis of partitioning of each solute enantiomer between the mobile and stationary phases.

*Recognition mechanism in resolution with CHBA 1*

CHBA 1 showed markedly stronger retention than the solute enantiomers in a silica gel column, as was evident from a comparison of the behaviour of CHBA 1

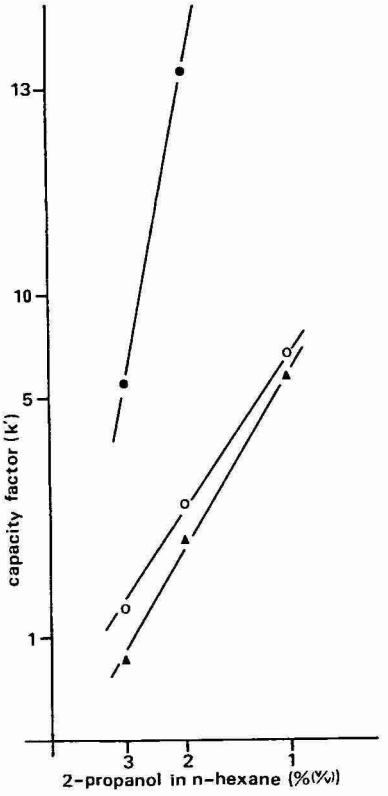


Fig. 1. Plot of the logarithm of the capacity factor ($k'$) *versus* logarithm of the concentration of 2-propanol in *n*-hexane. ●, Chiral hydrogen-bonding additive (CHBA), N-acetyl-L-valine-*tert.*-butylamide (1); ○, CHBA, N-acetyl-L-valine *tert.*-butyl ester (2); ▲, N-acetyl-L-phenylalanine *tert.*-butyl ester, as a typical solute to be resolved. The $k'$ value of CHBA 1 could not be determined when 2-propanol–*n*-hexane (1:99, v/v) was used, as the solute was not eluted within a reasonable time.

and the phenylalanine derivative, a typical solute. This comparison is shown in Fig. 1, which is a plot of the logarithm of the capacity ratios ($k'$) *versus* the logarithm of the concentration of 2-propanol in *n*-hexane. This strong adsorptivity on the silica gel surface stems from the presence of the two amide units constituting the so-called $C_5$ and $C_7$ conformational sites (see Fig. 3).

CHBA 1 is, in fact, strongly adsorbed on the surface of the silica gel. The adsorbed concentration, calculated from measurement of the amount of additive in the column, was approximately 0.46 mmol/g of the support. The ratio of the additive content of the mobile phase bulk to that of the adsorbed layer in the column was 1:20 at 20°C when chloroform–*n*-hexane (40:60, v/v), containing 15 m*M* of CHBA 1, was employed as the mobile phase solvent. Table I gives the optical resolutions obtained under the above conditions for racemic N-acetylamino acid *tert.*-butyl esters containing aromatic moieties; this is a UV detection requirement. The observed elution order of the solute enantiomers was not affected by the chromatographic conditions, such as column temperature and mobile phase composition[2]: the D-enantiomers were eluted faster than the L-enantiomers in all the solutes examined. Fig. 2 shows a typical resolution of the racemic N-acetyl-O-benzyltyrosine *tert.*-butyl ester.

We attach much importance to the association that occurs on the adsorbed layer of CHBA 1. Our guiding principle is that the additive molecule should be firmly adsorbed by hydrogen bonding on the silica gel surface and that the solute enantiomers associate with the adsorbed layer without displacement of CHBA 1[8,9]. The CHBA should assume a position with its less hindered face towards the surface of the gel, leaving the face having the isopropyl side-chain connected to an asymmetric carbon open for the bulk of the mobile phase.

It seems reasonable to expect that this CHBA interacts with the solute enantiomers to form diastereomeric dimers whose associative interactions proceed by bidentate NH ··· O=C hydrogen bonds on a silica gel surface. CHBA 1 is capable of generating two structures of the hydrogen-bond associates: "$C_5$–$C_7$" dimers, in which

TABLE I

OPTICAL RESOLUTION OF THE ENANTIOMERS OF RACEMIC N-ACETYLAMINO ACID *TERT.*-BUTYL ESTERS WITH CHIRAL HYDROGEN BONDING ADDITIVE (CHBA), N-ACETYL-L-VALINE-*TERT.*-BUTYLAMIDE (1) ON A SILICA GEL COLUMN

Chromatographic conditions: column, 25 × 0.4 cm I.D.; packing, LiChrosorb Si 60 (5 μm); mobile phase solvent, chloroform–*n*-hexane (40:60, v/v), containing 15 m*M* of CHBA 1, N-acetyl-L-valine-*tert.*-butylamide; flow-rate, 1 ml/min; column temperature, 20°C; detection, UV at 265 nm.

| *Amino acid* | *Capacity factor (k')* | | *Separation factor (α)** |
|---|---|---|---|
| | *D-* | *L-* | |
| N-*t*-BuTrp | 3.31 | 4.07 | 1.23 |
| S-BzlCys | 3.57 | 4.28 | 1.20 |
| α-PheGly | 4.22 | 5.10 | 1.21 |
| Phe | 4.30 | 5.56 | 1.29 |
| O-BzlTyr | 4.31 | 5.13 | 1.19 |

* $\alpha = k'_L/k'_D$.

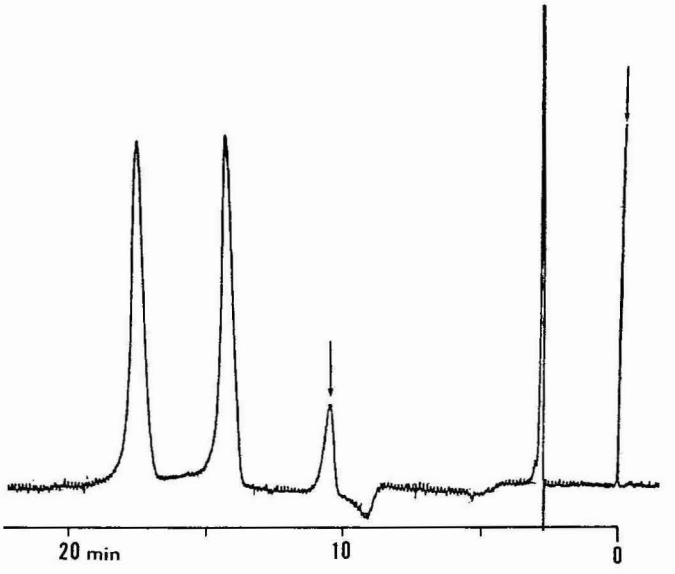


Fig. 2. Optical resolution of racemic N-acetyl-O-benzyltyrosine *tert.*-butyl ester with CHBA 1, N-acetyl-L-valine-*tert.*-butylamide. Chromatographic conditions: mobile phase solvent, chloroform–*n*-hexane (40:60, v/v), containing 15 m*M* of CHBA 1; flow-rate, 1 ml/min; column temperature, 20°C; detection, UV 250 nm; sample volume injected, 3 μl of 2% (v/v) chloroform solution. The order of emergence of the enantiomers was such that the D-enantiomer was eluted faster than the L-enantiomer. The peak indicated with an arrow was assumed to correspond to CHBA 1, displaced by the solute, because of its appearance at a $k'$ value of 2.81, regardless of the solutes to be resolved. This displaced additive cannot be a primary layer, firmly attached the surface of the silica, but the additive hydrogen-bonded to the primary layer.

the $C_7$ conformational site is offered by CHBA 1, and "$C_5$–$C_5$" dimers. These two dimers can be formed as the solute enantiomers pass through the column. The associated complex of the enantiomers may either lie flat on the surface or stand upright because the association competes in part with the adsorption of the CHBA. These two orientations may possibly be in equilibrium, rather than one of the two predominating. Assuming the above associations to occur in the adsorbed state of CHBA 1, as shown in Fig. 3, the associated complex of the L-enantiomers can adopt a more stable flat-lying posture, because their side-chains are oriented towards the bulk of the mobile phase, similar to that of CHBA 1. The D-enantiomers must, however, orient their large substituents towards the surface of the silica gel when in the fully hydrogen-bonded state and their probable lesser degree of adsorption may result in elution of the D-enantiomers first.

The above selectivity is presumed to be exerted by the silica gel surface on which CHBA is deposited, and hence may be independent of the "raw" enantioselectivity, *i.e.*, the difference in stability between the diastereomeric associated complexes observed in solution. This selectivity therefore must be termed "chromatographic enantioselectivity", as a direct allocation of raw enantioselectivity cannot be made for the stationary phase process, although it must be kept in mind that the selectivity contributes, at least in part, to the overall stationary phase process.

Immobilization of CHBA on the silica gel surface should make it possible to

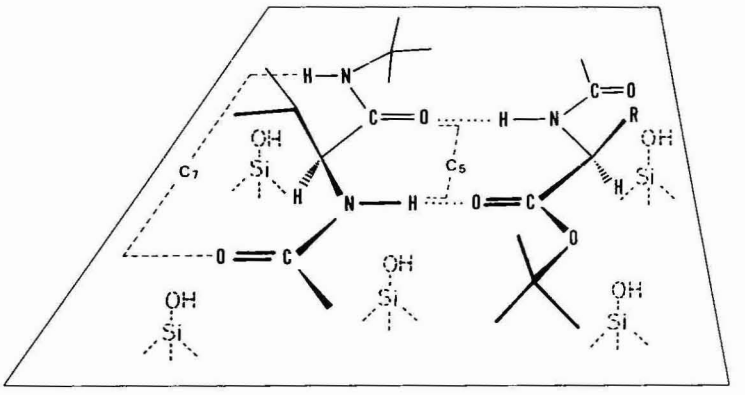

Fig. 3. The solute L-enantiomer–additive association occurring in the adsorbed state of CHBA 1, N-acetyl-L-valine-*tert.*-butylamide on a silica gel surface. The complexed L-enantiomers, whose associative interactions are bidentate NH · · · O=C hydrogen bonds, can provide a more stable flat-lying orientation because their side-chains are oriented toward the bulk of the mobile phase, similar to that of CHBA 1. The "$C_5$–$C_7$" dimers in which the $C_7$ conformational site is offered by CHBA 1 may be formed along with the "$C_5$–$C_5$" dimers in this figure, as the solute enantiomers pass through the column.

elicit the enantioselectivity of the hydrogen-bonded phase from the chiral mobile phase system. We therefore prepared the chiral stationary phase CSP 1, shown in Fig. 4, consisting of glutaryl-L-valine-*tert.*-butylamide monocarboxylic acid, containing the CHBA 1 structure ionically bound to 3-aminopropylsilica, and made an examination to determine whether the solute enantiomers are resolved in the same elution order as that by CHBA 1. CSP 1 showed, as expected, the same elution order, *i.e.*, the D-enantiomer is followed by the L-enantiomer, as shown in Table II. It seems reasonable to expect a recognition mechanism similar to that assumed for CHBA 1. The chiral moiety of the L-configuration is also adsorbed on the surface and forms the most stable associated complex with the L-enantiomer of the solute. The mobile phase solvent used contained not chloroform but 2-propanol as the stronger solvent component, as chloroform lacks the capability to elute the amino acid solutes in the absence of CHBA 1. It is evident that the elution order is independent of the mobile phase composition in chiral stationary phases, on the basis of hydrogen-bond associations, as has previously been reported[10].

The actual resolutions, expressed as the separation factor ($\alpha$), are determined by superimposing the raw enantioselectivity in the mobile phase process on the above stationary phase process. Chiral recognition in the mobile phase is perhaps not great enough to surpass the stereoselectivity of the stationary phase, as is implied by a comparison of the elution order and magnitude of the resolutions observed for CSP

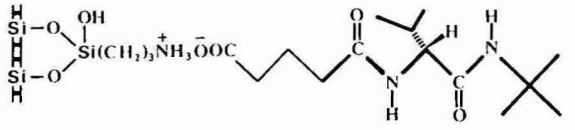

Fig. 4. Aminopropylsilica, modified *in situ* by glutaryl-L-valine-*tert.*-butylamide monocarboxylic acid (CSP 1).

TABLE II

OPTICAL RESOLUTION OF THE ENANTIOMERS OF RACEMIC N-ACETYLAMINO ACID *TERT.*-BUTYL ESTERS ON THE PACKED AMINO COLUMN, MODIFIED BY GLUTARYL-L-VALINE-*TERT.*-BUTYLAMIDE MONOCARBOXYLIC ACID

Chromatographic conditions: column, 50 × 0.1 cm I.D.; packing, 3-aminopropyl-silica [Nucleosil-5$NH_2$ (5 μm)], *in situ* modified by the chiral acid glutaryl-L-valine-*tert.*-butylamide monocarboxylic acid (CSP 1); mobile phase solvent; 2-propanol–*n*-hexane (1:99, v/v); flow-rate, 60 μl/min; column temperature, 20°C; detection, UV at 254 nm.

| *Amino acid* | *Capacity factor (k')* | | *Separation factor (α)** |
|---|---|---|---|
| | *D-* | *L-* | |
| N-*t*-BuTrp | 5.79 | 6.88 | 1.19 |
| S-BzlCys | 4.98 | 5.59 | 1.12 |
| α-PheGly | 6.10 | 6.81 | 1.12 |
| Phe | 5.50 | 6.43 | 1.17 |
| O-BzlTyr | 7.17 | 8.26 | 1.15 |

* $\alpha = k'_L/k'_D$.

1 and those for CHBA 1, although clarification as to whether the mobile phase process enhances or reduces the selectivity in the stationary surface was not possible in the present study.

*Recognition mechanism of resolution with CHBA 2*

CSP 2, obtained by substitution of the C-terminal amide unit with an ester unit, as shown in Fig. 5, was insensitive to solute enantiomers at the column temperatures used for CSP 1, such as 20°C. A lower column temperature of −27°C forced the resolution of the racemic solutes on the CSP 2 column in the same elution order as that observed for CSP 1. For example, racemic N-acetylvaline *tert.*-butyl ester was resolved at an α value of 1.11 ($k'_D$ = 2.37, $k'_L$ = 2.63), using dichloromethane–*n*-hexane (60:40, v/v) as the mobile phase solvent. Detection was effected by UV absorption at 230 nm. When CHBA 2, which is believed to be the immobilized moiety in CSP 2, was used instead of CHBA 1, the resolutions appeared to be induced by raw enantioselectivity in the mobile phase process, as the selectivity exerted by this molecule adsorbed on the silica gel surface need not be taken into consideration at ambient temperatures.

CHBA 2 is capable of resolving the solute enantiomers at a concentration of 0.1 *M* in chloroform–*n*-hexane (10:90, v/v), as shown in Table III. The observed order of emergence of the solute enantiomers was reverse of that obtained with

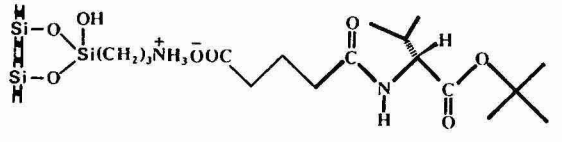

Fig. 5. Aminopropylsilica, modified *in situ* by glutaryl-L-valine *tert.*-butyl ester monocarboxylic acid (CSP 2).

TABLE III

OPTICAL RESOLUTION OF THE ENANTIOMERS OF RACEMIC N-ACETYLAMINO ACID *TERT.*-BUTYL ESTERS WITH CHBA, N-ACETYL-L-VALINE-*TERT.*-BUTYL ESTER (2) ON A SILICA GEL COLUMN

Chromatographic conditions: column, 25 × 0.1 cm I.D.; packing, Spherosil XOA-600 (5 μm); mobile phase solvent, chloroform–*n*-hexane (10:90, v/v), containing 0.1 *M* of CHBA 2; flow-rate, 30 μl/min; column temperature, 20°C; detection, UV at 254 nm.

| *Amino acid* | *Capacity factor (k')* | | *Separation factor (α)** |
|---|---|---|---|
| | *L-* | *D-* | |
| N-*t*-BuTrp | 9.51 | 10.73 | 1.13 |
| S-BzlCys | 9.03 | 10.08 | 1.12 |
| α-PheGly | 9.31 | 10.43 | 1.12 |
| Phe | 9.85 | 11.46 | 1.16 |
| O-BzlTyr | 12.49 | 15.24 | 1.22 |

* $\alpha = k'_L/k'_D$.

CHBA 1: the L-enantiomers were eluted faster than the D-enantiomers. NMR and IR spectra of the self-association of CHBA 2 in solution can thus be studied as an analogue of the solute–additive associations in the mobile phase in order to clarify the chiral recognition mechanism responsible for the enantioselectivities of CHBA 2. Diastereomeric associated complexes, similar to those observed in CHBA 2, are probably formed from pairs of alternate solutes and CHBA 2.

A concentration study of $^1H$ and $^{13}C$ NMR in carbon tetrachloride solutions showed that the diastereomeric dimers, as depicted in Fig. 6, whose associative interactions are bidentate NH ··· O=C (ester) hydrogen bonds, are formed in enantiomeric solutions[11]. The formation of these dimers in enantiomerically enriched mixtures is also indicated by a split of the amide NH resonance into two $^1H$ NMR signals for the D- and L-enantiomers, termed self-induced non-equivalence[12,13], in 0.1 *M* carbon tetrachloride solution.

The IR spectra confirm the stability difference between the homochiral (Fig. 6a) and the heterochiral hydrogen-bonded dimer (Fig. 6b) by an external comparison of the optically pure and racemic samples in carbon tetrachloride solution. In the

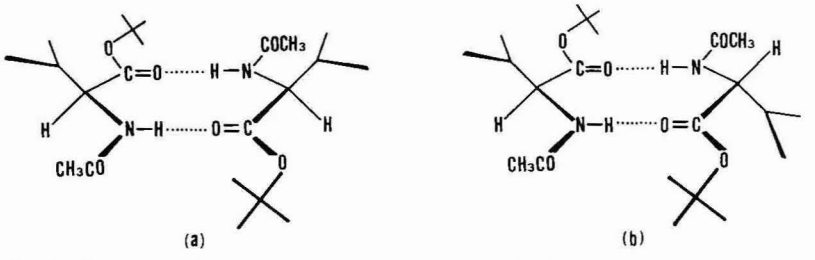


Fig. 6. Diastereomeric dimers interlinked via bidentate NH ··· O=C (ester) hydrogen bonds, proposed to account for the self-induced NMR non-equivalence of the enantiomeric carbon tetrachloride solution of the chiral N-acetylvaline *tert.*-butyl ester (2). The homochiral (L–L) dimer (a) is more stable than the heterochiral dimer (b).

L-enantiomeric solution, the intensity of the hydrogen-bonded NH stretching band (3360 $cm^{-1}$), corresponding to the intermolecular hydrogen bond[14] in the amide group, is stronger than that in the racemic mixture containing enantiomeric sample at concentrations exceeding 0.01 *M* at *ca.* 23°C. The homochiral hydrogen-bonded dimer formed in the enantiomerically pure solution was concluded to be more stable than the corresponding heterochiral dimer in the racemic solution. This stability difference is not affected by replacement of the co-solvent carbon tetrachloride with chloroform–hexane (10:90, v/v), as is indicated by the external comparison of intensity on the NH absorption, and hence the elution order observed with CHBA 2 is clearly explained in terms of the dominance of the mobile phase process: the most strongly complexed solute enantiomer, *i.e.*, the L-enantiomer in this instance, in the mobile phase is less strongly adsorbed on the stationary phase and is the first to be eluted.

The contrast in elution order between CHBA 1 and CHBA 2 is at least in part ascribable to the structure of CHBA 2, having only one $C_5$ conformational site for bidentate binding to the solute enantiomers. That is, surrender of the $C_5$ site to the solutes is strongly competitive with the adsorption of CHBA 2 itself.

It is of interest that all chiral hydrogen-bonding agents capable of inducing the formation of distereomeric solvates with enantiomeric molecules containing proton--releasing or proton-accepting groups are potentially capable of serving as chiral mobile phase additives, provided that there are appropriate means for detecting the elution of the solute enantiomers.

## CONCLUSIONS

Chiral mobile phase additives provide a chiral environment in the column and form an associated complex with each enantiomer. Hence there are two different chiral recognition mechanisms responsible for the enantioselectivity of the CHBA. If the CHBA is firmly adsorbed on the silica gel surface and acts as a chiral hydrogen-bonded phase with which the solutes can form diastereomeric associated complexes such as CHBA 1, the main driving force for the chiral recognition can be the steric effect exerted by the surface of the support adsorbing the solute–additive associates rather than the difference in stability between the complexes in the mobile phase bulk. This enantioselectivity must be termed "chromatographic enantioselectivity". However, if the CHBA adsorbed on the surface is displaced by the solutes or its adsorbed state cannot form diastereomeric associated complexes capable of generating optical resolutions of the solutes, such as CHBA 2, raw enantioselectivity in the mobile phase becomes the most important source in the chiral resolution. In this instance, the chromatography can function as a sophisticated means for clarification of the association stereoselectivity of chiral molecules.

## ACKNOWLEDGEMENTS

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REFERENCES

1 A. Dobashi and S. Hara, *Tetrahedron Lett.*, 24 (1983) 1509.
2 A. Dobashi and S. Hara, *J. Chromatogr.*, 267 (1983) 11.
3 A. Dobashi and S. Hara, *Anal. Chem.*, 55 (1983) 1805.
4 Y. Dobashi, A. Dobashi and S. Hara, *Tetrahedron Lett.*, 25 (1984) 329.
5 Y. Dobashi and S. Hara, *J. Am. Chem. Soc.*, 107 (1985) 3406.
6 G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, 46 (1964) 2935.
7 W. H. Pirkle and J. M. Finn, *J. Org. Chem.*, 46 (1981) 2935.
8 R. P. W. Scott and P. J. Kucera, *J. Chromatogr.*, 149 (1978) 93.
9 R. P. W. Scott, *Faraday Symp. Chem. Soc.*, 15 (1981) 49.
10 A. Dobashi, K. Oka and S. Hara, *J. Am. Chem. Soc.*, 102 (1980) 7122.
11 A. Dobashi, N. Saito, Y. Motoyama and S. Hara, *J. Am. Chem. Soc.*, in press.
12 W. H. Pirkle and D. J. Hoover, in E. L. Eliel and N. L. Allinger (Editors), *Topics in Stereochemistry*, Vol. 13, Wiley-Interscience, New York, 1982, p. 316.
13 G. R. Weisman, in J. D. Morrison (Editor), *Asymmetric Synthesis*, Vol. 1, Academic Press, New York, 1983, p. 153.
14 R. T. Ingwall, C. Gilon and M. Goodman, *Macromolecules*, 9 (1976) 802.

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CHROMSYMP. 704

# EFFECTS OF CONTACT POTENTIALS ON THE PULSED ELECTRON-CAPTURE DETECTOR

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## SUMMARY

Studies of the effect of the contact potential difference on the standing current and the response of the constant-frequency electron-capture detector are reported. The effect of temperature and carrier gas flow-rate on the contact potential difference is also shown. The space charge [P. L. Gobby, E. P. Grimsrud and S. W. Warden, *Anal. Chem.*, 52 (1980) 473] model proved useful in the interpretation of the experimental results.

## INTRODUCTION

In most commercial electron-capture detectors the anode and the cathode are made of different materials. The cathode is usually a radioactive source ($^{3}H$ or $^{63}Ni$) and the anode is usually made of stainless steel. The difference in the work functions of the electrodes materials constitutes the contact potential difference. The contact potential difference can range from fractions of a volt up to several volts, and such a potential difference can cause a detector current of in the nanoampere range, which, depending on the polarity of the potential difference, either opposes or enhances the current normally measured by an electrometer. The contact potential difference between the two clean metals does not depend significantly on the temperature, assuming that the metals are at the same temperature. However, the temperature can affect the adsorption–desorption phenomena of the charges on the metal surface, hence modifying the detector pulse-free current. The influence of the adsorbed material on the contact potential difference was described by Loeb[1]. He considered three different layers: adsorbed layers of ions, adsorbed layers of dipoles and adsorption by the Van der Waal's forces. These were said to create dipole layers at the metal surface, lowering or raising the contact potential difference, depending on the polarity.

Lovelock[2] pointed out that the contact potentials can cause an anomalous detector response in d.c. operation. He found that when the contact potential difference opposed the applied potential, the gas chromatographic (GC) peak had an unexpectedly large area, whereas if the potential difference enhanced the applied potential, a diminished response was observed. Lovelock, who proposed the pulse sampling mode of electron-capture detector operation, suggested that with this mode of operation the effects of the contact potentials are rarely, if ever, encountered.

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In 1980, Grimsrud and Warden[3] found that the effects of the contact potentials on the constant-frequency detector response were insignificant for short pulse periods ($t_p < 500$ $\mu$s). Grimsrud and Warden proposed a modification of the circuit for measuring the detector current, which consisted of the addition of a small potential, $E_{bias}$, to compensate for the effects of contact potential. The value of $E_{bias}$ was set so as to achieve zero-detector current when there was no pulse applied to the cell. Grimsrud and Connolly[4] believe that additional small but significant potentials can be created at the cell boundaries owing to the unequal rates at which the charges of opposite sign arrive at the electrodes, which is in accordance with Loeb's findings mentioned above.

In more recent work, Knighton and Grimsrud[5] considered the effects of the contact potential on detector operation. Their electron-capture detectors exhibited high standing currents without the application of any external field. They observed field-free currents of opposite polarities, ranging from a few tenths up to several nanoamperes. They also introduced a parameter $L'$, the rate constant for all electron losses other than those which are caused by a reaction with a sample, in which the effects of the contact potentials were taken into account.

Recently, Simon and Wells[6] thoroughly evaluated the effect of contact potentials on the detector standing current in a constant-current mode for two geometries of the detector, *viz.*, cylindrical and coaxial displaced cylinders. They used four different cell configurations, pulsing the pin or the outer cylinder with pulses of different polarity. They pointed out that there are two effects to be considered in the pulse-free period of detector operation: contact potential and space charge effects. Simon and Wells showed that when the field generated by the contact potential difference is in opposition to that generated by the applied pulses, it could overcome the field generated by the space charge for sufficiently long pulse periods. For small pulse periods, the space charge effects dominate, and for $t_p$ approaching zero the d.c. mode is approached. They also showed that for a contact potential difference opposing the potential applied to the cell, a local minimum in the current *versus* pulse frequency plot was observed. They attributed this phenomenon to the averaging of the electric fields generated by the space charge and the contact potential difference. If, on the other hand, the contact potential difference enhanced the potential applied to the cell, no local minimum in the plot was observed. They attributed this to the minimization of the space charge field in such a cell configuration. They discussed in a similar way the question of the displaced coaxial geometry.

In this work, the effects of contact potential on the standing current and on the response of the constant-frequency electron-capture detector were examined for various values and for two possible polarities of the contact potential difference.

## EXPERIMENTAL

A GCHF 18.3 gas chromatograph (G.D.R.) was used. A pin-cup electron-capture detector with a $^{63}$Ni ionization source of 10-mCi activity was built in our laboratory. The volume of the electron-capture detector was 1 cm$^3$. A 1/16-in. stainless-steel pin protruded up to half of the height of the cell, and the ionization source formed the outer cylinder. The pulse voltage generator was also built in our laboratory, with the following parameters: pulse amplitude, 50 V; pulse period range, 10–30 000 $\mu$s; and pulse duration range, 1–100 $\mu$s.

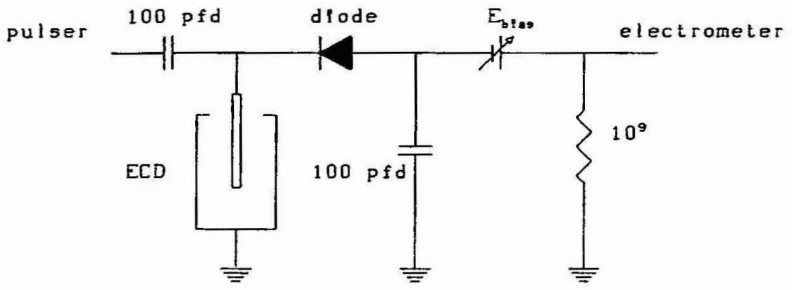


Fig. 1. Circuit for measuring the constant-frequency electron-capture detector current.

The circuit for measuring the detector current, which is depicted in Fig. 1, was similar to that proposed by Grimsrud and Warden[3], where a small potential, $E_{bias}$, was added to compensate for the effects of the contact potential. The detector was maintained at 200°C. A stainless-steel 1.5 m × 4 mm I.D. silica gel column, operating at ambient temperature, was used. The carrier gas was nitrogen, purified by passage through a two-stage filter packed with activated charcoal and 5 Å molecular sieve. The carrier gas flow-rate was maintained at 75 $cm^3$/min. The detector current was measured by an RFT 6350 electrometer (G.D.R.) and the chromatograms were plotted on a TZ-4100 recorder (Laboratorní Přístroje, Prague, Czechoslovakia). $SF_6$ with a specified purity of 99.7% was obtained from Merck-Schuchard. A mixture of 2.4 ppb (v/v) of $SF_6$ in nitrogen was prepared in 2-l stainless-steel bottles by the successive dilution method. This concentration was chosen so as to give a detector response of less than 10% of the standing current. On-column injections were made with help of a six-port valve, made by Valco (U.S.A.). Approximately 1 $cm^3$ of sample was injected several times in order to minimize the standard deviation of the detector response. A pulse duration of 9 $\mu$s was sufficient for the collection of all the thermal electrons during the pulse.

A small potential, $E_{bias}$, was used not only to compensate for the effects of the contact potential but also to set a desired value of the pulse-free current. The value of the pulse-free current measured without adding $E_{bias}$ to the measuring circuit was of the order of +108 pA in our detector, *i.e.*, with a polarity opposing that of the standing current. This value constituted 12% of the maximum detector current. The maximum detector current, $I_0$, was of the order of −830 pA, and the pseudo-recombination rate constant, $k_D$, was of the order of 540 $s^{-1}$.

## RESULTS AND DISCUSSION

The results indicating the effects of the contact potential on both the detector standing current, $I_b$, and the detector response to a sample, $R$, will be discussed in relation to the Gobby *et al.*[7] space charge model. Other possible electron-capture detector models were also considered, such as the modified Wentworth *et al.*[8] kinetic model, or the space charge model developed by Aue and Kapila[9], but neither of them provided a satisfactory explanation of the experimental data. The reason is obvious: those models were developed with different assumptions and for cell configuration other than that used in this study. The space charge model of Aue and Kapila, which

considers that the electron capture and negative ion migration cause the detector response, works successfully under the condition that the "centre of charge" is situated close to the cathode and far from the anode. This is not the case here. We found the Wentworth *et al.* kinetic model helpful when considering the effects of the contact potential under the condition that the electric field generated by the contact potentials enhanced the potential applied by the pulser, whereas for the opposite configuration of these fields the experimental data were difficult to reconcile with the kinetic model predictions.

Some important attributes of the Gobby *et al.* space charge model should be mentioned. The model is applied to an electron-capture detector in which ionization produces a uniform distribution of the ion–electron pairs throughout the cell. The pulse amplitude and the pulse width are chosen so that all the thermal electrons can be collected. In the pulse-free portion of the pulse period, the positive ions that are in excess after the removal of the electrons dissipate to all the grounded surfaces of the cell. The ions are driven by the space charge field which they themselves have generated. The thermal electrons produced between pulses form, together with the surrounding positive ions, a plasma region in which the charge neutrality is maintained. The plasma is surrounded by the positive-ion sheath, which separates it from the cell boundaries. The sizes of both the plasma and the ion sheath are subject to changes during the time after the pulse. The size of the plasma increases with time after the pulse, whereas for the size of the ion sheath the reverse is expected. It is believed that there is no gradient of the positive ion density throughout the cell, because the positive ions are lost at nearly the same rate by the recombination within the plasma and by the space-charge-driven migration in the positive-ion sheath zone.

*Battery current*

An electric field, generated by the contact potential difference, causes a mea-

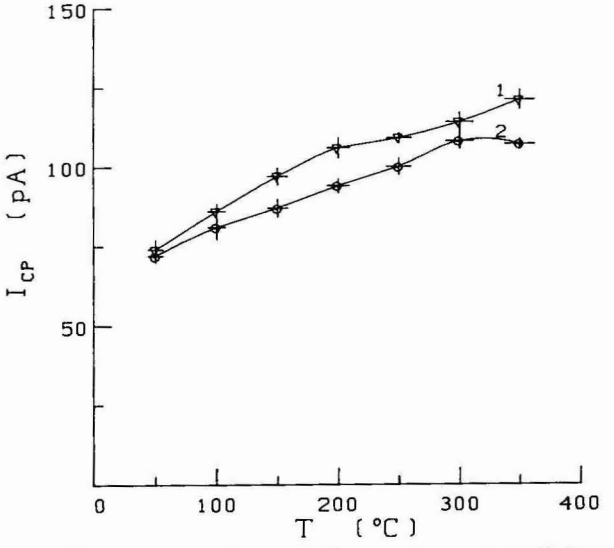


Fig. 2. Battery current, $I_{CP}$, *versus* detector temperature, $T$. Curve 1, decreasing $T$; curve 2, increasing $T$. The error bars shown are equal to 2 standard deviations.

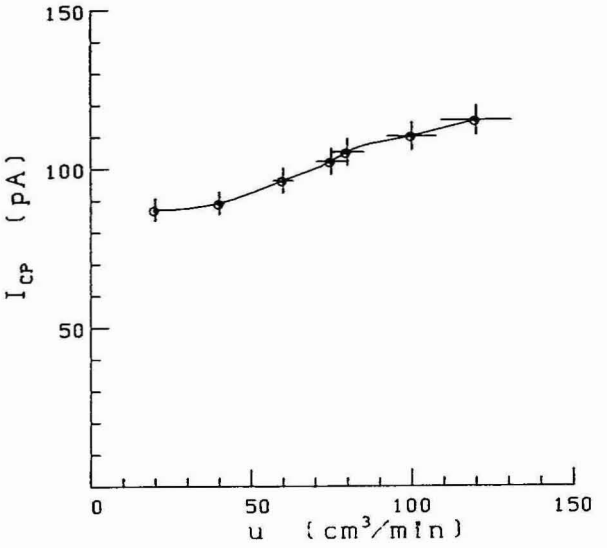


Fig. 3. Battery current, $I_{CP}$, *versus* carrier gas flow-rate, $u$. Error bars as in Fig. 2.

surable detector current, $I_{CP}$, with a polarity and size that vary with the experimental conditions. This current will be referred to as the battery current. It is measured with no pulse applied to the cell. Fig. 2 shows the relationship between $I_{CP}$ and the detector temperature, $T$. The results obtained varied with the way in which the detector temperature was changed. Increasing $T$ from 50 to 350°C resulted in curve 1, and when T was decreased the next day from 350 to 50°C curve 2 was obtained. The plots show that $I_{CP}$ is strongly temperature dependent.

Fig. 3 shows the relationship between $I_{CP}$ and carrier gas flow-rate, $u$. When $u$ was increased from 20 to 120 cm$^3$/min $I_{CP}$ increased by 30%. With the electric field generated solely by the contact potential difference, we can consider that our detector is working in the d.c. mode. An increase in carrier gas flow-rate can result in slightly higher values of the gas pressure in the detector and hence in a higher value of the current, as is shown in Fig. 3. It is more difficult to interpret the relationship between $I_{CP}$ and the detector temperature, as the picture is more complex. All the physical phenomena that determine the detector current are strongly temperature dependent. The detector temperature affects the ionization, recombination and electron-capture rate constants and also the electron mobility. Also, adsorption/desorption phenomena, which affect the contact potential difference, are sensitive to changes in temperature. All this makes it difficult to predict and interpret the $I_{CP}$ *vs.* detector temperature relationship.

*Standing current and electron concentration*

Fig. 4 shows the relationship between the detector standing current, $I_b$, and the pulse period, $t_p$, measured for three values of $I_{CP}$. The results obtained are in agreement with those of Simon and Wells[6]. A local minimum in the plot is observed if the $I_{CP}$ polarity is opposite to the $I_b$ polarity (curve 3). This phenomenon was found to occur in the coaxial cylinders geometry, as recently reported by Simon and Wells.

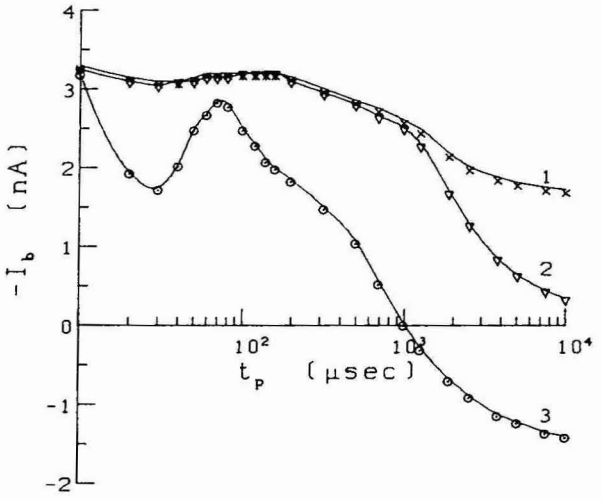


Fig. 4. Detector standing current, $-I_b$, *versus* pulse period, $t_p$, for three values of the battery current, $I_{CP}$: (1) −1.6 nA; (2) 0 nA; (3) +1.6 nA.

They attributed this effect to the pulse period-dependent competition between the electric fields generated by the space charge (positive ions) and by the contact potential. A $^{63}$Ni foil of greater activity was used in our measurements, which resulted in a higher value of the maximum detector standing current, $I_0$. Higher values of the standing current allow a more distinctive presentation of the "local minimum" effect, such as that depicted in Fig. 4.

The detector characteristics are often illustrated by the relationship between the electron concentration, $b$, and the pulse period, $t_p$, as presented in Fig. 5. The plots have different shapes, depending on the $I_{CP}$ value. For $I_{CP} = 0$ (curve 2), this relationship can be accurately described by the Wentworth *et al.*(or similarly Gobby *et al.*) equation for $b$ in the following form:

$$b = k_p R_\beta [1 - \exp(-k_D t_p)]/k_D \quad (1)$$

where $k_p R_\beta$ (mol $l^{-1}$ $s^{-1}$) is the rate of production of ion–electron pairs in the detector and $k_D$ ($s^{-1}$) is the pseudo-recombination rate constant, reflecting the loss of electrons by reactions other than electron capture by the sample molecules.

However, the other plots cannot be described by eqn. 1. The conclusion is that neither $k_p R_\beta$ nor $k_D$ can be determined if the effects of the contact potential are not compensated for. A knowledge of these rate constants is necessary for the evaluation of the electron-capture rate constant for the sample molecules and for optimization of the detector[10].

*Detector response*

The small potential, $E_{bias}$, which is used to compensate for the effects of the contact potential can be varied to cover the desired range of the battery current, $I_{CP}$. To illustrate the correlations between the detector response and the size of the battery current, $I_{CP}/I_0$ ($I_0$ = maximum detector current) rather than $I_{CP}$ was taken as the

independent variable. The ratio of $I_{CP}$ to $I_0$ is not so sensitive to changes in the detector geometry, the activity of the ionization source, temperature and pressure, which similarly affect either of two currents. The variability of $I_{CP}/I_0$ was chosen so as to reflect the real conditions of the detector operation.

The detector response was measured as the difference between the standing currents corresponding to the absence and the presence of a sample, at the maximum of a chromatographic peak. The ratio of two detector responses, $R_{CP}/R_0$, was chosen to reflect the effect of the contact potential difference on the detector response, where $R_{CP}$ is the detector response corresponding to a set value of the battery current, $I_{CP}$, and $R_0$ is the detector response at $I_{CP} = 0$. The correlations between detector response and battery current were measured at three different values of the supply parameter, $k_D t_p$. At $I_{CP} = 0$, the detector response approaches the maximum value at $k_D t_p = 1.7$. In addition to this value, also two others, the preceding ($k_D t_p = 1$) and the following ($k_D t_p = 3$) ones, were used.

Fig. 6 shows the effect of the contact potential on the detector response for the contact potential difference which generates the electric field enhancing that generated by the applied pulse. The relationship between the detector standing current, $I_b$, and battery current, $I_{CP}/I_0$, for three values of $k_D t_p$ is also shown. For $k_D t_p = 1$ and 1.7 the battery current, $I_{CP}$, only slightly affects the detector standing current. At $k_D t_p = 3$, an increase in $I_{CP}/I_0$ from 0 to 50% results in a 50% increase in the standing current. For all three $k_D t_p$ values, the detector response, expressed as the ratio $R_{CP}/R_0$, decreases nearly proportionally to the increase in the battery current, $I_{CP}$. An $E_{bias}$ of about 0.5 V is needed to achieve an $I_{CP}/I_0$ ratio of 50% in this configuration. Such a low value certainly could not influence the collection of the thermal electrons during the pulse, as it constitutes only a small fraction of the pulse amplitude (50 V). However, even such a low potential can significantly alter the

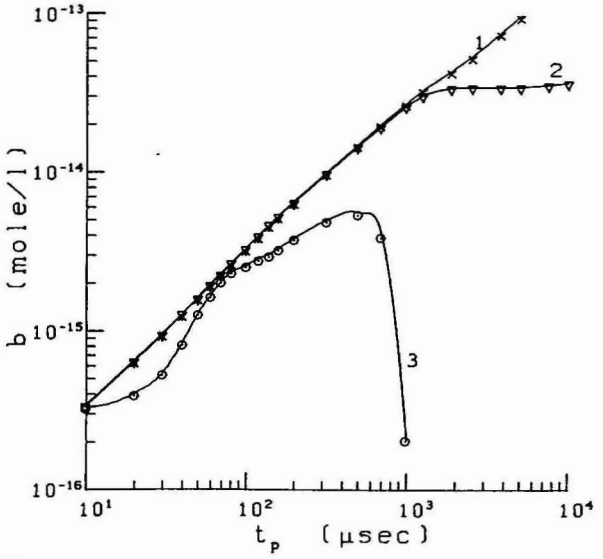


Fig. 5. Electron concentration, $b$, *versus* pulse period, $t_p$, for three values of the battery current, $I_{CP}$, as in Fig. 4.

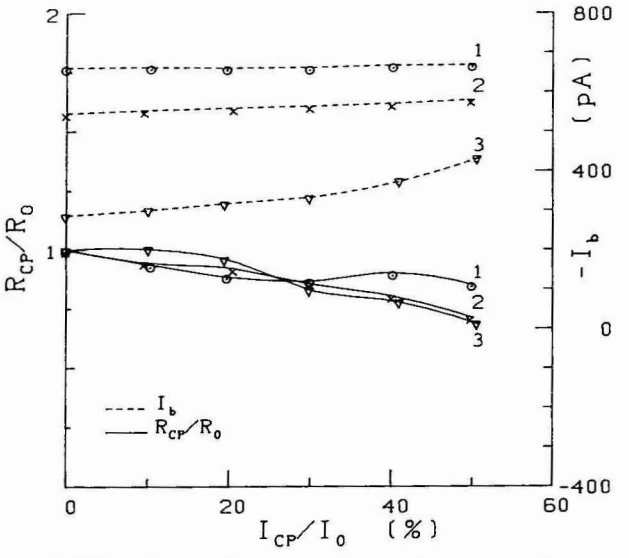


Fig. 6. Effect of contact potential on the detector standing current and the response for the contact potential difference, generating the electric field that enhances that applied to the cell. Supply parameter, $k_D t_p$ = (1) 1, (2) 1.7 and (3) 3.

detector kinetics in the remaining pulse-free portion of the pulse period, especially for higher values of the pulse period. There seem to be at least two reasons for the increase in the detector standing current with an increase in battery current. The first is that even low fields, created by the contact potential difference, will act to prevent the migration of the positive ions to the anode, an effect that undoubtedly leads to an increase in standing current. The second reason is that, for sufficiently long pulse-free periods, the positive ions that are in excess after the pulse can dissipate, driven by a space charge field to such on extent that the field generated by the contact potential difference can overcome the space charge field, resulting in the collection of some fraction of the electrons created by ionization. This effect is also more significant for higher values of $k_D t_p$ and $I_{CP}$ and has a similar influence on the standing current to the previous one. The collection of the thermal electrons in the periods between pulses results in a diminished time-averaged electron concentration in the detector. This, in turn, will be responsible for the smaller than normal (*i.e.*, at $I_{CP} = 0$) extent of the electron-capture reaction with the sample molecules, and hence a decrease in the detector response. The effect is less pronounced at lower values of $k_D t_p$, owing to non-linearity in the correlation between the electron concentration in the detector and the pulse period, $t_p$. For $k_D t_p$ values of 1, 1.7 and 3, the corresponding values of the electron concentration constitute 63, 82 and 95% of the maximum electron concentration, respectively. It is obvious that higher values of $k_D t_p$ create greater possibilities for the space charge dissipation.

The results become considerably more difficult to interpret when the electric field generated by the contact potential opposes that generated by the external pulse. Fig. 7 shows the influence of the contact potential on the detector standing current and on the corresponding response for three values of the supply parameter, $k_D t_p$.

As shown. the range over which the standing current and the response change is much greater than in the previous detector configuration. The detector standing current decreases as the battery current increases. The standing current changes sign, going through zero at a value of the battery current that is inversely proportional to the $k_D t_p$ value. This effect is due to the fact that the detector, in the pulse-free portion of the pulse, is operated in a "reversed field" mode, with the central pin becoming the cathode and the ionization source serving as the anode. We can no longer neglect the influence of the contact potential difference on the amplitude of the pulse, as in this instance the values of the $E_{bias}$ applied to cause some battery current are much higher than in the previous instance. About 35 V is needed to achieve an $I_{CP}/I_0$ of 50%. Such an $E_{bias}$ value becomes comparable to the amplitude of the pulse and can certainly reduce the collection of thermal electrons during the pulse.

In the periods between pulses, the migration of the excess of positive ions is favoured in the central pin direction, thus causing a reduction in the standing current. These two effects, becoming stronger with an increase in the battery current, combine to cause a strong decrease in the standing current following the increase in $I_{CP}/I_0$. At certain values of $I_{CP}/I_0$, the positive component of the standing current arising from the collection of the positive ions at the central pin becomes equal to the negative component arising from the collection of the thermal electrons. With sufficiently long periods between pulses, lower $I_{CP}$ values are required to achieve this balance, as greater densities of the positive ions and longer collection periods make the balance between these two current components easier. If the battery current is increased any further, the positive component of the standing current dominates over the negative component.

Whereas the explanation of the effect of the contact potential on the standing

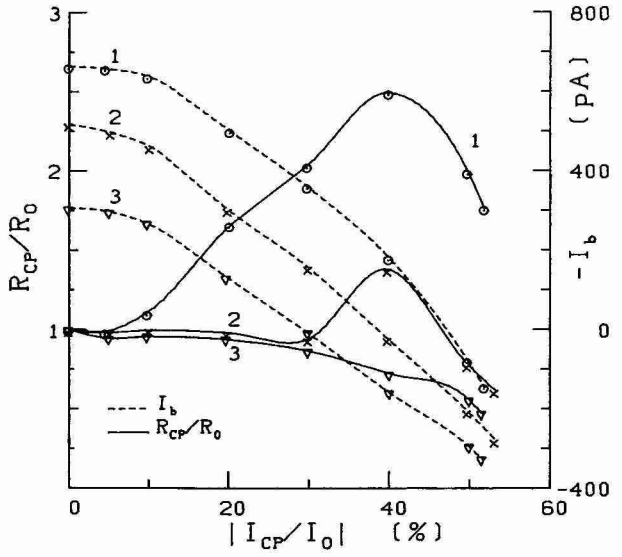


Fig. 7. Effect of contact potentials on the detector standing current and the response for the contact potential difference generating the electric field that opposes that applied to the cell. Supply parameter values as in Fig. 6.

current seems to be obvious, the picture becomes considerably more complicated if the influence of the contact potential difference on the response is considered. As shown in Fig. 7, at $k_D t_p = 1$, the plot of the correlation between detector response ($R_{CP}/R_0$) and battery current ($I_{CP}/I_0$) reaches a maximum. At $k_D t_p = 1$, the detector response at its maximum is 2.5 times greater than the response measured at $I_{CP} = 0$. It should be noted, however, that this value is still lower than the response measured at the optimum value of $k_D t_p$ and at $I_{CP} = 0$. At higher $k_D t_p$ values, the maximum disappears and, at $k_D t_p = 2$, the relationship between response and battery current becomes a monotonically decreasing function. Let us consider why the effect of the contact potential on the detector response changes its character depending on $k_D t_p$. As was said earlier, we can no longer neglect the influence of the battery current on the amplitude of the pulse. As the contact potential difference and, hence, the battery current increase, the net result is a decrease in the pulse amplitude. Therefore, fewer electrons are collected during the pulse leaving more electrons to be captured by the sample molecules, which results in an increased response. The picture changes if the excess of the positive ions can dissipate in the period between pulses to such an extent that some fraction of the thermal electrons can be collected. As a result, the concentration of the electrons and, hence, the response decrease. At higher values of $k_D t_p$, this effect becomes more pronounced, because even at low values of $I_{CP}/I_0$ the space charge can dissipate between pulses and in the remaining portion of this period a substantial collection of electrons takes place, and thus only a decrease in the response is observed.

## CONCLUSIONS

The results indicate that the effects of the contact potential cannot be neglected in the constant-frequency mode of operation of the electron-capture detector. These effects introduce significant changes in the shapes of the detector characteristics such as the relationship between electron concentration and pulse period. As the important detector parameters, such as pseudo-recombination rate constant, ionization rate constant or electron capture rate coefficient, can be evaluated by using the relationship between the concentration of electrons and the pulse period, it is essential that the effects of the contact potential be compensated for.

The contact potential difference affects the detector response in a polarity-dependent manner. Generally, the response decreases with increasing battery current (and contact potential difference). The exception is that, if the battery current polarity is opposite to that of the standing current, the maximum in the relationship between response and battery current is observed at $k_D t_p$ less than 1.7, a phenomenon that can be explained by using the space charge model of Gobby *et al.*[7].

If the effects of the contact potential are compensated for, our electron-capture detector becomes more reliable as its response to a given sample concentration becomes more reproducible, assuming that repeatable injections are made. Such a compensation should also facilitate interlaboratory comparisons.

## REFERENCES

1 L. B. Loeb, *Static Electrification*, Springer Verlag, Berlin, 1958.
2 J. E. Lovelock, *Anal. Chem.*, 35 (1963) 474.
3 E. P. Grimsrud and S. W. Warden, *Anal. Chem.*, 52 (1980) 1842.
4 E. P. Grimsrud and M. J. Connolly, *J. Chromatogr.*, 239 (1982) 397.
5 W. B. Knighton and E. P. Grimsrud, *J. Chromatogr.*, 288 (1984) 237.
6 R. Simon and G. Wells, *J. Chromatogr.*, 302 (1984) 221.
7 P. L. Gobby, E. P. Grimsrud and S. W. Warden, *Anal. Chem.*, 52 (1980) 473.
8 W. E. Wentworth, E. Chen and J. E. Lovelock, *J. Phys. Chem.*, 70 (1966) 445.
9 W. A. Aue and S. Kapila, *J. Chromatogr.*, 188 (1980) 1.
10 P. Rotocki and J. Lasa, *J. Chromatogr.*, 294 (1984) 51.

*Journal of Chromatography*, 349 (1985) 167–172
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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# USE OF CYCLODEXTRINS IN CHROMATOGRAPHY FOR SELECTIVE SEPARATIONS, PRE-CONCENTRATION AND PREPARATION OF DEFINED MIXTURES

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## SUMMARY

Interactions of cyclodextrins or cyclodextrin polymers with substances in the gaseous or liquid phase were studied under stationary and dynamic conditions. The results were utilized for chromatographic purposes, selective separations, pre-concentration and the preparation of well defined gaseous mixtures or solutions with low contents of the test substances. Positional isomers of benzene derivatives were separated by gas–solid chromatography. The inclusion complexes with substances in the gaseous state were obtained under stationary conditions and were used for the storage and preparation of mixtures with low contents of toluene. The formation of the cyclodextrin complexes was followed in the liquid–solid system under dynamic conditions. The gel capacities and the equilibrium constants for the inclusion complexes obtained from the breakthrough curves permit conclusions to be drawn concerning the use of cyclodextrins for selective pre-concentration.

## INTRODUCTION

The inclusion properties of cyclodextrins (CDs), *i.e.*, their ability to interact selectively with substances of various types on the basis of the shape and size of their molecules, provide extensive application possibilities in chromatography[1,2]. Selective interactions have been found in both liquid and gas chromatography. In liquid chromatography, cyclodextrins have been used as stationary phases in the form of gels, cyclodextrin–epichlorohydrin[3–6] or polyurethane[7,8] resins or as a polymer formed by polymerization of cyclodextrin with ethylene glycol–di(epoxypropyl) ether[9,10]. Chemically bonded phases with cyclodextrins have also been described[11–16]. Cyclodextrins have also been used as selective components of mobile phases[1,17–19]. In gas chromatography, the interactions of solid cyclodextrins[20], including O-methylated cyclodextrins[21,22] with gaseous components have been used in gas–solid chromatography, and cyclodextrins have been used as components of stationary phases in gas–liquid chromatography[23,24].

The formation of well defined inclusion complexes makes it possible to use cyclodextrins not only in selective separations of gaseous substances, but also in the storage of substances and the preparation of gaseous mixtures or solutions containing

trace amounts of a given component. Cyclodextrin polymers permit the selective concentration of substances from solutions, followed by their chromatographic determination. In continuation of our previous research, these application possibilities were studied.

## EXPERIMENTAL

α- or β-CD (ICN Pharmaceuticals, NY, U.S.A.) was deposited on the inert support, Chromosorb W (60–80 mesh), from a dimethylformamide solution. The weight ratio was 3 and 1.5 times that for the complete coverage for α- and β-CD, respectively. The solvent was evaporated at 110°C and a pressure of 13.3 kPa in a vacuum evaporator, and the preparation was dried under identical conditions for at least 10 h. The coverage was determined gravimetrically, after dissolution of a known weight of CD. The α- or β-CD polymer (CDP), prepared by cross-linking of CD with ethylene glycol–di(epoxypropyl) ether in a poly(vinylacetate) medium, was kindly provided by Professor Szejtli (Chinoin, Budapest). The α-CDP contained 45% α-CD, with a grain size of 0.09–0.123 mm and a swollen capacity of 5.0 ml/g of sorbent. The content of β-CD in β-CDP was 49%, the grain size 0.09–0.125 mm and the swollen capacity 45 ml/g. The swollen gels were packed in 50 × 4 mm columns.

The chromatographic measurements were carried out on a CHROM-5 instrument (Laboratorní Přístroje, Prague, Czechoslovakia), equipped with a flame-ionization detector and a TZ 4221 recorder, or on a Packard 428 chromatograph (Packard-Becker, Delft, The Netherlands).

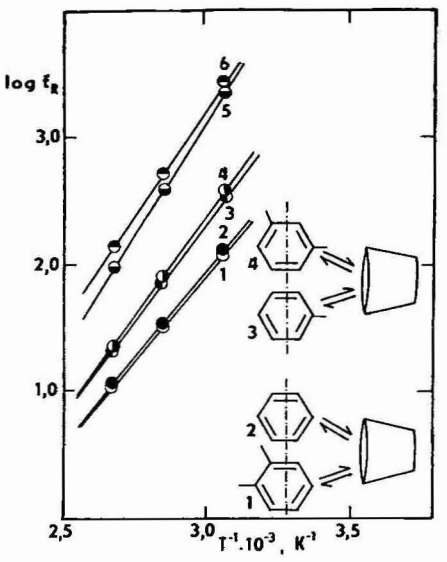
## RESULTS AND DISCUSSION

### *Separation of positional isomers*

It has been demonstrated that the selectivity of the separation process based on inclusion can also be used when a solid cyclodextrin interacts with a gaseous substance[25]. This fact has been used for the separation of positional isomers, especially in the benzene series.

The dimensions of the benzene molecule are close to those of the α-CD cavity. Therefore, substituents in various positions on the benzene ring strongly affect this interaction. The character of the interaction is demonstrated in Fig. 1, depicting the temperature dependence of the logarithms of the retention times of benzene, toluene, ethylbenzene and *o*-, *m*- and *p*-xylene; the orientation of the molecules during interaction is also indicated. A comparison of these dependences also indicates a common character of the interactions of benzene and *o*-xylene, toluene and *m*-xylene and ethylbenzene and *p*-xylene, which can be explained by the same orientation of the molecules during the interaction, with only part of the molecule penetrating into the cavity. As follows from the log $t'_R$ values, the stability of the intermediate complexes increases in the series benzene, *o*-xylene < toluene, *m*-xylene < ethylbenzene, *p*-xylene.

A practical consequence of this specific interaction is the separation of *o*-, *m*- and *p*-xylene (see Fig. 2) at 100°C on a stationary phase containing 3.5% of α-CD deposited on Chromosorb W.



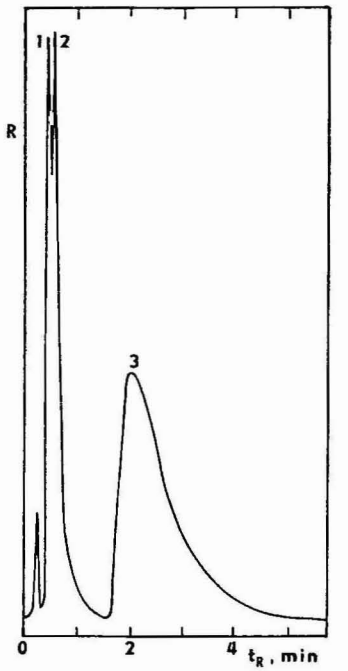




Fig. 1. Temperature dependence of the reduced retention times of aromatics and graphical representation of a possible configuration in the inclusion interaction with the α-CD cavity. 1, *o*-Xylene; 2, benzene; 3, toluene; 4, *m*-xylene; 5, ethylbenzene; 6, *p*-xylene.

Fig. 2. Separation of the xylene isomers on an α-CD phase. Column temperature, 100°C; flow-rate ($N_2$), 22 ml/min. 1, *o*-Xylene; 2, *m*-xylene; 3, *p*-xylene.

*Use of complexes formed by inclusion of substances from the gaseous phase*

By exposure of sorbents coated with α- or β-CD to media with a known pressure of the vapour of a substance (usually the saturated vapour at a given temperature), sufficiently stable complexes can be reproducibly prepared that dissociate in aqueous solution or at an elevated temperature and release the original component, the guest. The time dependence of the stability of these inclusion complexes is shown in Fig. 3 for α- and β-CD with toluene. When the complex was stored in a stoppered bottle at 4°C, the stability remained virtually unchanged for over 1 month. The same complex was less stable at 25°C, and the toluene content decreased by *ca.* 15% during the same period. On transferring the complex to an aqueous solution the toluene content decreased to about 25% of the initial value in 1 month.

It follows that inclusion from the gaseous phase permits the simple preparation of inclusion complexes that can be stored for long periods without appreciable decomposition. These complexes can be utilized in many ways. For example, standard mixtures of substances can be prepared for trace gas chromatographic analysis. In view of their low equilibrium constants, complexes with low contents of test components can be obtained that yield aqueous solutions with a known, trace content of a substance that is normally sparingly soluble in water. For example, saturation of a sorbent with 3.5% of α-CD by toluene vapour at 25°C permits the preparation of

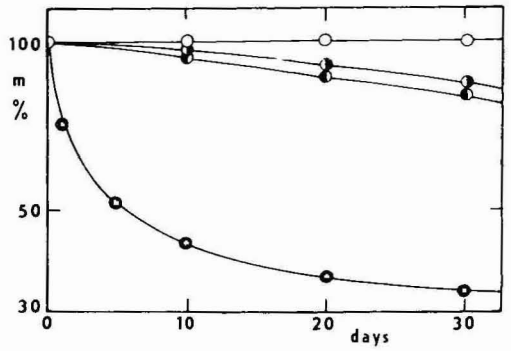



Fig. 3. Time dependence of the stability of the inclusion complexes of α- and β-CD with toluene. $m$ = Relative toluene content in the complex: ○, β-CD–toluene stored at 4°C; ◑, the same complex stored at 25°C; ◐, α-CD–toluene at 25°C; ●, β-CD–toluene dissolved in water at 25°C.

a phase that, on dissolution of 100 mg in 100 ml of water, yields a solution with a toluene concentration of 15 $\mu g\ l^{-1}$, with a 5% relative precision.

Another possibility is the use of thermal decomposition of the inclusion complex. A solid phase is then injected into the injection port. Temperatures of 120–150°C are required to release the volatile component quantitatively. The complexes formed by inclusion from the gaseous phase can thus be used to advantage for calibration of the detection devices at very low concentrations. The relative standard deviation of these methods of injection is 5–10%. A drawback is the possible thermal degradation of the cyclodextrin itself at higher temperatures, as the degradation products may affect the chromatographic packing. The new calibration method can be used in the chromatographic determination of trace pollutants in the atmosphere.

*Use of complexes formed by inclusion from solutions*

The inclusion properties of CDPs were studied by using selected phenolic compounds as models. The measurements were carried out dynamically with a CDP

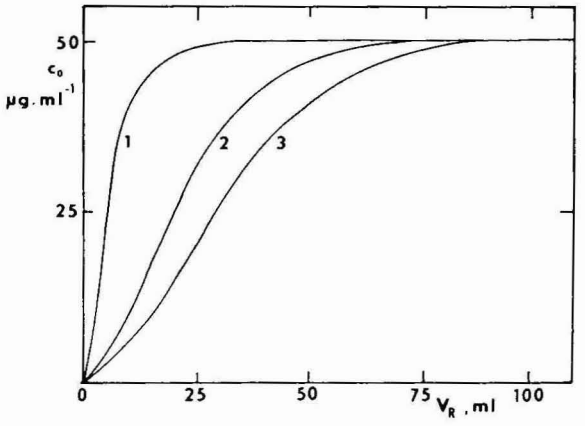



Fig. 4. Dependence of the outlet concentration of nitrophenols on the elution volume for α-CDP using a 50 × 4 mm column, pH = 7.00, $I$ = 0.05. 1, *o*-Nitrophenol; 2, *m*-nitrophenol; 3, *p*-nitrophenol.

TABLE I

CAPACITIES ($Q'$) AND EQUILIBRIUM CONSTANTS ($K_{eq}$) FOR α-CDP and β-CDP GELS

$K_{eq}$ is defined as the dissociation constant of the inclusion complex (see ref. 25)

| *Guest* | *α-CDP* | | *β-CDP* | |
|---|---|---|---|---|
| | $Q'$ ($mg\ g^{-1}$) | $K_{eq}$ ($mol^{-1}$) | $Q'$ ($mg\ g^{-1}$) | $K_{eq}$ ($mol^{-1}$) |
| Phenol | – | – | 1.49 | 34.4 |
| *o*-Cresol | 1.66 | 30.1 | 2.04 | 47.3 |
| *m*-Cresol | 2.25 | 41.6 | 2.06 | 47.8 |
| *p*-Cresol | 2.78 | 50.5 | 2.88 | 67.4 |
| *o*-Nitrophenol | 1.81 | 35.7 | 2.53 (1.06)** | 58.6 (48.7)** |
| *m*-Nitrophenol | 6.43 (6.53)* | 131 (133)* | 4.97 )2.08)** | 118 (96.2)** |
| *p*-Nitrophenol | 8.85 (9.50)* | 184 (198)* | 6.74 (2.82)** | 162 (131)** |
| α-Naphthol | 6.75 | 138 | 20.3 | 549 |
| *p*-Chlorophenol | 16.1 | 357 | 10.4 | 260 |
| 3,4-Xylenol | – | – | 5.08 | 121 |

* For pH 3.5.

** For $c_0 = 25\ mg\ l^{-1}$.

column and a mobile phase containing a potential guest as the saturating component. Fig. 4 shows, as an example, the outlet concentrations of *o*-, *m*- and *p*-nitrophenol as a function of the elution volume. From the experimental breakthrough curves the capacities of α- and β-CDP and the equilibrium constants of the complexes formed were found and are given in Table I for phenol and some of its derivatives. It can be seen that the α-CDP capacity increases for phenols substituted in the *para*-position; with β-CDP considerable increase in the capacity for 1-naphthol is observed.

These findings can be used for the selective trapping of some trace substances in waters[26]. For these purposes, a simple apparatus was used, where the flow-rate through a CDP column was controlled by the gas overpressure at the sample surface. The inclusion complexes formed were decomposed by an increase in temperature (*ca.* 170°C) and analysed gas chromatographically, or they were transferred in solution and determined spectrophotometrically. When the trapping efficiencies were determined for model solutions of trace substances, it was found that β-CDP had an 89% efficiency for 1-naphthol and only 34% for phenol. It follows from the literature on ion-exchanger sorbents[27,28], *e.g.*, XAD-2 (1-naphthol, 91%; phenol, 40%), that the efficiency of cyclodextrin polymers is comparable to that of ion exchangers. Moreover, both CDPs exhibit selective properties that are useful, especially in analyses of multi-component pollutant mixtures in water. The use of β-CDP may be of practical significance for the selective trapping of voluminous molecules, *e.g.*, some pesticides and polyaromatics.

## CONCLUSION

Inclusion, as a specific phenomenon in the interaction of a host in the gaseous or liquid phase with a solid or dissolved cyclodextrin, offers many possibilities for application in gas and liquid chromatography:

(1) gas chromatography with a solid phase containing cyclodextrin permits some specific separations, especially of positional isomers;

(2) inclusion complexes of cyclodextrins with a known stoichiometry can be used in gas chromatography for the preparation of defined gaseous mixtures or aqueous solutions of trace substances;

(3) polymeric cyclodextrins can be used for the trapping of certain trace substances in waters.

## REFERENCES

1 W. L. Hinze, *Sep. Purif. Methods*, 10 (1981) 159.
2 E. Smolková-Keulemansová, *J. Chromatogr.*, 251 (1982) 17.
3 J. Solms and R. H. Egli, *Helv. Chim. Acta*, 48 (1965) 1225.
4 Societe des Produits Nestlé, *Neth. Pat. Appl.*, 6 505 361, 1964.
5 N. Wiedenhof, *Stärke*, 21 (1969) 163.
6 J. L. Hoffman, *Anal. Biochem.*, 33 (1970) 299.
7 Y. Mizobuchi, M. Tanaka and T. Shono, *J. Chromatogr.*, 194 (1980) 153.
8 Y. Mizobuchi, M. Tanaka and T. Shono, *J. Chromatogr.*, 208 (1981) 35.
9 B. Zsadon, M. Szilasi, F. Tödös, K. H. Otta, É. Fenyvesi and J. Szejtli, *Acta Chim. Acad. Sci. Hung.*, 100 (1979) 265.
10 B. Zsadon, M. Szilasi, F. Tödös, É. Fenyvesi and J. Szejtle, *Stärke*, 31 (1979) 11.
11 M. Tanaka, Y. Mizobuchi, T. Sonoda and T. Shono, *Anal. Lett.*, 14 (1981) 281.
12 M. Tanaka, Y. Kawaguchi, M. Nakae, Y. Mizobuchi and T. Shono, *J. Chromatogr.*, 299 (1984) 341.
13 M. Tanaka, Y. Kawaguchi, T. Niinae and T. Shono, *J. Chromatogr.*, 314 (1984) 193.
14 M. Tanaka, Y. Kawaguchi, T. Shono, M. Uebori and Y. Kuge, *J. Chromatogr.*, 301 (1984) 345.
15 D. W. Armstrong, W. DeMond, A. Alak, W. L. Hinze, T. E. Riehl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234.
16 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 237.
17 D. Sybilska, J. Lipkowski and J. Wójcikowski, *J. Chromatogr.*, 253 (1982) 95.
18 T. Sakai, Y. Niinuma, S. Yanagihara and K. Ushio, *J. Chromatogr.*, 276 (1983) 182.
19 Y. Nobuhara, S. Hirano and Y. Nakanishi, *J. Chromatogr.*, 258 (1983) 276.
20 E. Smolková-Keulemansová, H. Králová, S. Krýsl and L. Feltl, *J. Chromatogr.*, 241 (1982) 3.
21 J. Mráz, L. Feltl and E. Smolková-Keulemansová, *J. Chromatogr.*, 286 (1984) 17.
22 M. Tanaka, S. Kawano and T. Shono, *Fresenius' Z. Anal. Chem.*, 316 (1983) 54.
23 D. Sybilska and T. Kościelski, *J. Chromatogr.*, 261 (1983) 357.
24 T. Kościelski, D. Sybilska, L. Feltl and E. Smolková-Keulemansová, *J. Chromatogr.*, 286 (1984) 23.
25 S. Krýsl, *Ph.D. Thesis*, Charles University, Prague, 1984.
26 S. Krýsl, *Method of Trapping of Volatile Substances, Czech. Pat. Appl.*, PV 05254-84, 1984.
27 R. C. Chang and I. S. Fritz, *Talanta*, 25 (1978) 659.
28 G. A. Junk, J. J. Richard, M. D. Grieser, D. Witiak, M. D. Arguello, R. Vick, H. I. Svec, J. Fritz and G. V. Calder, *J. Chromatogr.*, 99 (1974) 745.

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CHROMSYMP. 642

# CATALYTIC HYDROGENATION AND EXCHANGE REACTIONS ON GAS CHROMATOGRAPHIC COLUMNS, IN THE PRESENCE OF SUPPORTED $(\eta^5\text{-}C_5H_5)NiOs_3(\mu\text{-}H)_3(CO)_9$

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## SUMMARY

The heterometallic cluster $(\eta^5\text{-}C_5H_5)NiOs_3(\mu\text{-}H)_3(CO)_9$ is decomposed under $H_2$ to metal particles on the inert support of a gas chromatographic column. The column is used as a catalytic reactor to hydrogenate C–C and C–O multiple bonds and to study the H-for-Cl substitution reactions on chlorinated hydrocarbons. The performance of such a column and its possible use are briefly discussed.

## INTRODUCTION

Attention has recently been given to the heterometallic clusters as potential homogeneous catalysts[1–3] or as precursors of stoichiometrically defined heterogeneous catalysts[1–4]. Indeed, the presence of different metals is thought to allow a more efficient and controlled activation of small substrate molecules[5,6].

The catalytic hydrogenation reactions have extensively been studied. Some have been carried out in the presence of clusters both under homogeneous[7] and in heterogeneous conditions[8]. Moreover, the study of hydrogen–deuterium exchange processes has allowed some conclusions about the occurrence of "true cluster catalysis" under homogeneous conditions[9]. The deuteration of some intermediates or products has also permitted some hypotheses on the reaction mechanisms[10].

On the other hand, the use of on-column hydrogen–deuterium or hydrogen–tritium exchange reactions for labelling purposes and for radioactivity determination by gas chromatography (GC) of compounds containing labile tritium is well established[11–14]. These reactions show that a GC column may be used not only as a powerful separation and analysis tool, but also as a versatile "chemical reactor", offering remarkable possibilities both in catalytic hydrogenation processes and in selective exchange reactions aimed at obtaining labelled "target molecules". There are many analogies in the processes (*e.g.*, substrate–support interactions and chemisorption, hydrogen chemisorption and activation, etc.) occurring within a GC column and in an heterogeneous stationary-bed reactor.

We have already reported that the heterometallic cluster $(\eta^5\text{-}C_5H_5)NiOs_3(\mu\text{-}H)_3(CO)_9$ (refs. 15–17) (complex 1) is active and selective in the hydrogenation

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and isomerization of acetylenes and ethylenes[15] and of dienes under homogeneous conditions, where the complex behaves as a "cluster catalyst"[10]. When supported and thermally treated on $\gamma$-$Al_2O_3$, complex 1 is active in the hydrogenation of acetylene and benzene, and in the methanation of CO and $CO_2$ under atmospheric pressure of dihydrogen[18,19]. It also behaves as an hydrogenation catalyst for dienes when supported by GC materials, packed into a GC column and used in a GC apparatus under a stream of dihydrogen[20]; depending on the "activation" procedure and upon the "ageing" of the supported system, results typical of homogeneous or of heterogeneous catalysis are obtained.

We have extended our investigations into the behaviour of complex 1 when supported on a GC column to include the reactivity of a variety of unsaturated small molecules (acetylenes, ethylenes, aromatic hydrocarbons, organic molecules with functional groups, carbon oxides) in the presence of dihydrogen. We have also attempted on-column exchange reactions on saturated and unsaturated substrates, aimed at obtaining specific (labelled or not) "target molecules".

The results of these experiments are discussed and compared with those of the hydrogenation reactions under homogeneous conditions or on $\gamma$-$Al_2O_3$, in the presence of complex 1.

## EXPERIMENTAL

### *Materials*

The solvents used were carefully dehydrated over sodium. Ultrapure $N_2$ or $H_2$ (SIAD) were generally used as carrier gases; in some experiments, ultrapure acetylene, carbon monoxide and mixtures of the latter with hydrogen or helium were used (SIAD). The substrate molecules tested were commercial products (Fluka, Merck) and were checked for impurities before use both by GC and by $^1H$ NMR spectroscopy. For the deuteration and tritiation experiments, $^2H_2$ and $^3H_2O$ (Farmitalia-Carlo Erba, and Amersham) were used, respectively.

### *Analysis of the reactants and products*

A Carlo Erba 4200 gas chromatograph equipped with a flame ionization detector was used in order to check the purity of the compounds to be hydrogenated; these tests were also helpful for determining the retention times of both the starting materials and the products. A JEOL JNM GX 270 Fourier transform $^1H$ NMR spectrometer was also used for the above purposes. The deuterated derivatives were identified by means of a single-focusing Hitachi–Perkin-Elmer RMU 6H instrument [operated in electron impact (EI) mode at 70 eV] or by means of a Kratos MS-50 apparatus, coupled with a Carbo Erba 4200 FID gas chromatograph.

### *Catalytic experiments in the gas chromatograph*

The apparatus consisted of two coupled gas chromatographs. A Carlo Erba Fractovap Model B was used as both injection system and "chemical reactor". It was couipled by means of a thermostatted junction to the Carlo Erba 4200 flame ionization and hot-wire detectors, acting as both flow regulator and analytical unit. The gas flowing through the system was the carrier and the reagent at the same time.

The catalytic column (1 m × 6 mm I.D.) was filled with 12 g of silanized

Chromosorb P (Johns Manville), 60–80 mesh, which had been previously wetted with a light petroleum (b.p. 40–70°C) solution containing 35 mg of complex 1 and then dried in a Rotavap evaporator under reduced pressure at room temperature. The column was then heated at 155°C for 20 h in a stream of dihydrogen (25 ml/min), while carefully controlling the possible sublimation of part of the cluster. The combined effect of heat and $H_2$ resulted in decomposition of the cluster to metal particles (of predictable stoichiometry, see ref. 19), so that a "pure heterogeneous" catalyst was obtained by this procedure. The total modification of complex 1 was confirmed by extracting the material with chloroform. No soluble compounds could be detected.

No catalytic effect was observed on pure Chromosorb P at temperatures up to 230°C, the maximum operating temperature for the catalytic system.

The retention times for the identification of the hydrogenated products were obtained by injecting real samples of the hydrogenated substances into the catalytic column. Retention times on the catalytic column were of the same order of magnitude as the dead-time of the column: about 70 s.

A schematic diagram of the apparatus is shown in Fig. 1.

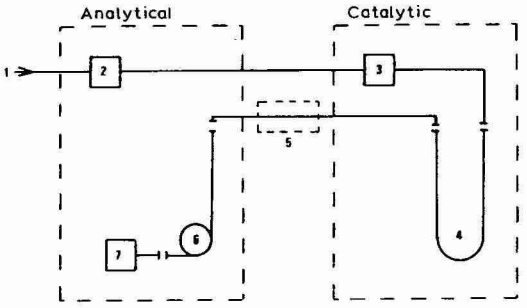


Fig. 1. Diagram of the apparatus used for catalysis experiments: 1 = carrier reactant inlet; 2 = flow controller; 3 = injection port; 4 = catalytic column; 5 = heated connection line; 6 = analytical column; 7 = detector.

For the analysis of saturated and unsaturated hydrocarbons, the analytical instrument was operated with a 2 m × 6 mm I.D., *n*-octane/Porasil C, 80–100 mesh column; carrier gas, $H_2$ (25 ml/min); temperature, 10°C for 6 min, then 5°C/min up to 155°C. In some cases, the temperature program was modified or shortened to allow a better separation of the reaction products or simply to save time.

The detection of diphenylacetylene, phenylacetylene and their hydrogenation products was carried out using a 2 m × 6 mm I.D., SE-30 (5% on Chromosorb W AW, 60–80 mesh) column; carrier gas, $H_2$ (46 ml/min); temperature program, 60°C for 6 min, then 10°C/min up to 240°C.

Acetonitrile, nitrobenzene, benzene, toluene and their derivatives were separated and detected with a 2 m × 6 mm I.D., Carbowax 20M (4% on Chromosorb W AW, 60–80 mesh) column; carrier gas, $H_2$ (100 ml/min); temperature program, 60°C (injection), then 2°C/min up to 190°C.

## RESULTS AND DISCUSSION

### *Hydrogenation experiments*

*Alkynes.* The following alkynes have been tested: acetylene, *tert.*-butylacetylene, phenylacetylene, diphenylacetylene, 1- and 2-pentyne, 1- and 3-hexyne.

At 200°C, acetylene is quantitatively hydrogenated: ethane is the main product, some butane and trace amounts of benzene and cyclohexane also being observed. Blank experiments showed that the acetylene used was free from these compounds. No cracking to methane was observed. Under the same conditions, ethylene is fully hydrogenated to ethane and no methane is observed. In the temperture range of 40–200°C, *tert.*-butylacetylene is hydrogenated to 2,2-dimethylbutane in 100% yields. Once more, no cracking products are observed.

In the temperature range 60–200°C, phenylacetylene is hydrogenated to the extent of about 90%. Ethylbenzene is the main product and, depending on the temperature used, variable yields of a minor product, tentatively identified as ethylcyclohexane, are formed (above 150°C). By contrast, only a 4–5% yield of diphenylethane is observed when diphenylacetylene is processed under the same conditions (and also at 230°C). This is probably due to the fact that the high-boiling alkyne (b.p. 300.2°C) is retained to some degree by the catalytic column.

Hydrogenation of 1- and 2-pentyne and 1- and 3-hexyne to pentane or hexane, respectively, takes place in 98–99% yield; 1–2% of cracking products is observed. Complex 1, under homogeneous conditions[10], is efficient in the hydrogenation of the terminal alkynes and less efficient in that of the internal alkynes; alkenes are formed, and hence the homogeneous system shows greater selectivity.

*Aromatic hydrocarbons.* At 200°C, benzene yields about 10% of cyclohexane; no linear products are observed. Toluene, under comparable conditions, is hydrogenated to the extent of only 1% to methylcyclohexane. Again, no linear products are formed.

*Substrates with functional groups.* In the range 100–200°C, there are indications of the partial reduction of nitrobenzene to aniline. Indeed, when injected into the catalytic column, the compounds can be recovered only in part, but aniline cannot be observed. Injections of water (acting as a weak nucleophile and as a weak acid) to free the adsorbed aniline were unsuccessful.

Acetonitrile behaves like nitrobenzene. However, the ethylamine is retained in the column and water injections are, once again, inadequate to free it.

*Acetone.* Acetone reacts with hydrogen producing mainly propane and isopropanol. The latter can be hydrogenated, on the same column and under the same conditions, to propane. The product distribution as a function of temperature, and the mechanisms of these reactions, are under investigation.

*CO, $CO_2$ and $CS_2$. Reaction of CO with $H_2$ in a stream of helium.* Commercial carbon monoxide (bottles) and carbon monoxide obtained from the thermal decomposition of $Ni(CO)_4$ were tested. At room temperature, no hydrogenation was observed, whereas in the range 160–210°C low yields of methane (a few percent) were found. Carbon dioxide was not appreciably hydrogenated under these conditions, whereas carbon disulphide gave rise to small yields of methane.

The absence of carbon dioxide hydrogenation under the conditions described here may be due to the difficulty in reaching the water gas shift equilibrium during

the short contact times, in a system which is by definition not in equilibrium (see ref. 19 and the discussion below).

We have attempted to obtain water gas shift reaction (WGSR) products at 200°C by using as reactant and carrier gas carbon monoxide–helium (1:9) (50 ml/min). However, when water was injected no dihydrogen could be detected, neither the hot-wire detector nor by a flow ionization chamber (using $^3H_2O$). Interestingly, and somewhat surprisingly, *methane* could be detected when water was injected in the above mixture (25 ml/min).

Thus, at least in the system here described, the formation of methane seems to be due to the reaction of carbon monoxide with water. In view of the industrial importance of this reaction, further studies are in progress.

*Comparison of the hydrogenation results with those obtained in a pulse reactor*

In the pulse reactor described in refs. 18 and 19, containing complex 1 supported on $\gamma$-alumina (activated generally at 250°C for 4 h in a stream of dihydrogen) and operated in the temperature range 52–240°C, acetylene is hydrogenated to ethane (about 85%) and to ethylene (about 12%); some methane is also observed (2–3%); the conversion for each cycle is 0.5–0.7 mol of substrate per mol of active metals[19]. Benzene is hydrogenated to cyclohexane (98%) and some linear hexane (0.8%) with 2.0% conversion for each cycle.

Finally carbon monoxide and dioxide are hydrogenated (0.2–0.3% conversion) to 100% methane and (0.09–0.3% conversion) to 90% methane and 10% carbon monoxide, respectively[19]. Thus, complex 1 on $\gamma$-alumina is apparently more efficient than when supported on Chromosorb. However, in the present system, neither cracking nor linear products are observed from the hydrogenation of acetylene and of the aromatic hydrocarbons.

There is strong evidence that the (very efficient) methanation of carbon dioxide on the complex $1/\gamma$-$Al_2O_3$ catalyst occurs in two steps, *i.e.*, the reaction at high temperatures of $CO_2$ with $H_2$ to give CO and $H_2O$ (WGSR)[9], followed by the hydrogenation of CO to methane. However, on the catalytic column described here methane is obtained upon reaction of CO with *water*, and this fact is not contrary to the findings on the system complex $1/\gamma$-$Al_2O_3$.

*On-column exchange reactions*

*Hydrogen–chlorine exchange.* These reactions occur for some saturated substrates, with varying degrees of difficulty, depending probably upon the nature (and reactivity) of the molecules. Thus, methane is obtained from dichloromethane (30%), chloroform (12%), carbon tetrachloride (4%) and methyl chloride (0.8%), at 230°C in a stream of dihydrogen. No silane, but only hydrogen chloride was observed in the hydrogenation of tetrachlorosilane, probably because, as in the case of amines, the support material in the catalytic column is not inert enough for such reactive molecules.

The hydrogenation of $C_2{}^2H_4$ and the possible hydrogen–deuterium exchange on this substrate have also been studied. At 200°C, the reaction with $H_2$ is complete, but only 28% of the hydrogenation products consists of $C_2{}^2H_4H_2$; besides, a H-for-$^2H$ exchange reaction occurs, and the derivatives $C_2{}^2H_3H_3$ (26%) and $C_2{}^2HH_5$ (46%) are formed (GC–mass spectrometric analyses), thus indicating that, at least

at relatively high temperatures, exchange processes also take place with considerable efficiency.

The catalytic system described here, based on a GC column used as chemical reactor, shows some advantage when compared with the "classical" homogeneous and heterogeneous systems: (i) simple construction and relatively "mild" operating conditions (low $H_2$ pressures and reaction temperatures generally lower than those used in the heterogeneous pulse reactions[19], with yields relatively high when considering the short contact times on the column), and (ii) good versatility coupled with the possibility of selectively collecting the (known) hydrogenation products.

The use of two gas chromatographs is obviously not necessary. Any thermostatic system coupled with a reliable injection device may be substituted for the first instrument. Coupling of both catalytic and analytical columns in the same instrument is not recommended, because the temperature requirements for the two columns may be different. Moreover, previous experiments had shown that, at least when complex 1 is not completely decomposed, partial sublimation into the analytical column occurs, with loss of efficiency[20].

The versatility of the system is evidenced by the great variety of operating conditions and of substrates one can work with in a limited period of time. However, there are some technical difficulties with high-boiling compounds and/or with molecules having functional groups. Some of these are probably due to the lack of complete inertness of the support. They could be overcome by choosing a material different from silanized Chromosorb P and/or by shortening the contact time through the use of a shorter catalytic column. A large increase in the flow-rate of $H_2$ might be useful in principle, but would cause detection and identification problems.

The method proposed is comparable (with regard to its efficiency) to the homogeneous[10] and heterogeneous[19] systems described elsewhere and based on complex 1, at least if we consider the hydrogenation of acetylenes, dienes and benzene. However, as expected for a heterogeneous catalysis, low selectivity is observed.

Lower efficiency in the methanation of carbon monoxide and dioxide than is observed with complex $1/\gamma\text{-}Al_2O_3$ in a pulse reactor is observed. This fact is probably due to the short contact times allowed by the flow-rate of the carrier and reactant gas required for a good separation of the products.

A final advantage of the chromatographic system is the possibility of coupling the catalytic column with a preparative gas chromatograph: this would permit one to collect selectively the "labelled" molecules and to separate them from other products. This would be of particular relevance for labelled molecules of pharmacological or of biological interest.

The potential applications of a method allowing the catalytic hydrogenation, identification and selective collection of the products in the same apparatus are obvious and are the subject of active research in our laboratories.

ACKNOWLEDGEMENTS

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## REFERENCES


1 W. L. Gladfelter and G. L. Geoffroy, *Adv. Organomet. Chem.*, 18 (1980) 207.
2 G. Henrici-Olivé and S. Olivé, *Coordination and Catalysis*, Monographs in Modern Chemistry 9, Verlag Chemie, New York, 1977.
3 G. Henrici-Olivé, *The Chemistry of the Catalyzed Hydrogenation of Carbon Monoxide*, Springer, Berlin, 1984.
4 B. F. G. Johnson (Editor), *Transition Metal Clusters*, Wiley, New York, 1980.
5 E. L. Muetterties, *Bull. Soc. Chim. Belg.*, 85 (1976) 451.
6 E. L. Muetterties, T. N. Rhodin, E. Band, C. F. Brucker and W. R. Pretzer, *Chem. Rev.*, 79 (1979) 91.
7 A. P. Humphries and H. D. Kaesz, *Prog. Inorg. Chem.*, 25 (1979) 146 and references therein.
8 R. Whyman, in B. F. G. Johnson (Editor), *Transition Metal Clusters*, Wiley, New York, 1980, p. 545.
9 R. M. Laine, *J. Mol. Catal.*, 14 (1982) 137; and references cited therein.
10 M. Castiglioni, R. Giordano, E. Sappa, A. Tiripicchio and M. Tiripicchio Camellini, *J. Chem. Soc., Dalton Trans.*, in press; and references cited therein.
11 M. Senn, W. J. Richter and A. L. Burlingame, *J. Am. Chem. Soc.*, 87 (1965) 680.
12 J. Tadmor, *J. Inorg. Nucl. Chem.*, 23 (1961) 158.
13 P. Volpe and M. Castiglioni, *J. Chromatogr.*, 114 (1975) 23.
14 M. Castiglioni and P. Volpe, *Radiochim. Acta*, 34 (1983) 167.
15 M. Castiglioni, E. Sappa, M. Valle, M. Lanfranchi and A. Tiripicchio, *J. Organomet. Chem.*, 241 (1983) 99.
16 M. R. Churchill and C. Bueno, *Inorg. Chem.*, 22 (1983) 1510.
17 G. Lavigne, F. Papageorgiou, C. Bergounhou and J. J. Bonnet, *Inorg. Chem.*, 22 (1983) 2485.
18 P. Moggi, G. Albanesi, G. Predieri and E. Sappa, *J. Organomet. Chem.*, 252 (1983) C89.
19 G. Albanesi, P. Moggi, G. Predieri and E. Sappa, *Appl. Catal.*, submitted for publication.
20 M. Castiglioni, R. Giordano, E. Sappa, G. Predieri and A. Tiripicchio, *J. Organomet. Chem.*, 270 (1984) C7.

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CHROMSYMP. 681

# PRE-CONCENTRATION OF ORGANIC POLLUTANTS

# POTENTIAL INTERFERENCE FROM THE USE OF STYRENE COPOLYMER ADSORBENTS

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## SUMMARY

The characterization of volatile products released on heating from Porapak Q, Chromosorb 101, 102, and 103 is reported. The structures of these compounds are verified by gas chromatography–mass spectrometry.

The polymers were also extracted with dichloromethane. Thermal and extraction clean-up methods are compared, with the aim of establishing the best conditions for minimizing interference in air-sampling schemes.

## INTRODUCTION

In previous papers[1,2] we have pointed out that great care should be used in the choice of the conditioning temperature when styrene copolymers are employed as air samplers. The temperature limit is particularly important if reproducible retention data are to be obtained and if the breakthrough is to be determined accurately. Moreover, thermal treatment at the temperature limit, for at least 3 h, ensures good cleaning of the polymeric surface, reducing the possibility of interference during the thermal desorption process. The temperature limit is the manufacturers' recommended maximum temperature at which the polymers can be heated without decomposition.

In this study, the characterization of volatile products released at the temperature limits from Porapak Q, Chromosorb 101, 102, and 103 is reported. The structures of the organics are verified by gas chromatography–mass spectrometry (GC–MS). The origin of these materials is investigated, with reference to the chromatographic analysis of polymer sample extracts. Thermal and extraction procedures are compared with the aim of establishing the best conditions for minimizing interference in air-sampling schemes.

## EXPERIMENTAL

Chromosorb 101, 102 (60–80 mesh), Chromosorb 103, and Porapak Q (80–100 mesh) were supplied by Supelchem (Milan, Italy). The solvents used were of the highest commercially available grade.

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A 1.5-g sample of each polymer was heated at the temperature limit for 6 h in a stainless-steel apparatus under ultra-pure nitrogen, flowing at 100 ml/min. The released products were trapped at liquid nitrogen temperature and dissolved in dichloromethane (2 ml). The same amounts of each polymer were extracted for 48-h periods in a Soxhlet apparatus with dichloromethane (40 ml). Solvent volumes were reduced to 2 ml. Both heating and extraction samples were submitted for GC and GC–MS analysis. GC analysis was performed with an OV-1 fused-silica capillary columns (25 m × 0.32 mm I.D.) installed in a Carlo Erba HRGC 5160 Mega Series, equipped with a flame ionization detector. Hydrogen was used as carrier gas, and nitrogen as make-up gas. Samples of 0.5 μl of dichloromethane solutions were injected. All GC–MS analyses were performed on a Finnigan 4500 mass spectrometer. Chromatographic separations were accomplished using an OV-101 3% on Supelcoport (80–100 mesh) glass column (6 ft. × 2 mm I.D.). The temperature conditions were: isothermal 60°C for 6 min, 60–80°C at 8°C/min, 80–140°C at 4°C/min, 140–270°C at 8°C/min. The flow-rate of the helium carrier gas was 20 ml/min and the inlet temperature was 250°C. Sample of 0.5–1 μl were injected. Mass spectrometer operating parameters were the following: scan time, 1.95 s; electron energy, 70 eV; multiplier voltage, 1500 kV; inlet temperature, 250°C. Total ion chromatograms were collected, and individual components spectra were compared with computerized library mass spectra to provide qualitative identifications.

## RESULTS AND DISCUSSION

Several different batches of each polymer were investigated in order to obtain representative data. The polymers studied and their physical properties are listed in Table I[3,4].

GC–MS measurements showed that, on heating, the polymers release a variety of materials. Fig. 1 illustrates, as an example, a reconstructed total ion chromatogram of the volatile products evolved from Porapak Q. As confirmed by MS, typical contaminants in this polymer are alkyl derivatives of benzene and styrene.

A more complete listing of the identified compounds for all the polymers is reported in Table II. Contaminants vary according to polymer type, although there is a similarity between Chromosorb 101, 102, and 103. Porapak Q showed a predominance of ethylethenylbenzene isomers and dimethylpropenylbenzene. Chromosorb 102 displayed a predominance of ethenylbenzene and ethylethenylbenzene

TABLE I

PHYSICAL PROPERTIES OF ADSORBENTS

EVB = ethylvinylbenzene; DVB = divinylbenzene; STY = styrene.

| *Polymer* | *Composition* | *Surface area ($m^2/g$)* | *Temperature limit (°C)* |
|---|---|---|---|
| Porapak Q | EVB–DVB | 500–600 | 250 |
| Chromosorb 101 | STY–DVB | < 50 | 275 |
| Chromosorb 102 | STY–DVB | 300–400 | 250 |
| Chromosorb 103 | Cross-linked polystyrene | 15–25 | 275 |

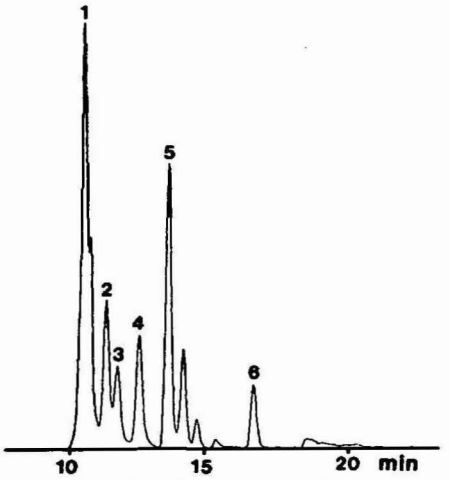


Fig. 1. Total ion chromatogram of volatile products evolved from Porapak Q. For GC–MS conditions see Experimental. Peaks: 1 = 1,3- and 1,4-ethylstyrene isomers; 2 = 1,3-divinylbenzene; 3 = 1,4-divinylbenzene; 4 = 3-methylindene; 5 = 1,1-dimethyl-2-propenylbenzene; 6 = 1- or 2-methylnaphthalene.

isomers. Chromosorb 101 and 103 presented chromatograms with more peaks, *i.e.* a higher contaminant level. It must be remembered that the temperature limit of such polymers is greater than that of other polymers. However, Chromosorb 103 showed a predominance of ethenylbenzene, ethylethenylbenzene and dimethylethenylbenzene isomers, whereas Chromosorb 101 evidenced a predominance of ethenylbenzene, ethylethenylbenzene isomers, and ethenylbenzene dimer. It is interesting to note that this polymer also evolved two higher-boiling compounds, *i.e.* dibutylphthalate and dicyclohexylphthalate esters.

TABLE II

CHEMICAL CHARACTERIZATION OF VOLATILE PRODUCTS RELEASED FROM POLYMERS ON HEATING

PQ = Porapak Q; CH101 = Chromosorb 101; CH102 = Chromosorb 102; CH103 = Chromosorb 103.

| *Compound* | *PQ* | *CH101* | *CH102* | *CH103* |
|---|---|---|---|---|
| 1,3-Diethylbenzene (or 1,2- or 1,4-) | | | ★ | ▲ |
| Triethylbenzene (1,3,5- or 1,2,4-) | | ■ | | ▲ |
| Ethenylbenzene | | ■ | ★ | ▲ |
| 1-Methylethenylbenzene | | ■ | | |
| Ethenylethylbenzene (1,3- and 1,4-isomers) | ● | ■ | ★ | ▲ |
| Dimethylethenylbenzene (1,2,4- or 1,3,4-) | | | ★ | ▲ |
| Diethenylbenzene (1,3- and 1,4-) | ● | | | |
| 1,1-Dimethyl-2-propenylbenzene | ● | | | |
| 3-Methylindene | ● | ■ | | |
| Naphthalene | | ■ | | ▲ |
| Methylnaphthalene (1- or 2-) | ● | ■ | | |
| Ethenylbenzene dimer | | ■ | | |
| Dibutylphthalate | | ■ | | |
| Dicyclohexylphthalate | | ■ | | |

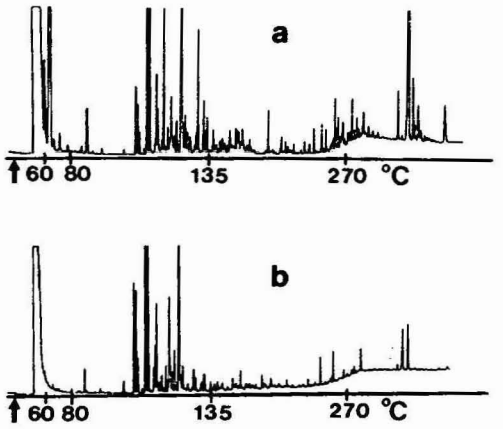


Fig. 2. Glass capillary chromatograms of extractable organics in (a) Chromosorb 101, (b) Chromosorb 102. For chromatographic conditions see Experimental. Attenuation, × 32.

Long-chain esters are widely used as plasticizers in polymer manufacture[5]; mass spectra of these compounds displayed a strong characteristic peak at mass 149.

A weak peak, which can be attributed to dibutylphthalate, appear for Chromosorb 103 after a heating period longer than 6 h. Therefore, such a polymer was further heated. GC analysis showed that phthalic esters are completely released within 12 h of heating. In fact, in a previous paper, it was pointed out that Chromosorb 103 released only a few products after 48 h[6]. Porapak Q and Chromosorb 102 did not show phthalic esters under any heating conditions.

The quantitation of volatile (or extracted) products was not performed, because the levels of organics vary from batch to batch. Furthermore, the aim of this work is to provide a satisfactory clean-up procedure suitable for every polymer batch.

The residual organics released from polymers may be derived from the manufacturing process. To investigate this point, extraction with dichloromethane was

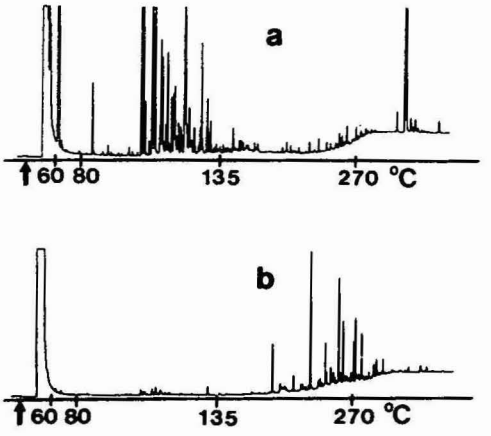


Fig. 3. Glass capillary chromatograms of extractable organics in (a) Chromosorb 103, (b) Porapak Q. For chromatographic conditions see Experimental. Attenuation, × 32.

performed. Hunt and Pangaro[7] characterized organic residues in some XAD resins by an extraction method. It must be remembered that extraction with solvents is widely used as an alternative clean-up procedure[8].

Aliphatic hydrocarbons and their halogen derivatives are indeed good solvents for monomers but not for polymers.

GC capillary analysis of extractable organics is reported in Figs. 2 and 3. Extractions were quantitative after 48 h. In fact, the chromatograms of the polymers already extracted for this period did not show any peaks. Compounds identified by GC–MS carried out on extraction samples are listed in Table III. The majority of these compounds were found in thermal test samples; Chromosorb 101 and 103 extracts showed that the same components and released on heating. Chromosorb 102 extracts had a few more components than the heated sample (*i.e.* naphthalene). Porapak Q extracts are quite different from samples obtained on heating. In fact, the identified compounds are two phthalic esters and some long-chain saturated hydrocarbons. All these products are used as additives in polymer manufacture[5].

Styrene–divinylbenzene copolymers are widely employed as air samplers, although many authors have described disadvantages associated with high blank levels[9]. The manufacturing process leaves a good deal of monomeric material trapped interstitially in the porous structure of the polymer. Such a structure does not seem to be involved in the release of organics during clean-up treatments. In fact, surface area measurements, carried out before and after treatments, gave invariable values[6]. In a previous paper[2] it was pointed out that thermal treatment at the temperature limit for at least 3 h assures good cleaning of the polymeric surface. In fact, the chromatograms of previously treated polymer showed only a few peaks. Since MS confirmations gave similar results, both clean-up procedures seem to be satisfactory.

To confirm this point, similar amounts of each polymer were first extracted and afterwards conditioned at the temperature limit for 3 h. Volatile products released

TABLE III

CHEMICAL CHARACTERIZATION OF EXTRACTABLE ORGANICS

| *Compound* | *PQ* | *CH101* | *CH102* | *CH103* |
|---|---|---|---|---|
| 1,3-Diethylbenzene (or 1,2- or 1,4-) | | | ★ | ▲ |
| Triethylbenzene (1,3,5- or 1,2,4-) | | ■ | | ▲ |
| Ethenylbenzene | | ■ | ★ | ▲ |
| 1-Methylethenylbenzene | | ■ | | |
| Ethenylethylbenzene (1,3- and 1,4-isomers) | | ■ | ★ | ▲ |
| Dimethylethenylbenzene (1,2,4- or 1,3,4-) | | | ★ | ▲ |
| Diethenylbenzene (1,3- and 1,4-) | | | | |
| 1,1-Dimethyl-2-propenylbenzene | | | | |
| 3-Methylindene | | ■ | | |
| Naphthalene | | ■ | ★ | ▲ |
| Methylnaphthalene (1- or 2-) | | ■ | | |
| Ethenylbenzene dimer | | ■ | | |
| Dibutylphthalate | ● | ■ | | |
| *n*-Butyl-2-methylpropylphthalate | ● | | | |
| Dicyclohexylphthalate | | ■ | | ▲ |
| $n$-$C_{14}$–$n$-$C_{18}$ saturated hydrocarbons | ● | | | |

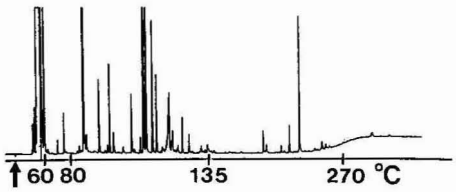


Fig. 4. Glass capillary chromatogram of volatile products evolved from previously extracted Chromosorb 101. For chromatographic conditions see Experimental. Attenuation, × 32.

on heating were trapped, as described in the Experimental section. The chromatogram obtained for Chromosorb 101 (Fig. 4) shows that the polymer still contained appreciable levels of organic residues. Other polymers gave identical results. Therefore, the extraction clean-up procedure is less suitable than the thermal procedure; contaminant materials are largely extractable, but thermal treatment at the temperature limit provides a more rigorous clean-up of polymeric surfaces.

The appearance of residual artifacts in adsorbents employed in air-sampling schemes can pose a significant problem in the characterization of an environmental sample, expecially during the thermal desorption process. Fig. 5 shows chromatograms obtained during thermal desorption of a Chromosorb 103 tube. In these experiments, the sampling tubes, filled with *ca.* 0.2 g of polymer, were inserted through

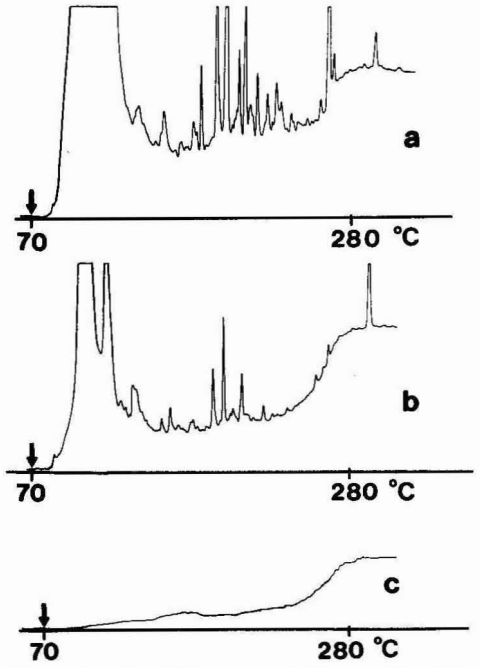


Fig. 5. Blanks of Chromosorb 103 sampling tube obtained during thermal desorption. (a) Polymer pretreated at 200°C for 1 h; (b) polymer extracted with dichloromethane; (c) polymer pretreated at temperature limit for 6 h. Experimental conditions: 3% OV-101, 6 ft. × 2 mm I.D. glass-packed column; temperature, 70–280°C at 8°C/min; detector, 300°C; attenuation, × 32.

a thermostated aluminium block (250°C). It is possible to note that poor blanks are obtained if the polymer is conditioned at a temperature lower than the limit and for a shorter time, as practiced by many workers[10,11]. Poor blanks are also obtained if a polymer is only extracted. Chromosorb 103 provides satisfactory blanks if the sampling tube is conditioned at the temperature limit for almost 6 h. Porapak Q, Chromosorb 101 and 102 gave similar results, but thermal treatment at the temperature limit for at least 3 h is enough.

## CONCLUSION

The characterization of volatiles released by heating Porapak Q, Chromosorb 101, 102, and 103 showed that such potential contaminants are manufacturing residues. The majority of them can also be extracted with dichloromethane. Such artifacts can seriously interfere in the subsequent GC analysis if the polymer clean-up procedure is inadequate.

The best conditions for minimizing contamination in air-sampling schemes is to pretreat adsorbents at their proper temperature limit for a suitable period of time, depending on the nature of the polymer.

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## REFERENCES

1 S. Coppi, A. Betti, G. Blo and C. Bighi, *J. Chromatogr.*, 267 (1983) 91.
2 A. Betti, S. Coppi, G. Blo and C. Bighi, *Ann. Chim. (Rome)*, 74 (1984) 349.
3 *Porapak Porous Polymer Beads GC Column Packing Materials*, Water Assoc., MA, U.S.A.
4 *Chromosorb Century Series Porous Polymer Supports*, Johns-Manville, Denver, CO, U.S.A.
5 M. Baccaredda Boy, *Materie Plastiche ed Elastomeri*, Casa Editrice Ambrosiana, Milan, 1976.
6 S. Coppi and A. Betti, *J. Chromatogr.*, 330 (1985) 55.
7 G. Hunt and N. Pangaro, *Anal. Chem.*, 54 (1982) 369.
8 R. L. Grob, *Modern Practice of Gas Chromatography*, Wiley, New York, 1977.
9 A. J. Núñez, L. F. Gonzáles and J. Janák, *J. Chromatogr.*, 300 (1984) 127.
10 A. Cert and J. Bahima, *J. Chromatogr. Sci.*, 22 (1984) 7.
11 P. Lövkvist and J. A. Jönsson, *J. Chromatogr.*, 286 (1984) 279.

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CHROMSYMP. 665

# EFFECT OF THE MESH-SIZE DISTRIBUTION OF POROUS POLYMER BEADS ON EFFICIENCY AND SELECTIVITY OF STATIONARY PHASES

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## SUMMARY

The proposed method for the classification and characterization of the gas chromatographic behaviour of stationary phases of porous polymer beads is influenced by the mesh-size distribution of the different commercial batches, which changes the number of theoretical plates, the efficiency, and the selectivity of the columns.

The true distribution of the diameters of many batches of Porapak and Chromosorb "Century Series" PPB was therefore measured and compared with the nominal mesh range. Appreciable variation in the diameters between different batches was observed, and the effective size distribution was sometimes very different from the nominal values.

The gas chromatographic behaviour of columns packed with porous polymer beads of the same type but different sizes was investigated at various temperatures and column lengths by determining the theoretical plate number, the retention values, and the selectivity of the columns.

## INTRODUCTION

Stationary phases of porous polymer beads (PPB) are suitable for the analysis of inorganic gases, light hydrocarbons and polar liquid compounds[1–6]. Porapak (Waters Assoc., Framingham, MA, U.S.A.) and Chromosorb Century Series (Johns-Manville, Denver, CO, U.S.A.) PPB are widely used. Different types of the two stationary phases are produced in order to permit a satisfactory separation of many compounds of different polarity. Similar behaviour of some Porapak and Chromosorb PPB was claimed, and this should allow the use of either product with similar results. Probably owing to different preparation techniques, the gas chromatographic (GC) behaviour of Porapak and Chromosorb PPB made from the same type of polymer was found to be more or less different[7].

From the point of view of the user, some of the observed differences are more useful than the claimed similarities, because the two PPB series can be used to increase the choice of stationary phases, mainly when mixed columns are applied to the fast and complete resolution of complex mixtures[8].

The characterization of Chromosorb and Porapak PPB has been studied pre-

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viously, and classifications based on retention relative to reference compounds[9–11], retention indices with respect to light paraffins[12], and comparison with standard polarity phases (squalane and apolane)[13,14] have been proposed. Liquid stationary phases with a known chemical composition are expected to behave in a predictable manner, the only possible variations being due to temperature and to the influence of the solid support used; therefore, the proposed classifications of liquid phases[15,16] permit the behaviour of a given column to be predicted within very narrow limits.

Some problems are still encountered with polymeric liquid phases (silicones, polyglycols, etc.) where the actual distribution of molecular weights may differ from the nominal value (*e.g.* in the Carbowax series) and from the value of the batch used for the classification. The same problem arises with PPB, as the various batches or lots may differ in molecular weight, cross-linking ratio, amount of polarity modifier, pore-size distribution, real mesh size, specific surface area of the beads and column packing density.

The effects of the size of the polymer particles and of the column-packing density on retention times and shape of the peaks have scarcely been investigated. The retention times listed by the manufacturers do not refer to specific mesh sizes, and extensive research on the GC behaviour as a function of the diameter of the beads has not been reported. Mesh sizes 50–80, 80–100 and 100–120 have generally been used, but under different experimental conditions and for various analytical purposes. A comparison based only on literature data is therefore impossible. Moreover, the effective distribution of particle diameters in a batch of PPB of a given "nominal" mesh size is probably measured by the manufacturer but is not available to the users.

In order to investigate the effect of mesh size on GC behaviour, the effective diameter distribution of Porapak and Chromosorb PPB was measured, and analyses of standard gas mixtures were accomplished by using columns filled with various sizes and known amounts of these packings, thus permitting the evaluation of the column-packing density.

## EXPERIMENTAL

The effective particle sizes could not be checked by sieving the various batches of PPB, because sieves with a mesh range smaller than 20 mesh units were not commercially available, the accuracy of the existing sieves was not high enough, and electrostatic charge effects may produce agglomeration of the beads and a greater "apparent" mesh size. Therefore, the particle-size distribution was obtained by measuring the diameter of the spherical particles on 60-times-enlarged photos taken with an optical microscope (Reichert Zetopan). A graduated reference slide (1 mm divided in 100 parts) was photographed with the samples in order to permit absolute diameter measurement, independent of the reproducibility of the photo enlargement. Photos of 10–20 samples, each containing 50–200 particles, were used to measure the size distribution of each polymer batch.

The various commercial mesh ranges correspond to slightly different metric diameters, depending on the classification (Tyler, U.S., Institute of Mining and Metallurgy, etc.)[17,18]. Fig. 1 is a plot of mesh sizes *vs.* diameter in micrometres, taken from different sources. As the producers of the tested beads are U.S. companies, the

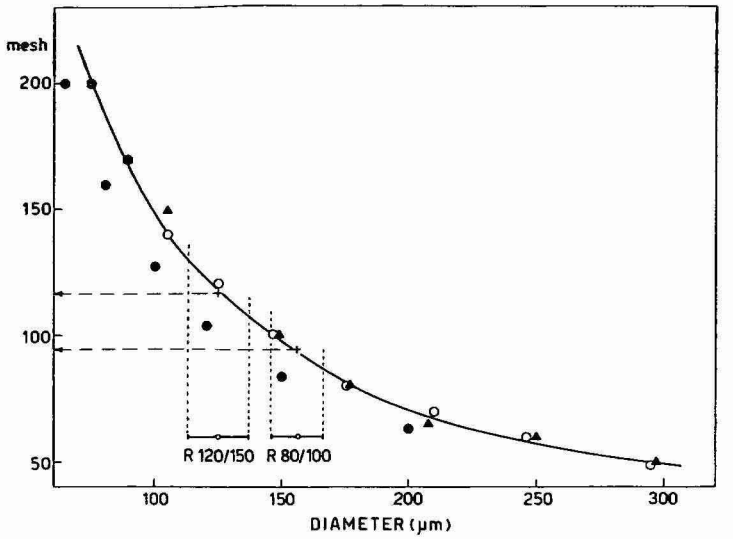


Fig. 1. Correlation between diameter and mesh size in different classifications: (○) U.S., (▲) Tyler, and (●) Institute of Mining and Metallurgy (IMM) (from refs. 17 and 18). Actual diameter ranges of Porapak R PPB used for comparison are shown (see text).

U.S./Tyler scale was used for calculation of the diameters corresponding to the mesh ranges.

The mean value between the greatest and the smallest diameter of each particle was calculated for polymers having non-spherical beads (Chromosorb 102 and 104 and Porapak Q in some mesh ranges). The histograms showing the distribution of some PPB batches are presented in Figs. 2 and 3. The average diameter, $D$, and the standard deviation, $\sigma$, are also shown and compared with the nominal mesh range. A general summary of the results is shown in Table I and Fig. 4. As a general rule, Porapak PPB have spherical shapes and a narrow range of diameter, whereas Chromosorb types have a more irregular appearance. Variable amounts of the various

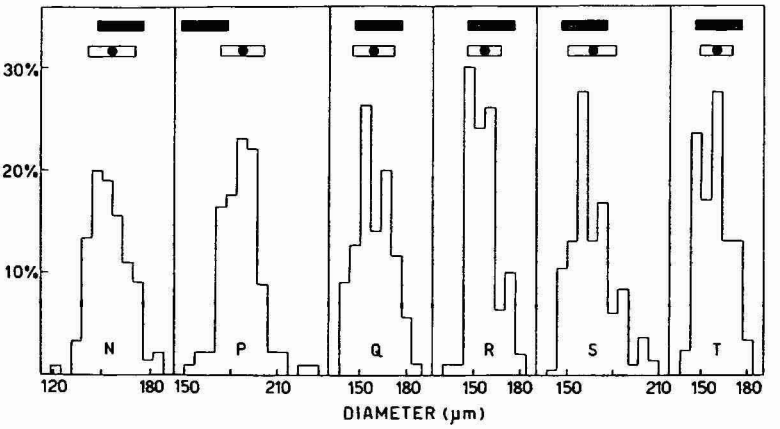


Fig. 2. Histograms showing the diameter distribution of some batches of 80–100 mesh Porapaks. Nominal interval (▣), average diameter (●) and $\sigma$ interval (□).

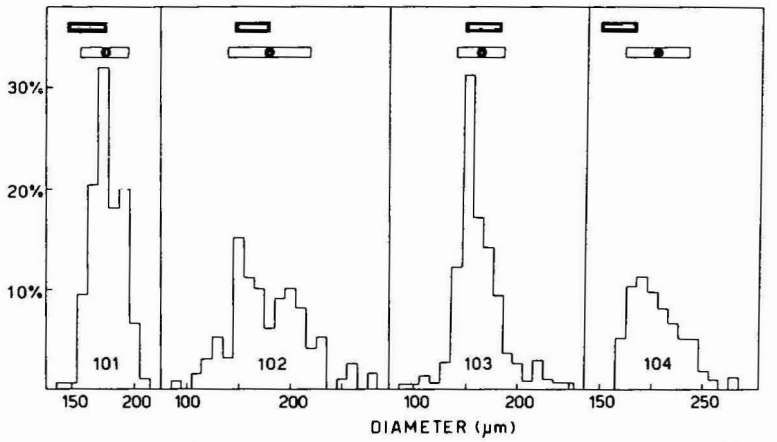


Fig. 3. Histograms showing the diameter distribution of some batches of 80–100 mesh Chromosorb "Century Series". For symbols see Fig. 2.

PPB (from 14 to 100%, with an average of 66 ± 24%) fall within the nominal mesh range.

The effect of the mesh-size distribution on the efficiency of the column was investigated by carrying out analyses of standard mixtures of methane, carbon dioxide, ethyne, ethene and ethane at temperatures ranging from 25 to 60°C, with a flow-rate of 22 $cm^3$/min of helium. Tables II and III show the number of theoretical plates per metre, averaged for all the tested batches of every PPB type and for the whole temperature range. The difference between two columns filled with the same batch (P I and P II), the effect of the total column length (R 80–100, 3, 2, and 1 m), and the influence of the mesh size (R 80–100 and R 120–150) are also shown in Table II.

The differences between the various compounds analysed can be correlated with the different elution order and asymmetry of the peaks, depending on the polarity of the PPB, whereas the values of the $\sigma$ depend on both the fluctuations between different runs and on the effects of temperature on retention time and peak shape. The columns with the narrowest mesh range showed the greatest efficiency.

The comparison between the behaviour of Porapak R 80–100 and 120–150 mesh (nominal values) was further investigated. The batches of these types of Porapak were chosen because of their narrow and symmetrical diameter distribution, confirmed by the high number of theoretical plates and by the low $\sigma$ values. Whereas the 80–100 batches had an average diameter nearly coincident with the centre of the nominal mesh range, the 120–150 batch distribution was centred on the lowest value of the nominal interval, and therefore corresponds to a true 100–130 mesh range (see Fig. 1). The theoretical plate number of the smallest size batch is *ca.* 30% greater for equal column lengths.

Fig. 5 shows the linear behaviour of the values for the logarithm of the net retention value, $V_N$, obtained by multiplying the adjusted retention volume, $V'_R$, by the column pressure-gradient correction factor of Martin and James[19],

$$j = \frac{3(P_i - P_0)^2 - 1}{2(P_i - P_0)^3 - 1}$$

TABLE I

DIAMETER DISTRIBUTION OF SOME BATCHES OF VARIOUS PPB

| *PPB type* | *Nominal value* | | *Batch (lot)* | *Average diam. (μm)* | *±σ(%)* | *Minimum diam. (μm)* | *Nominal mesh range (%)* | | | *Maximum diam. (μm)* |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Mesh* | *Diam. (μm)* | | | | | *Above* | *Within* | *Below* | |
| *Porapak* | | | | | | | | | | |
| N | 80–100 | 147–175 | 1201 | 157 ± 15 | 9.6 | 121 | 18 | 79 | 3 | 215 |
| | | | 1210 | 143 ± 10 | 7.0 | 120 | 54 | 46 | – | 165 |
| | | | 1128 | 155 ± 16 | 10.3 | 125 | 28 | 63 | 9 | 190 |
| P | 80–100 | 147–175 | 1769 | 187 ± 14 | 7.5 | 153 | – | 20 | 80 | 247 |
| Q | 50–80 | 175–295 | 1016 | 216 ± 17 | 7.9 | 186 | – | 100 | – | 263 |
| | | | 1250 | 197 ± 23 | 11.9 | 154 | 18 | 82 | – | 269 |
| | 80–100 | 147–175 | 1297 | 159 ± 11 | 6.9 | 141 | 9 | 84 | 7 | 186 |
| | | | 1297* | 165 ± 14 | 8.5 | 147 | – | 88 | 12 | 201 |
| | | | 1477 | 153 ± 10 | 6.5 | 125 | 15 | 84 | 1 | 180 |
| | | | 1457 | 157 ± 7 | 4.4 | 140 | 22 | 87 | 1 | 180 |
| | 100–120 | 124–147 | 1444 | 134 ± 11 | 8.2 | 102 | 22 | 62 | 16 | 154 |
| | 120–150 | 97–124 | 410 | 121 ± 10 | 8.3 | 102 | – | 68 | 32 | 154 |
| | | | 516 | 100 ± 8 | 8.0 | 70 | 16 | 84 | – | 180 |
| | 150–200 | 74–97 | 651 | 95 ± 6 | 6.3 | 83 | – | 69 | 31 | 115 |
| | | | 1423 | 85 ± 7 | 8.2 | 65 | 3 | 91 | 6 | 100 |
| R | 80–100 | 147–175 | 609 | 156 ± 10 | 6.4 | 134 | 2 | 96 | 2 | 181 |
| | 120–150 | 97–124 | 425 | 125 ± 12 | 9.6 | 121 | 18 | 79 | 3 | 215 |
| S | 80–100 | 147–175 | 684 | 167 ± 15 | 9.0 | 147 | – | 81 | 19 | 208 |
| | | | 686 | 151 ± 15 | 10.0 | 135 | 35 | 63 | 2 | 185 |
| T | 80–100 | 147–175 | 1797 | 159 ± 10 | 6.4 | 140 | 2 | 95 | 3 | 180 |
| *Chromosorb* | | | | | | | | | | |
| 101 | 80–100 | 147–175 | 70 | 183 ± 20 | 10.9 | 80 | 2 | 31 | 67 | 250 |
| 102* | | | 31274 | 178 ± 38 | 21.3 | 90 | 16 | 36 | 48 | 290 |
| | | | 2409 | 184 ± 29 | 15.8 | 145 | 41 | 59 | 22 | 265 |
| | | | 261 | 181 ± 26 | 14.4 | 135 | – | 53 | 47 | 225 |
| | | | 102 | 154 ± 14 | 9.1 | 100 | 21 | 62 | 17 | 190 |
| 103 | | | 26 | 161 ± 23 | 14.3 | 80 | 15 | 65 | 20 | 290 |
| 104* | | | 2415 | 163 ± 30 | 18.4 | 110 | 30 | 35 | 35 | 230 |
| 105 | | | 10 | 129 ± 25 | 19.8 | 80 | 67 | 30 | 3 | 220 |
| 107 | | | 3 | 182 ± 20 | 11.0 | 130 | – | 14 | 86 | 210 |

* Irregularly shaped beads.

as a function of the reciprocal of the absolute temperature, $T$. The values for the 100–130 mesh columns are higher than the corresponding values for the 80–100 mesh columns; the effect of mesh size is not the same for all of the compounds tested and, therefore, the classification according to $I$ values is also slightly influenced (Fig. 6).

Owing to the fact that the packing density of the columns depends on the average diameter of the PPB, the columns filled with 80–100 Porapak R contained an average of 4.3 ± 0.05 g of stationary phase, and those filled with 100–130 mesh Porapak R contained 4.5 ± 0.04 g. It is known that the highest possible packing density (obtained with a face-centred cubic lattice) fills *ca.* 74% of the available volume when all the spheres have the same diameter. By taking into account the different diameters of the PPB, a slightly disordered structure results and, therefore,

TABLE II

NUMBER OF THEORETICAL PLATES PER UNIT LENGTH ($n$/m), AND PERCENTAGE STANDARD DEVIATION OF THE VALUES ($\sigma$) FOR THE VARIOUS COMPOUNDS ON DIFFERENT PORAPAK TYPES

Column length, 3 m; mesh size, 80–100 (except for Porapak R, where indicated).

| *Porapak* | *N* | | *PI* | | *PII* | | *Q* | | *S* | | *T* | | *R 80–100* | | | | | | *R 120–150** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *1m* | | *2m* | | *3m* | | *3m* | |
| | | | | | | | | | | | | | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ |
| Methane | 580 | 7 | 500 | 14 | 530 | 10 | 420 | 12 | 580 | 15 | 740 | 14 | 410 | 13 | 510 | 13 | 710 | 7 | 920 | 7 |
| Carbon dioxide | 1230 | 3.7 | 850 | 16 | 890 | 18 | 590 | 20 | 1220 | 7 | 1060 | 7 | 830 | 15 | 900 | 9 | 1030 | 9 | 1390 | 3 |
| Ethene | 1190 | 12 | 1160 | 16 | 1030 | 21 | 360 | 11 | 1190 | 9 | 1080 | 20 | 1100 | 7 | 1050 | 9 | 1060 | 7 | 1520 | 4 |
| Ethyne | 1170 | 10 | 1090 | 20 | 1040 | 23 | 430 | 18 | 1090 | 14 | 1040 | 17 | 1120 | 8 | 1080 | 10 | 1200 | 3 | 1480 | 3 |
| Ethane | 1150 | 11 | 1190 | 16 | 1200 | 16 | 590 | 9 | 950 | 11 | 1020 | 13 | 1010 | 7 | 1030 | 13 | 1140 | 7 | 1330 | 5 |

* Nominal values; true mesh range 100–130 (see text).

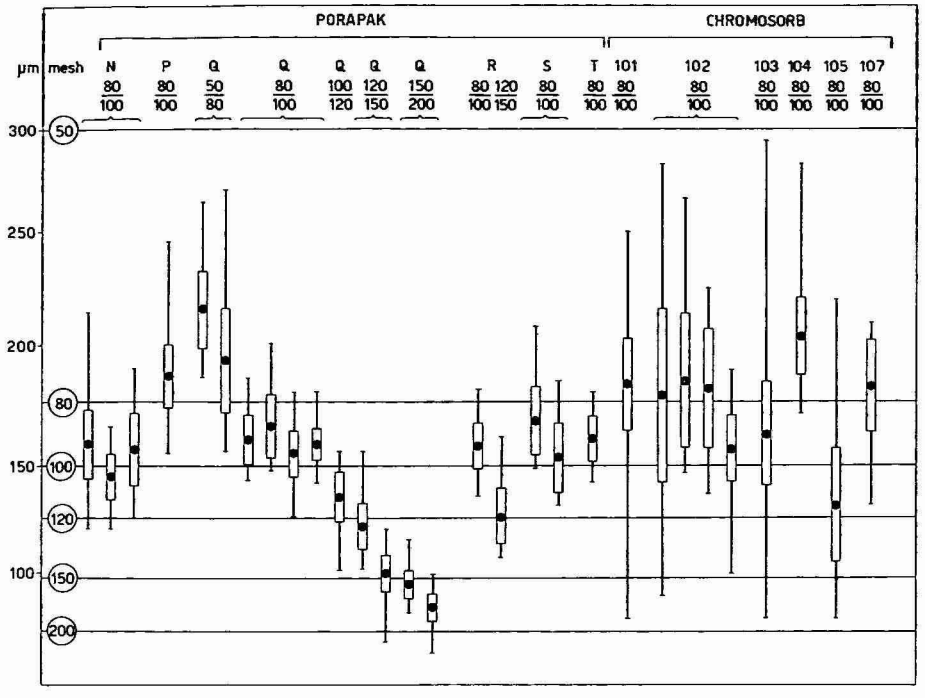


Fig. 4. Average diameter (●), standard deviation (□) and max.–min. diameter range (|——|) of many different PPB, compared with the nominal mesh ranges.

a theoretical calculation of the void volume of the columns is difficult. On the basis of the weight of stationary phases, and under the assumption that the density, $\rho$, of Porapak R is independent of the mesh size, it can be concluded that the filling efficiency of the 100–130 mesh batch is *ca.* 5% higher than that of the 80–100 mesh-batch.

The use of the specific retention volume, $V_g = 273\ V_N/W_L T_C$ (where $W_L$ is the weight of liquid phase in the column), that corresponds to the volume of gas required to move one half the sample through a theoretical column containing 1 g of liquid phase, may not be justified for a PPB stationary phase, because it is not clear whether the separation mechanism involves the entire volume of the beads or a surface layer of varying depth, depending on the diameter, shape, and mobility of the sample molecules. In addition, the relative importance of purely diffusive phenomena, which probably predominate at low temperatures, is not known with respect to absorption and solution of the sample component in the beads, which increases with increasing temperature and thermal softening of the cross-linked polymer.

Therefore, in order to take into account the different amounts of PPB in the columns filled with different mesh ranges, and therefore the column-packing density, the net retention volumes per gram of solid stationary phase, $V_S = V_N/W$ (where $W$ is the total weight of the PPB), were calculated. The $V_S$ values at 30 and 60°C for the various gases and the values of the ratio $V_S$(80–100):$V_S$(100–130), averaged for the $V_S$ values from 25 to 60°C at 5°C intervals, are shown in Table IV. The change of $V_S$

TABLE III

NUMBER OF THEORETICAL PLATES PER UNIT LENGTH ($n$/m) AND PERCENTAGE STANDARD DEVIATION OF THE VALUES AVERAGED OVER ALL TEMPERATURES ($\sigma$), FOR THE VARIOUS COMPOUNDS ON DIFFERENT CHROMOSORB TYPES

Column length 3 m, mesh size 80–100.

| *Compound* | *Chromosorb* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 101 | | *102* | | *103* | | *104* | | *105* | |
| | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ | *n/m* | $\sigma$ |
| Methane | 490 | 21 | 560 | 8 | 370 | 15 | 460 | 15 | 930 | 8 |
| Carbon dioxide | 780 | 15 | 760 | 8 | 310 | 28 | 940 | 7 | 870 | 6 |
| Ethene | 810 | 10 | 810 | 10 | 220 | 24 | 500 | 16 | 990 | 5 |
| Ethyne | 820 | 15 | 860 | 12 | 370 | 13 | 880 | 11 | 990 | 4 |
| Ethane | 660 | 7 | 880 | 6 | 280 | 22 | 970 | 8 | 950 | 3 |

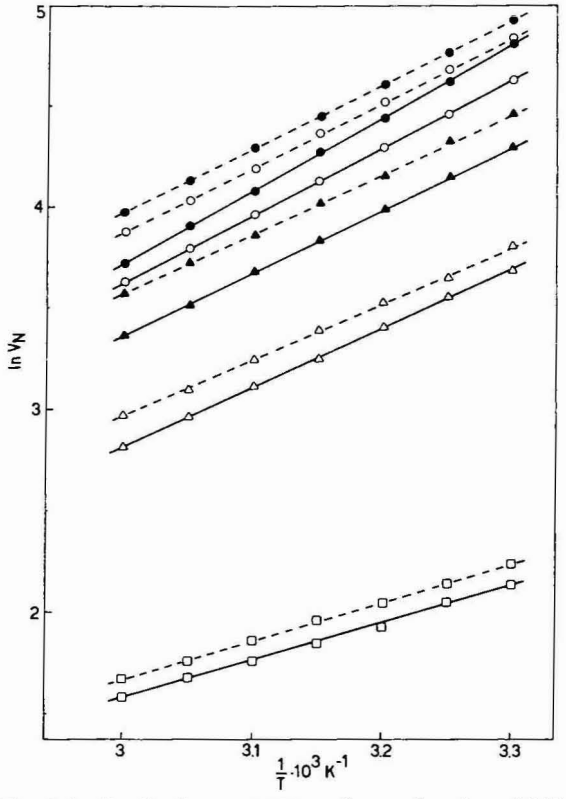


Fig. 5. ln $V_N$ of reference compounds as a function of $1/T$ on Porapak columns of different mesh size. Curves: ——, 80–100 mesh; – – – –, 100–130 mesh). ●, ethyne; ○, ethane; ▲, ethene; △, carbon dioxide; □, methane.

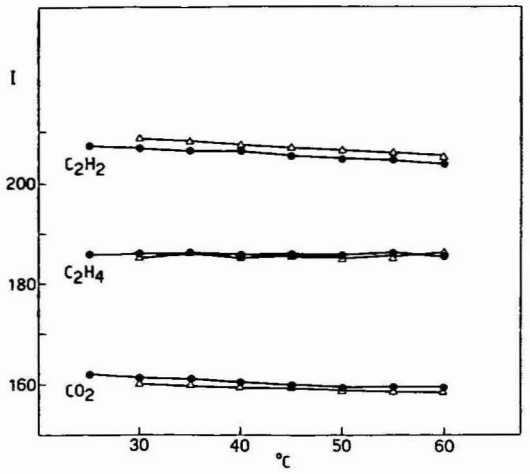


Fig. 6. Retention index values of reference compounds as a function of temperature and mesh size. ●, 80–100 mesh; △, 100–130 mesh.

values with temperature is great, reflecting the variation in the retention time, but their ratio remains nearly constant for each compound, as shown by the small values of the standard deviation, $\sigma$.

Table IV also shows that the relative increase of $V_S$ when changing from 80–100 to 100–130 mesh

$$\Delta V_S(\text{rel}) = \frac{V_S\,(100\text{–}130) - V_S\,(80\text{–}100)}{V_S\,(80\text{–}100)}\,100$$

increases with increasing retention time, but not in proportion to it. The change of the adsorption enthalpies, $\Delta H^\circ$[10,20,21], which gives an idea of the molecular interactions and can be calculated from the slope of the plot ln $V_N$ (or $V_S$) against $1/T$ from the equation

$$\ln V_S = \frac{-\Delta H^\circ}{RT} + q$$

is between 2 and 4% when changing from 80–100 to 100–130 mesh. This variation is of the order of magnitude of the experimental error and therefore the interactions of the two types of Porapak R can be assumed to be the same.

The different sensitivities of the retention volumes of the various compounds to the change of mesh size may be explained in terms of different depths of diffusion into the beads, depending on the molecular dimensions and free path with respect to the pore diameter.

The observed difference in the behaviour of batches with different mesh sizes cannot be explained merely by differences in chemical composition and specific surface areas caused by non-reproducible syntheses, because the $\Delta V_s$(rel) values between various batches of Porapak R 80–100 with average diameters close to the centre of

TABLE IV

EFFECT OF MESH SIZE ON THE BEHAVIOUR OF PORAPAK R COLUMNS

For symbols see text.

| | $V_S(80–100)$ | | $\Delta V_S(rel)$ (%) various 80–100 | $V_S$ (100–130) | | $\frac{V_S\ (80–100)}{V_S\ (100–130)}$ (average %) | $\pm\sigma$ (%) | $\Delta V_S(rel)$ (80–100 vs. 100–130) (%) | $-\Delta H^\circ$ (80–100) (kcal mol$^{-1}$) | $-\Delta H^\circ$ (100–130) (kcal mol$^{-1}$) | $\frac{\Delta V_S}{\Delta \bar{m}}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30°C | 60°C | | 30°C | 60°C | | | | | | |
| Methane | 1.98 | 1.14 | ±1.7 | 2.09 | 1.20 | 94.07 ± 1.05 | 1.12 | 5.55 | 3.64 | 3.72 | 0.27 |
| Carbon dioxide | 9.33 | 3.98 | ±1.9 | 9.89 | 4.34 | 92.04 ± 1.02 | 1.09 | 6.00 | 5.73 | 5.60 | 0.36 |
| Ethene | 17.16 | 6.77 | ±2.2 | 19.12 | 7.89 | 87.87 ± 0.96 | 1.09 | 11.42 | 6.21 | 6.00 | 0.55 |
| Ethane | 24.07 | 9.12 | ±2.4 | 27.70 | 10.76 | 85.36 ± 0.79 | 0.93 | 15.08 | 6.53 | 6.41 | 0.66 |
| Ethyne | 28.72 | 9.83 | ±3.8 | 34.84 | 12.05 | 81.77 ± 0.42 | 0.51 | 21.31 | 7.22 | 7.20 | 0.83 |

the nominal mesh range were appreciably smaller than the observed variation between 80–100 and 100–130 mesh (see Table IV, second column).

The observed increase in $\Delta V_s$(rel) follows the behaviour of the $b$ term of the Van der Waals' equation (due to the finite volume of the molecules and to their general incompressibility)[22], which increases in the order methane < carbon dioxide < ethyne < ethene < ethane, except for ethyne, which shows a greater increase in $V_S$, but can be correlated with the linear and rigid structure of the molecule. Therefore, the $V_S$(rel) increase can be correlated with the increasing difficulty of the sample molecules to permeate the bead volume, which reduces their interaction with the large beads, decreasing the retention times on the 80–100 polymer. One can expect that a complete interaction of all sample molecules, independent of their size, with the entire bead should only be possible for very small PPB, as confirmed by the increasing theoretical plate number. On the other hand, very small mesh sizes cause a high packing density and require a very high input pressure.

From the practical point of view, the effect of mesh-size variation on the classification of the PPB can be evaluated by taking into account that, in terms of retention index values, the observed $\Delta V_S$(rel) between 80–100 and 100–130 correspond to *ca.* ±2 index units (IU) at 30°C and ±1.5 IU at 60°C. By dividing $\Delta V_S$(rel) by the difference between the average mesh size of the two Porapak R batches, $\Delta\bar{m}$, (156 μm corresponding to 95 mesh, 125 μm to 117 mesh) the percentage variation of the retention for each mesh unit is obtained (see Table IV).

The effect of a variation of *ca.* 0.1 IU per mesh on the PPB characterization based on $I$ values is negligible when the average diameter of any given PPB batch falls within the nominal mesh range and for the most widely used 80–100 mesh PPB. In many of the batches tested (see Table I and Fig. 4) the $\Delta\bar{m}$ between the true mesh average and the centre of the nominal interval is smaller than 20 and, therefore, the variation in $I$ is probably within ±2 IU. On the other hand, when different mesh-size intervals are compared, and when compounds showing opposite changes of their $I$ values (*e.g.* carbon dioxide and ethyne in the test mixture) are analysed, differences in the $I$ values of *ca.* 5–10 IU can be expected that are two to three times greater than the observed differences between various batches of the same mesh size. In this instance, the validity of any classification based on retention times or indices may be strongly reduced when different batches of unknown mesh-size distribution are compared.

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REFERENCES

1 O. L. Hollis, *Anal. Chem.*,38 (1966) 309.
2 O. L. Hollis, *J. Chromatogr. Sci.*, 11 (1973) 335.
3 S. B. Dave, *J. Chromatogr. Sci.*, 7 (1969) 389.
4 S. B. Dave, *Ind. Eng. Chem. Prod. Res. Dev.*, 14 (1975) 85.
5 *Tech. Bull. FF-194 A*, Johns-Manville, Denver, CO, 1967.
6 *Tech. Bull. FF-202 A*, Johns-Manville, Denver, CO, 1980.

7 G. Castello and G. D'Amato, *J. Chromatogr.*, 248 (1982) 391.
8 G. Castello and G. D'Amato, *Ann. Chim. (Roma)*, 69 (1979) 541.
9 G. Castello, G. D'Amato and G. Canciani, *Ann. Chim. (Roma)*, 68 (1978) 255.
10 G. Castello and G. D'Amato, *J. Chromatogr.*, 196 (1980) 245.
11 G. Castello and G. D'Amato, *J. Chromatogr.*, 212 (1981) 261.
12 G. Castello and G. D'Amato, *J. Chromatogr.*, 243 (1982) 25.
13 G. Castello and G. D'Amato, *J. Chromatogr.*, 254 (1983) 69.
14 G. Castello and G. D'Amato, *J. Chromatogr.*, 269 (1983) 153.
15 L. Rohrschneider, *J. Chromatogr.*, 22 (1966) 6.
16 W. O. McReynolds, *J. Chromatogr. Sci.*, 8 (1970) 685.
17 C. D. Hodgman, R. C. Weast and S. M. Selby, *Handbook of Chemistry and Physics*, 38th ed., Chemical Rubber Publishing Co., Cleveland, OH, 1956.
18 R. H. Perry and C. H. Chilton, *Chemical Engineers' Handbook*, 5th ed., McGraw-Hill, New York, 1973.
19 A. J. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
20 F. M. Zado and J. Fabecic, *J. Chromatogr.*, 51 (1970) 37.
21 J. Czubryt and H. D. Gesser, *J. Chromatogr.*, 59 (1971) 1.
22 R. C. Weast (Editor), *CRC Handbook of Chemistry and Physics*, 58th ed., CRC Press, Cleveland, OH, 1978, D-178.

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| MONTH | O 1985 | N 1985 | D 1985 | J | F | |
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# Microprocessor Programming and Applications for Scientists and Engineers

*by* **R.R. SMARDZEWSKI**, *Surface Chemistry Branch, Naval Research Laboratory, Washington, DC, USA*

This is a much-needed, clear and straightforward explanation of the fundamentals of microprocessing including machine-language programming and its research applications. Most of the literature on the general subject of microprocessors has been written with the electrical engineer or computer hobbyist in mind. This book, however, has been written by a research chemist for those scientists, engineers and hobbyists outside the electrical engineering community, who seek a basic understanding of microprocessors and their applications in laboratory environments.

First principles and elementary concepts are illustrated throughout the text by a series of 35 programmed instructional experiments. The particular microprocessor chosen for examination is the popular 6502 which is currently employed in a variety of microcomputing systems (Apple, Acorn, BBC, Commodore, Rockwell, and others). Actual programming examples contained in the text demonstrate the basic principles and ideas of 6502 machine-language programming and applications in research/laboratory situations.

The book is a must for the modern scientist and engineer working in physical chemistry, physics, analytical chemistry, applied engineering, design, CAD-CAM, process control, and bioengineering. As *no prior background in computer science or programming* is assumed, it will serve as an ideal elementary text for the beginner. At the same time, the advanced machine-language programmer will find it an invaluable reference handbook.

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